Final Report

AQUAFIN CRC-SOUTHERN BLUEFIN TUNA AQUACULTURE SUBPROGRAM: APPLICATION OF THE USE OF DIETARY SUPPLEMENTS FOR IMPROVING FLESH QUALITY ATTRIBUTES OF FARMED SBT

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Aquafin CRC Project 2.2(2); FRDC Project No. 2004/209
Aquafin CRC-Southern Bluefin Tuna Aquaculture Subprogram: Application of the use of dietary supplements for improving the flesh quality attributes of farmed SBT

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OBJECTIVES

1. Adapt existing assays for oxidative stress from southern bluefin tuna (SBT) muscle extracts and mammalian cell cultures to SBT cultures.
2. Develop a supplement, delivered by a manufactured pellet, which can be fed to tuna that are being fed predominately on baitfish on a commercial farm.
3. Investigate the effects of simulated oxidative stress on SBT cell cultures and identify the most effective concentrations and combinations of antioxidants that combat this stress.
4. Test the effectiveness on farm vitamin supplement delivery systems in raising tissue level of antioxidants and increasing quality characteristic and shelf life.
5. For the purpose of SBT surrogate experiments, manipulate and control the level of lipid oxidation in commercial fish feeds.
6. Compare the flesh quality of SBT fed high vitamin supplement to SBT raised using existing industry standard farming practice.
7. Use a surrogate for SBT to assess the effects of oxidized feeds on flesh quality and shelf life.
8. Measure endogenous antioxidant vitamin levels in baitfish typically used in the tuna industry.
9. Use a surrogate fed oxidised feeds to test the protective effects of the antioxidants identified in cell culture experiments of the manufactured pellet.

OUTCOMES ACHIEVED TO DATE

This research on the application of the use of dietary supplements for improving the flesh quality attributes of farmed SBT has been focused on improving the colour shelf life of the sashimi product. Previous outcomes of our research group (2001/248) and supporting data from associated CRC project groups have led to the current interest in evaluating the use of a supplementary feed for tuna farming that can be used to extend shelf life.

Our previous research with SBT has examined the use of increased levels of dietary antioxidants to slow the browning process of tuna meat using fortified pellets and coated baitfish as a nutrient delivery system. We have established that feeding SBT pellets fortified with boosted levels of vitamins E and C and selenium will raise the level of these natural antioxidants in the fish muscle. Having demonstrated that higher levels of these antioxidants in the muscle consistently result in an extension of the colour shelf life of sashimi grade tuna meat; we also demonstrated that feeding SBT pellets fortified with a higher level of vitamin E alone is not as effective in extending the shelf life of SBT meat as a combination of higher levels of vitamins E and C. In addition, we found that pellet diets fortified with antioxidants are by far the most effective delivery system for these nutrients and therefore the most effective way to improve the shelf life of farmed SBT flesh. With that background research in place and the concurrent acceptance within the industry that additional antioxidant nutrients have a benefit the current project focused on gaining a better understanding of the application of available antioxidant nutrient and providing the industry will a reliable feed supplement to deliver additional nutrients to SBT. Within the current project the outcomes achieved have included the development and application of cell culture techniques through the adaptation of existing assays for oxidative stress from SBT muscle extracts and mammalian cell cultures to fish cultures. In this way the effects of simulated oxidative stress on SBT cell cultures and has enabled a close examination of oxidative stress processes at a cell level and identification of likely effective concentrations and combinations of novel antioxidants that combat this stress.

In concert with this research we have, for the purpose of SBT surrogate experiments, manipulated and controlled the level of lipid oxidation in commercial fish feeds fed to yellowtail kingfish (*Seriola lalandi*). This subsequently led to incorporation of these feeds into surrogate experiment investigating the amelioration of oxidative stress in a fish surrogate for SBT. These experiments have set a platform for research that may be appropriate should a reliable supply of fingerling – juvenile SBT become available in the future.

Through the investigation of the use of locally caught baitfish as a component of SBT diet we have demonstrated the benefits that fresh caught baitfish feed can have on SBT flesh quality. This has resulted in quantification of endogenous antioxidant vitamin levels in baitfish typically used in the tuna industry. In combination with results from project 2004/211 (Nutritional profiles of baitfish: effects of harvest and post-harvest processes on quality of local baitfish for feeding SBT) a project that stemmed partly from the need identified in the

Product Quality Projects – a great deal of information relating to the endogenous levels of antioxidant nutrients is now available for use within the industry.
Never the less development of a supplement, to be delivered by a manufactured pellet, which could be fed to SBT that are being fed predominately on baitfish was a focus of the current project. It has been concluded that supplement pellets (using the currently available physical characteristics and pellet composition) will give at best limited success in the delivery of additional supplementary nutrients to farmed SBT. The outcome achieved by this project in this area has been the development of a vitamin injection system. This system has incorporate meat marinating technology and the use of commercially available vitamin supplements to deliver to industry a method of creating a commercially viable supplementary diet for SBT. By refining the process of injection of antioxidant nutrients into baitfish a supplementary diet is now available that can be frozen and stored prior to being fed to SBT as required.
The testing of the effectiveness of this on farm vitamin supplement delivery systems in raising tissue level of antioxidants and increasing quality characteristic and shelf life is now taking place in a commercial arrangement between researchers and the industry. In pursuing technical project goals this project has been very effective within the education and training.
This project was a “coal face” project with the principal scientists based in the same location as the industry. This close liaison was very successful in increasing industry awareness of the science of product quality issues and scientist’s awareness of industry financial and non-financial business in the decision making context. This valuable relationship between scientist and industry was the key to identifying the appropriate solution for providing supplementary nutrients to farmed SBT and allowing the transition of successful PhD candidates, D’Antignana and Douglas, into ongoing positions within research and industry (respectively). In addition solid links were achieved between SBT quality characteristics, feed and the market through traditional mechanisms of workshops, meetings and conferences, international collaboration and through education (i.e. successful completion of Honours and PhD projects). Most notably, Dr D’Antignana's employment in an ongoing role of relevance to the Australian Southern Bluefin Tuna Industry Association (ASBTIA) and the aquaculture and fishing industry, ensures that important science/industry relationships are maintained.

Project contribution to the achievement of the outcomes in the Aquafin CRC Commonwealth Agreement:

An ability of producers to manipulate tuna quality to the requirements of the producer-to-market chain.

Experiments investigating the use of antioxidant vitamins have contributed significantly to the industry’s understanding of the “quality” of farmed SBT, the nature of oxidative stress and strategies that can reduce this type of stress in SBT production. Subsequently and consequently, the ability of producers to manipulate SBT quality characteristics to meet the requirements of the market is improved. This has come about by the combination of solid practical and

theoretical research into the action and use of antioxidant nutrients, gained through fish cell line research and practically based applied research with surrogates and SBT.

Tuna farmers capable of supplying a premium quality product

Investigations focused on the quality characteristics of fat and colour in concert with the concentrated effort on understanding the action and use of antioxidant nutrients have provided additional methods of quantifying these important product characteristics enabling manipulating of them throughout the production and cold chain process. Application of the results of this study is providing SBT producers greater capability to continue to supply a premium quality product to the competitive worldwide tuna market.

General Note:
Within this project's research portfolio independent studies were carried out by PhD candidates Janene Thompson and Alex Korte.

Janene Thompson's thesis (Investigating the enzymatic mechanisms of protection against oxidative stress in farmed Southern Bluefin tuna) has been reviewed and following acceptance of minor corrections will provide additional supporting information to this volume.

Alex Korte's thesis (Dietary and cellular antioxidants and antioxidant enzymes and their role in improving product quality and shelf life in Southern Bluefin tuna) is still to be submitted for examination and following review and acceptance, will provide additional supporting information to this volume.

NON TECHNICAL SUMMARY

Antioxidant supplemented pellet diets

Within this study the use of a high vitamin commercial pellets or a gel encapsulated pellet supplement has been found to be unsuccessful as a nutrient delivery system for SBT as a **supplement** diet with baitfish. When considering the results detailed here it should be noted that the benefits of a suitable weaning period in terms of improved pellet intake, for this study, are unknown as no opportunity to establish the pellet supplement as a recognised food with the SBT prior to the commencement of feeding baitfish was available. This result does in no way imply that pelleted diets are an unsuitable nutrient source for SBT. However while baitfish remain the primary source of nutrition for farmed SBT, supplement feeding with pellets is likely to continue to produce variable and somewhat unpredictable results.

Use of baitfish for feeding SBT

Within 12 weeks muscle vitamin E and C concentration of SBT fed on Australian sardines only (and predominantly fresh Australian sardines) was found higher than any of the other mixes of baitfish fed where frozen stored product was used Muscle oxidation parameters were also found to be lower in fish fed fresh Australian Sardines The use of frozen baitfish of variable chemical composition and the effect of unpredictable capture handling and storage conditions on the nutritional quality of the baitfish diet has been shown to have an effect on SBT flesh quality parameters. The use of Australian sardines as a fresh feed source for SBT has been demonstrated to provide antioxidant vitamins that result in improved product colour shelf life when compared to diet containing predominantly frozen stored baitfish. However the low fat level of Australian sardines means that other imported species of baitfish (all of which are stored frozen) are required to achieve the required SBT fat accretion within the farming season. Evidence from this research and other projects, (FRDC Final report (FRDC Project No. 2004/211 & FRDC Project No. 2000/221.20, SBT Aquaculture Subprogram) within the Aquafin CRC indicate that frozen stored baitfish are low in antioxidant vitamins (E and C) after short periods of frozen storage. Based on these results provision of supplementary antioxidant vitamins to “farmed” SBT is recommended.

Baitfish Liquid Vitamin Premix (LVP) injection

Baitfish Liquid Vitamin Premix (LVP) injection has been developed and shown that meat marination technology can be used to inject dietary supplements into baitfish fed to SBT. Specifically the technology is capable of delivering a prescribed dose of ascorbic acid (AA) and α-tocopherol acetate (α-toc ace) directly into baitfish, producing injected baitfish with added vitamins that can be fed either fresh or as frozen blocks. Preliminary research has shown that SBT fed LVP baitfish have substantially higher levels of the added nutrients than SBT fed a standard baitfish diet. This technology offers a practical method of delivering supplements to SBT which was previously unavailable using the predominant diet, baitfish. It is expected that this technology will be used to improve the health, growth and flesh quality characteristics of farmed SBT.

Diet trial with the surrogate yellowtail kingfish

These studies showed that vitamin E added to the diet in a synthetic form was able to be taken up by the surrogate fish. Although this resulted in treatment differences for flesh vitamin E levels there was no measurable effect on oxidation indicators or post mortem quality indicators (chemical or sensory).

By the use of surrogate experiments we were also able to show that there is some interaction between the nutrient components previously adding to the diets of SBT (i.e. vitamin E, C and selenium). Although the evidence does not directly indicate a synergistic effect on the in-vivo activity of these nutrients on oxidative processes there is evident that additions of more than one of the components can influence the tissue level of another. It was also shown that there were production improvements (growth and lower deaths) in surrogates that received

additional antioxidant vitamins. This is relevant information for the SBT industry and should be further explored. Should juvenile (fingerling to 1kg) SBT become available they would be a much more appropriate surrogate for this type of investigation and may give results more directly relevant to the current SBT ranching operation.

Development of a SBT cell line and its use to screen dietary antioxidants to improve fish flesh quality

A broad range of antioxidants have been screened using the developed cell line. Two commercially available products, NatuRose® (a carotenoid extract from *Haematococcus* microalgae) and Vin*life*® (a grape seed extract) were both identified as very effective antioxidants in the *in vitro* system. The use of these products might be appropriate for incorporation with the developed baitfish injection system.

KEYWORDS

Southern bluefin tuna, yellowtail kingfish, oxidation, antioxidant, vitamin, muscle colour, shelf life, quality, sashimi, diet.

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1.0 GENERAL INTRODUCTION

1.1 BACKGROUND

Oxidative stress is a major cause of poor flesh quality and reduced shelf life in fish. The main symptom is the peroxidation of cellular lipids to form lipid hydroperoxides, which degrade to form rancid odour and flavour compounds and initiate further lipid peroxidation. Fish, both wild and farmed, are especially susceptible to these processes because of a high proportion of polyunsaturated fatty acids (PUFA). In farmed fish, the problem is potentially worse, (1) because farmed fish may be accidently fed rancid baitfish containing high levels of lipid hydroperoxides, and (2) because the aim in fish farming, especially in the farming of southern bluefin tuna (SBT), is to produce fish high in lipids (up to 40% lipid in the otoro or belly flap of the fish). This high lipid level is achieved by feeding baitfish that is also high in lipids, thus compounding the risk of oxidative stress.

Fortunately fish, like other animals, have mechanisms to protect themselves against oxidative stress. These mechanisms involve dietary antioxidants (e.g. vitamin E, vitamin C, selenium and carotenoids like astaxanthin and canthaxanthin), cellular antioxidants (e.g. glutathione and thioredoxin) and antioxidant enzymes (e.g. superoxide dismutase, catalase, glutathione peroxidase and thioredoxin peroxidase). Little is known about these mechanisms in SBT. However, results we obtained from field trials under semi-commercial conditions in 2002 suggest that vitamin supplementation of the diet of SBT increases colour shelf life and stabilises the activity of glutathione peroxidase (one of the key antioxidant enzymes) during storage of muscle samples post-mortem (Thompson *et al*. 2006). This suggests that dietary antioxidants may spare cellular antioxidants which in turn may stabilise the antioxidant enzymes and this may explain the improved colour shelf life. Thus, dietary vitamin and mineral supplements have the potential to improve product quality directly and also indirectly by interacting with the antioxidant enzymes (Buchanan and Thomas, 2008).

Ideally it would be desirable to expand field trials with SBT to test (1) various concentrations and combinations of dietary antioxidants to determine which are the most effective, and (2) the capacity of dietary antioxidants to augment the protection provided by the cellular antioxidants and the antioxidant enzymes. However, field trials of this nature with SBT are expensive and logistically difficult. Thus, we have developed an *in vitro* system using SBT cell culture. With the cell culture system, it has been possible to (1) to investigate the interactions between the various protective mechanisms, i.e., the dietary antioxidants, the cellular antioxidants and the antioxidant enzymes, and (2) to determine the most effective concentrations and combinations of the dietary antioxidants.

The advantages of the in vitro cell culture system are (1) rapid screening of a large number of different concentrations and combinations of antioxidants in a short period of time, at minimal cost compared with the cost of experiments with live SBT, (2) the potential to identify the most effective concentrations and

combinations of antioxidants to be used as dietary supplements in SBT aquaculture, (3) a better understanding of the mechanisms underlying the impact of oxidative stress on tuna flesh quality and shelf life, and (4) basic scientific information to support a targeted approach to the development of dietary supplements to extend shelf life in SBT.

In parallel with the in vitro cell culture experiments we carried out two in vivo experiments with Yellowtail kingfish (YTK) as a surrogate species for SBT. The first experiment was to determine what level of lipid oxidation in either baitfish or specially-formulated pellets is likely to cause problems in terms of flesh quality and/or shelf life. The results we obtain for YTK will give an indication of what to expect for SBT. In order to quantify the effects of the oxidised feeds we used the TBARS (thiobarbituric acid reactive substances) assay to determine the malondialdehyde concentration in the flesh of the YTK. (Note: that malondialdehyde is one of the first stable products of lipid peroxidation and is a commonly used indicator of oxidative stress). Simultaneously, with determining the malondialdehyde concentration, we carried out flesh quality analyses such as colour shelf life on YTK fillets stored under market conditions.

In combination with in vitro experiments with the SBT cell cultures, we will test the most promising concentrations and combinations of dietary antioxidants on YTK. With YTK fed either baitfish or pellets containing various levels of oxidised lipids, we have examined how effective the various antioxidants are at protecting YTK against the oxidative stress caused by the oxidised lipids in the feed. In this way we will have a shortlist of the most effective antioxidants which we can then test on farmed SBT. Our work provides baseline information on acceptable levels of lipid oxidation in baitfish.

Within the current research being carried out with the SBT farming industry it was clearly identified that the development of a supplement feed for SBT that has the capacity to deliver specific nutrients to the fish, at any time during the production cycle, is a high priority (Project 2.2 Product Quality 2001/248; 2004/209 and Project A1.4. Nutrition 2001/249).

Baitfish Injection (vitamin supplement adoption commercialisation)
The current tuna Product Quality Project (specifically 2004/209) undertook research assessing supplement diets that involved providing specific nutrients to SBT by: (a) manipulating the baitfish species combinations in SBT diets; (b) coating baitfish diets with vitamin liquids and or powders; and (c) using pelleted diets as a supplement fed as a small percentage of the total ration. These nutrient delivery systems were found to be able to deliver specific nutrients to SBT; however they all have associated problems: (a), baitfish combinations are limited in the range of vitamin concentrations they can deliver, (b) coating systems are inherently inefficient as much of the added nutrient is lost prior to the fish consuming the diet and (c) the supplement pellet diet required a

considerable weaning effort and even following this, the pellet intake has not been high enough to effectively deliver added vitamins to the SBT.

More recently and on the back of some preliminary research several years ago (Steven Clarke pers. comm.) some promise has been seen in the development of a pellet encapsulation system based on fish gelatin or gelatin-like polysaccharide (available through Skretting Australia) but this system also had problems that precluded it from being immediately used for delivering supplement nutrients to SBT. However, independent of the ongoing pellet or nutrient encapsulation research, discussions with tuna farm operators revealed that there was renewed interest in the possibility of injecting a baitfish diet with specific nutrients. During attendance at recent conferences (Alltech, Kentucky USA and World Aquaculture, Florence Italy) it was noted that there have been significant advances made in the development of water soluble vitamins. Nutritional product providers confirmed that water soluble Vitamin A, B1, E and C are currently available.

On contacting the food technology company Convenience Food Systems (CFS) it was noted that the specifications of “off the shelf” meat injection systems appeared to have an immediately transferable application to the injection of baitfish feeds. On realizing that there was potential to match the needs of tuna farming production with goods and services available in the market, further consultation with tuna farming companies was carried out in order to obtain support and a commitment of in-kind contribution to the research required for this farming production problem.

In tandem with this consultation a baitfish injection pilot trial was carried out at the Department of Primary Industries Meat Research facility at Werribee in Victoria. This trial involved injecting fresh Australian sardines (*Sardinops neopilchardus*) and two frozen baitfish species, Atlantic herring (*Clupea harengus*) and sanma (*Cololabis saira*) with soluble vitamin E at 5% of the weight of the fish in order to achieve a target feed concentration of 300mg.kg^{-1} vitamin E.

The results of this trial indicated that, by using standard injection equipment and default settings, the baitfish can retain 5% soluble vitamin E (by weight) with concentrations of ~300mg.kg^{-1} at a throughput of up to 2 tonne baitfish / 1.5 hours. This result was encouraging and in combination with the enthusiasm of the interested SBT farming companies, Convenience Food Systems and DSM Nutritional Products, provided the impetus for an extension to this project 2004/209.

1.2 NEED

This research aimed to improve the product quality (the colour and flavour shelf life) of farmed SBT, which is perceived by the Japanese market to be shorter-lasting than wild tuna. The main aim in farming SBT is to produce a product that is high in lipid, and this is achieved by feeding baitfish that is itself high in lipid. The lipid in cold stored baitfish may be rancid and if fed to SBT may promote accelerated rancidity in the farmed SBT product. Improving the shelf life of farmed SBT provides an advantage for the Australian product, which is a high fat product, by extending the post mortem window of sale (i.e. shelf life).

Previous experiments on the Tuna Research Farm by Thomas and Buchanan and others, (Thomas, 2007, Project No. 2001/248) have demonstrated that:

- feeding SBT pellets fortified with boosted levels of vitamin E, C and selenium will raise the level of these natural anti-oxidants in the fish muscle and the appropriate colour of sashimi grade tuna flesh is retained for longer.
- pellet feeds with higher levels of vitamin E, C, and selenium were more effective in extending the shelf life of SBT flesh than those with a higher level of vitamin E alone.
- baitfish coated with vitamin E, C and selenium are effective in raising the fish tissue level of vitamin C but less effective at raising the tissue level of vitamin E and selenium.
- SBT with a higher tissue level of vitamin C has a slightly extended shelf life compared to those not fed fortified baitfish.
- a combination of higher levels of vitamin E, C, and selenium increases colour shelf life and stabilises the muscle activity of glutathione peroxidase (one of the key antioxidant enzymes).

There was now a need to improve the shelf life of farmed SBT, which could be achieved by (1) gaining better understand oxidative protection systems in SBT and (2) adapting our field experiment results and diagnostic tools to the commercial environment.

This was achieved by:

- developing an alternative method of anti-oxidant delivery which can be used within the existing commercial feeding system.
- use of in vitro cell culture techniques to gain a better understanding of oxidative protection system in SBT and as a rapid and cost-effective method of assessing vitamin supplement formulations that have the potential to improve flesh quality and extend shelf life.
- the use of surrogate experiments to obtain basic information on the level of oxidative products that require specific antioxidant feed supplementation use and as an intermediate step in the application of *in vitro* findings to commercial SBT farming.

This research provides valuable information on the effects of oxidized feed on flesh quality and also baseline information on the oxidative status (i.e. degree of rancidity) of farmed SBT fed currently available feeds.

The research was focused on product quality and continued our investigations into the physiology, nutrition and muscle biochemistry of SBT that underpins developments in tuna husbandry. This project utilised project 2001/248 results including the adopted PhD student's research. The present project recruited a PhD student and supported collaborative ties between Flinders University (Adelaide), CSIRO (Geelong), SARDI and the South Australian SBT industry.
This research addressed the South Australian Fisheries and Aquaculture Research and Development Strategy 2002-2007 in the following areas:
Program 2 Industry Production –
Post Harvest enhancement/supply chain management,
Farm husbandry and the nutrition of farmed species.

Baitfish Injection (vitamin supplement commercialisation)
Results of the two projects on dietary supplementation (2001/248 & 2004/209) established that there were losses in vitamin content of stored baitfish. Baitfish quality projects (2000/221.20 and 2004/211) further quantified this finding and gave direction for the handling procedures that reduce losses. The accumulated information in this area indicates there is an immediate need to provide the SBT industry with a means to provide farmed SBT with additional micro nutrients that employs a reliable and repeatable delivery method.

We have demonstrated (2001/248 & 2004/209) that there are benefits in feeding additional vitamins and selenium to SBT in that total antioxidant status of the fish and product shelf life is improved. In addition, trimming losses on portioned meat cuts, due to surface oxidation, are reduced. We have determined that the best delivery system for added nutrients is a pellet, however farmed SBT are currently fed baitfish diets and it has proved very difficult to achieve suitable intake of a vitamin-fortified pellet that can be fed in combination with the standard baitfish ration.

By tapping in to the meat industry injection technology researchers, working with industry, have been able to create a fortified baitfish diet that provides additional required nutrients, and is readily accepted by the fish, for as long as they continue to feed. This has provided farmers with a method of delivering nutrients to their fish at specific times during the growing season, so that they might obtain maximum effect and cost benefit from the nutrients delivered by the diet.

As the diet injected was baitfish, the SBT can achieve the maximum growth (fat accretion rate) currently possible and also have the benefit of the improved quality characteristics that result from additional antioxidants - as demonstrated through the results of research within projects 2001/248 & 2004/209.

In addition by injecting baitfish with vitamins that act as antioxidants, it was determined that baitfish deterioration during storage (2000/221.20 and 2004/211) was reduced thus providing a better quality stored feed for SBT.

There was also an expectation from industry that through application of this research SBT production would increase through improved growth and reduced

mortality. This possibility remains to be explored and in the absence opportunity for more rigorous experimental design than is possible within the commercial farming environment, will take some time determine.

1.3 OBJECTIVES

1. Adapt existing assays for oxidative stress from southern bluefin tuna (SBT) muscle extracts and mammalian cell cultures to SBT cultures.
2. Develop a supplement, delivered by a manufactured pellet, which can be fed to tuna that are being fed predominately on baitfish on a commercial farm.
3. Investigate the effects of simulated oxidative stress on SBT cell cultures and identify the most effective concentrations and combinations of antioxidants that combat this stress.
4. Test the effectiveness on farm vitamin supplement delivery systems in raising tissue level of antioxidants and increasing quality characteristic and shelf life.
5. For the purpose of SBT surrogate experiments, manipulate and control the level of lipid oxidation in commercial fish feeds.
6. Compare the flesh quality of SBT fed high vitamin supplement to SBT raised using existing industry standard farming practice.
7. Use a surrogate for SBT to assess the effects of oxidized feeds on flesh quality and shelf life.
8. Measure endogenous antioxidant vitamin levels in baitfish typically used in the tuna industry.
9. Use a surrogate fed oxidised feeds to test the protective effects of the antioxidants identified in cell culture experiments of the manufactured pellet.

1.4 STRUCTURE OF THE REPORT

This report is written as a series of individual chapters. Where contributions from PhD candidate investigations are appropriate, results of their investigations have been included in the relevant chapters. The information provided here highlights the potential and opportunities for the manipulation of product quality through the modulation of SBT dietary supplements. The results of the baitfish injection work (extension project 2004/209) will be reported separately as a supplementary report. The Non Technical Summary and Outcomes have been included in this volume.

1.5 REFERENCES

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Thompson, J.L., Thomas, P.M. and Schuller K.A. (2006), 'Purification and properties of a glutathione peroxidase from southern bluefin tuna (*Thunnus maccoyii*) liver', Comparative Biochemistry and Physiology Part B, Biochemistry and Molecular Biology, 144(1): 86-93.

2.0 Mixed energy baitfish diet: use and effects on SBT flesh quality characteristics

2.1 2005: Effects of varying the fat and protein level of commercial baitfish diets on the chemical composition and quality characteristics of farmed southern bluefin tuna (*Thunnus maccoyii*) flesh.

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2.1.1 Introduction

It has previously been established by the Tuna Quality Project group that the addition of vitamin E and C as antioxidants to southern bluefin tuna (SBT) diets (pellet and baitfish) will raise the muscle concentration of these antioxidants and subsequently result in significant improvements in the colour shelf life of fresh and frozen sashimi. These findings are significant because farmed SBT have been noted as having a short colour shelf life relative to wild caught tuna species, and providing a dietary supplement of antioxidants has been shown to increase colour shelf life. We have established that the most efficient way to increase the vitamin E and C concentration of farmed SBT muscle is to use pellets fortified with *dl*-α-tocopherol acetate and *l*-ascorbic acid monophosphate. Although pelleted feed development has progressed to the point of providing an alternative diet, from an economic perspective the exclusive use of pelleted feeds for SBT farming is currently not a viable proposition. At this point in time, the use of Australian sardines as feed for SBT is seen as the major opportunity to improve the cost-efficiency of production, provided that performance and product quality characteristics are maintained.

The present research was carried out in order to provide a direct comparison between farmed SBT fed a diet of Australian sardines (*Sardinops neopilchardus*) only, with SBT fed on baitfish mixes that had been combined, following a specific design (van-Barneveld *et al*. 2009) to vary the total fat and protein delivered to the fish.

2.1.2 Methods

SBT were purse-seined by commercial operators in the Great Australian Bight in March 2005. From 5 - 10 April 2005 approximately 200 SBT were transferred to each of four 32m Ø cages on the DI Fishing Tuna Farm in Boston Bay, Port Lincoln, South Australia. These SBT were fed diets with various fat and protein levels (Table 2.1), for 6-week periods in a predetermined order (Table 2.2) throughout an 18-week experiment. All tuna were fed to satiation twice daily, six days per week for 18 weeks.

Table 2.1. Sequence of 6 week baitfish feed periods over 18 weeks. Diets were High Protein / Low Fat (HP/LF), Medium Protein / Medium Fat (MP/MF), Low Protein / High Fat (LP/HF) and fresh Australian Sardines only (AS).

Cage Number	0-6wks	7-12wks	13-18wks
10	MP/MF	MP/MF	MP/MF
11	LP/HF	MP/MF	HP/LF
12	HP/LF	MP/MF	LP/HF
13	AS	AS	AS

Table 2.2. The estimated percentage protein and lipid content, and percentage species composition of each feed type, High Protein Low Fat (HP/LF) Medium Protein Medium Fat (MP/MF), Low Protein High Fat (LP/HF) and fresh Australian sardines only (AS).

Baitfish mix	Protein	Lipid	US sardine *Sardinops sagax*	Redbait *Emmelichthys nitidus nitidus*	Australian sardine *Sardinops neopilchardus*
HP/LF	19.5	4.0	15	30	55
MP/MF	18.5	7.3	40	35	25
LP/HF	17.5	10.5	65	35	0
AS	20.4	~2.0	0	0	100

Fish Sampling

On 5 April prior to the commencement of experimental diets, 10 SBT were hook harvested from cage 10 to collect baseline data. At the end of each 6 week feed period, ten fish from each experimental cage were net harvested, then immediately killed following standard industry practice. Weight and length measurement were taken and a muscle sample was excised from each fish using a 17mm Ø stainless steel coring tool inserted into the bleed wound. The muscle samples were promptly stored on ice until they were processed for analysis upon return to the shore-based laboratory later on the same day. Condition Index (CI) of the fish was calculated on whole fish wet weight (gilled and gutted weight / 0.87) as:

$$CI = \frac{Weight(kg)}{Length(m)^3}$$

Analytical Procedures

Muscle collected from each fish was chopped and mixed to a fine mince. Sub-samples of the homogenate were immediately stored at 4^0C for colour and aged

TBARS assessment and at - 80^0C for fresh TBARS and micronutrient analysis. The vitamin E (α-tocopherol) concentration was determined by a HPLC method based on the method of Huo *et al.* (1999). Vitamin C was determined by a technique based on the HPLC fluorescence detection method of Brown and Miller (1992) at the laboratory at the Lincoln Marine Science Centre. For the measurement of TBARS approximately 1g of tissue was homogenized by mortar and pestle and then added to 5ml of 0.6M perchloric acid for an extraction period of 1 hour. 500 µl of the resulting supernatant was added to 500µl of thiobarbituric acid in screw-top test tubes. The tubes were placed in a water bath at 100°C for 35 minutes to generate a colour reaction. The sample solution was then read against a blank in a spectrophotometer at 540nm. The concentration of TBARS (mg.kg-^{1} tissue ww) was determined against a tetraethoxypropane standard curve.

Selenium level was determined using a method based on the fluorimetric technique of Watkinson (1966) modified as per Paynter *et al.* (1993). Colour shelf life was measured on the day of harvest and then every 24 hr for up to 5 days (samples were then retained until 8 days post harvest for aged TBARS analysis). Each fresh muscle sample was placed in duplicate small plastic wells (1x colour, 1 x TBARS) and blindly assigned to an individually numbered white tile, covered with clear plastic cling wrap and stored at 4°C. At each measurement time a panel of 5 people gave a subjective colour rating to each sample using a predetermined grading system ranging from red to brown, using scores between 0 and 6 (Table 2.3).

Table 2.3. Definition of scores given every 24 hours to describe visual colour changes in SBT muscle stored at 4^0C for up to 5 days post harvest.

Score	Colour change description
0	Fresh red meat - pre “bloomed”.
1	Bright red meat - “bloomed” (brighter red than fresh red).
2	Change in red intensity i.e. a dulling of the bloomed meat.
3	Either a significant darkening of the meat or margins beginning to brown.
4	Either a significant darkening of the meat i.e. heading towards red/black or brownness spreading from the margins/brown “marbling”.
5	Either a blackening of the meat or an overall browning of the meat but, for either, still with a pink/red undertone.
6	Either a black sample or a brown/green sample; no red/pink undertone visible.

At the same time an objective colour measurement was taken from the surface (cling wrap temporarily removed) of the centre of each sample using a Minolta colour meter. CIE a* and b* values for the angle of hue and chroma were calculated using the following equations:

$$Hue = \arctan(b^*/a^*)$$

$$a^* > 0 b > 0^*$$

and

$$Chroma = \sqrt{(a^{*2} + b^{*2})}$$

The hue angle is a descriptor of what is generally understood to be the true colour, and the chroma is the intensity or degree of saturation of the colour.

Statistics

Analysis of variance (ANOVA) was performed using computer package Sigma Stat, version 3.1. Significant differences between treatments were determined by Tukey – Kramer HSD. Where necessary data were log transformed to satisfy normality and homogeneity of variance requirements. Unless otherwise stated a significance level of $\alpha < 0.05$ was used for all statistical tests.

2.1.3 Results and Discussion

The CI of the fish sampled at transfer to the farming cages was 17.7 $\pm$ 0.4. Over the treatment period there was an increase in CI of the sampled SBT groups, however there were no differences in CI between any of the fish groups at any of the harvest times (Table 2.4).

Table 2.4. Condition index of SBT harvested at 6, 12 and 18 weeks after being fed mixed baitfish diets or AS only (Table 2). Values are mean $\pm$ SE (n = 10). Absence of superscripts = no significant differences $P > 0.5$

Cage	Diet sequence (6 week intervals)	0-6wks	7-12wks	13-18wks
10	MPMF / MPMF / MPMF	22.2 ± 0.9	26.3 ± 1.2	25.4 ± 0.5
11	LPHF / MPMF / HPLF	21.9 ± 0.6	25.4 ± 0.6	25.1 ± 0.4
12	HPLF / MPMF / LPHF	20.6 ± 0.8	24.4 ± 0.5	24.8 ± 0.5
13	AS / AS / AS	20.3 ± 0.9	24.4 ± 0.4	24.8 ± 0.4

The mean muscle vitamin E concentration of the SBT at the time of transfer was 5.1 $mg.kg^{-1}$ (Figure 2.1). Following 6 weeks of feeding the muscle vitamin E concentration of all the groups was either the same or lower than the level at transfer. Following 12 and 18 weeks of feeding, the muscle vitamin E concentration of the AS group was significantly higher than all the other mixed baitfish fed groups which were all less than 4 $mg.kg^{-1}$. Following 18 weeks of feeding the AS group had a muscle vitamin E concentration of 8.2 $mg.kg^{-1}$.

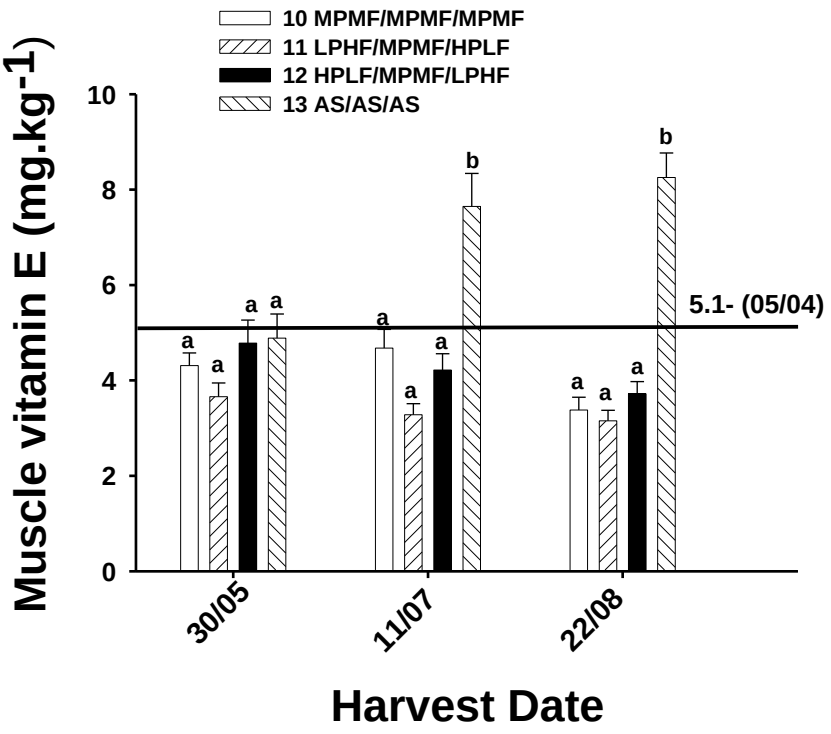

Figure 2.1. Muscle vitamin E concentration of SBT fed different baitfish diet combinations during the 18-week treatment period. Value at transfer (not included in comparisons) indicated by bold horizontal line. Diets were High Protein Low Fat (HP/LF), Medium Protein Medium Fat (MP/MF), Low Protein High Fat (LP/HF) and Fresh Australian sardines Only (AS). Values, mean ± SE (n=10) at each harvest not significantly different (P>0.05) to one another share superscript letters. Diet composition changed following 6 weeks of feeding except for groups in cages 10 & 13 (Table 2.1).

The muscle concentration of vitamin C showed a similar pattern to that described for vitamin E (Figure 2.2) in that the vitamin C concentration of the muscle of the AS group was significantly higher than all the other groups following 12 and 18 weeks of feeding. However, the muscle vitamin C concentration of the fish at the time of transfer to the farming pontoons was very low at 2.1 $mg.kg^{-1}$. This low level may have been due to stress and or low feed intake associated with capture and transfer of the fish to the farming pontoons. Following six weeks of feeding the muscle vitamin C concentration of all the treatment groups was well above the level measured at transfer and remained that way up to and including the harvest at 18 weeks.

There appeared to be a slight drop in the muscle vitamin C concentration of all the treatment groups between the 12 and 18 week harvests which may have been due to a slowing of the feed intake that is generally associated with the end of the farming season and lower water temperature. Previous research by this project group has demonstrated that if the dietary intake of vitamin C is reduced the vitamin C concentration of the SBT muscle will drop quite rapidly. In the current research the vitamin C dose would automatically be reduced with the lower feed intake associated with the seasonal decline in feed intake.

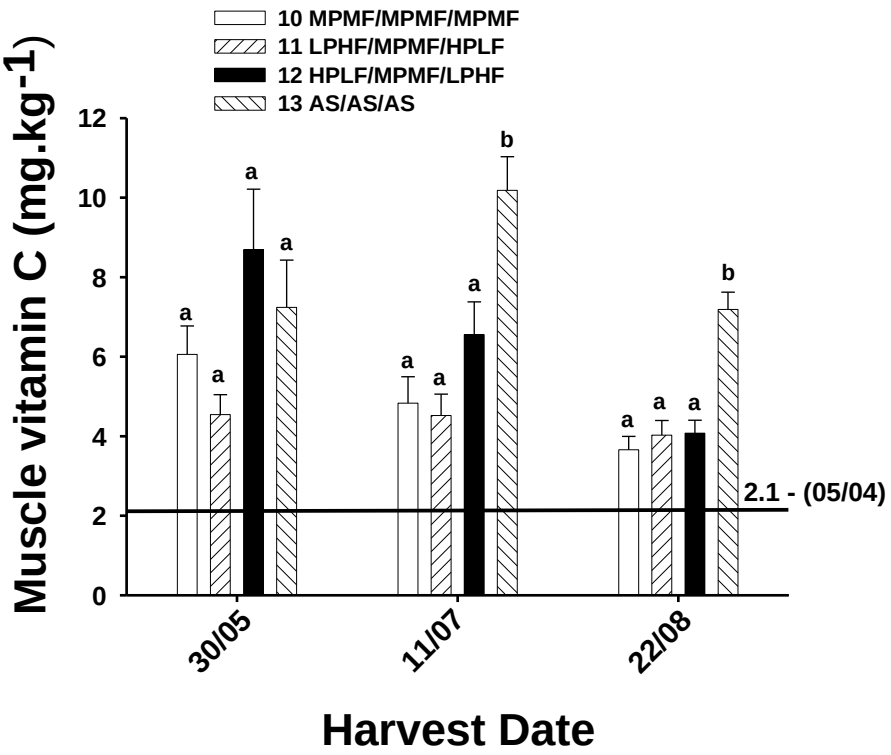

Figure 2.2. Muscle vitamin C concentration of SBT fed different baitfish diet combinations during the 18 week treatment period. Value at transfer (not included in comparisons) indicated by bold horizontal line. Diet composition changed for each group following 6 weeks of feeding except for groups in cage 10 & 13 (Table 2.1). Values, mean ± SE (n=10) at each harvest not significantly different (P>0.05) to one another share superscript letters.

The muscle selenium concentration of the fish at transfer was 0.82 mg.kg^{-1}. There was little change in the muscle selenium concentration of the fish groups over the treatment period, however there was a slight trend indicating a reduction in muscle selenium concentration of all groups over time (Fig.2.3). This trend has been observed in previous farming seasons in SBT fed on pilchard diets without supplementation; however the pattern is not consistent from year to year. At the last harvest for this experiment (22/08/2005), the post mortem change in TBARS, of the SBT treatment groups were all low, however the fish fed the AS diet had a significantly lower muscle TBARS concentration than any of the other diet groups, which were all similar to one another (Fig.2.4c).

The muscle TBARS concentrations of all groups increased during the 8 days of cold storage. Following 4 days of cold storage there were no differences between muscle TBARS concentration of any of the diet groups. Following 8 days of storage the AS diet group had a significantly lower muscle TBARS concentration than the mixed baitfish fed groups that had the high fat baitfish diet included during the treatment period. This pattern of change in muscle TBARS concentration was similar at each harvest during the treatment period with the AS group tending to have a lower muscle TBARS concentration than those groups

that had the high fat baitfish included in their diet during the treatment period (Figs. 2.4a – 2.4c).

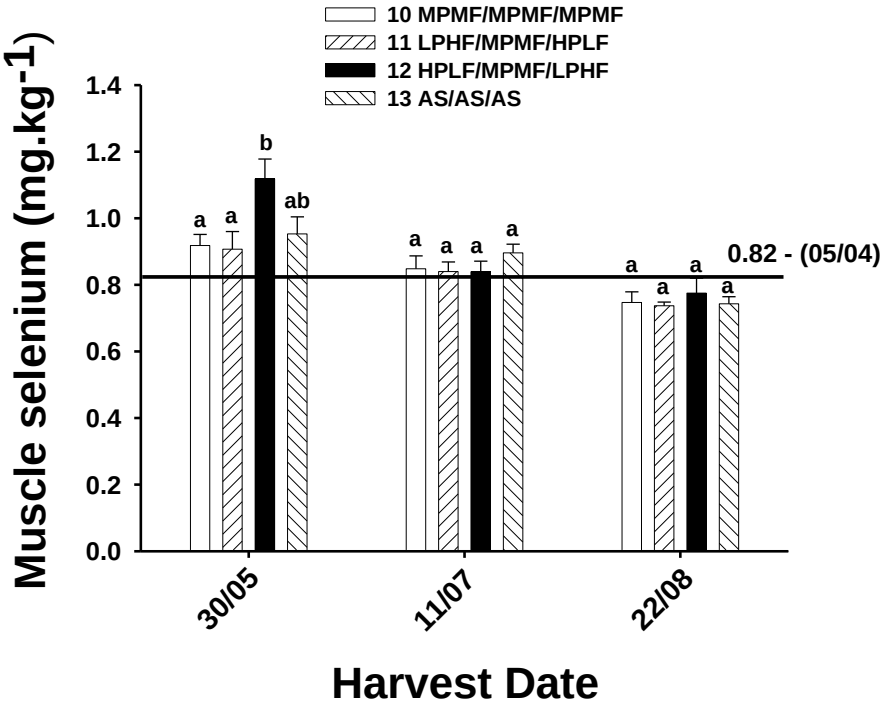

Figure 2.3. Muscle selenium concentration of SBT fed different baitfish diet combinations during the 18 week treatment period. Value at transfer (not included in comparisons) indicated by bold horizontal line. Diet composition changed for each group following 6 weeks of feeding except for groups in cages 10 & 13 (Table 2.1). Values, mean ± SE (n=10) at each harvest not significantly different (P>0.05) to one another share superscript letters.

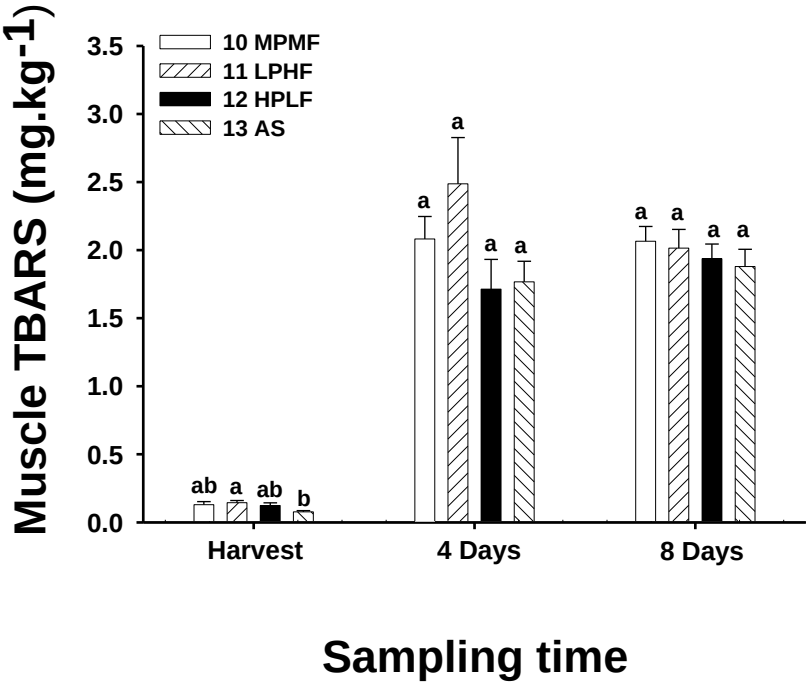

Figure 2.4a. Muscle TBARS concentration of SBT at the day of harvest (30/05/2005) and at 4 & 8 days post harvest following 6 weeks of mixed baitfish diets (Table 2.1). Values, mean ± SE (n=10) at each harvest not significantly different (P>0.05) to one another share superscript letters.

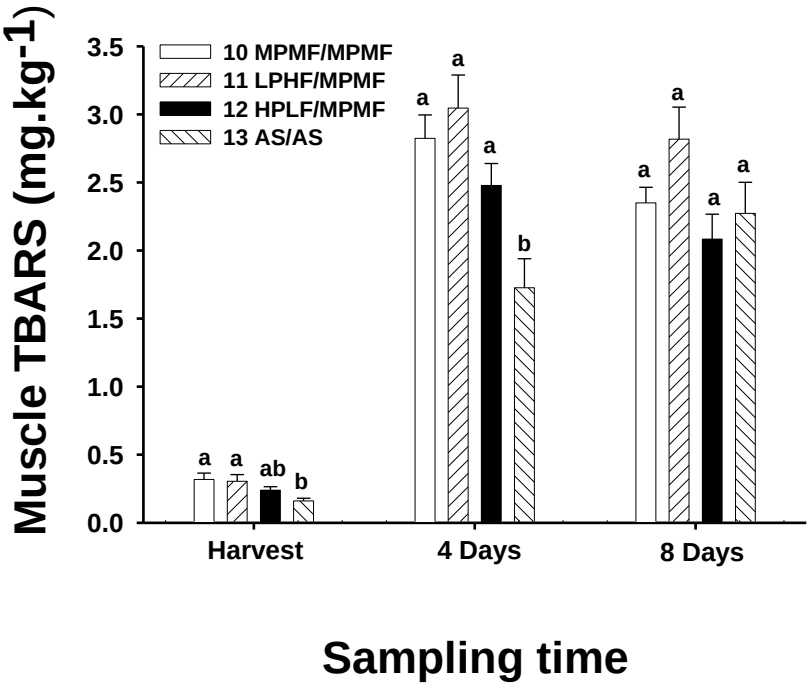

Figure 2.4b. Muscle TBARS concentration of SBT at the day of harvest (11/07/2005) and at 4 & 8 days post harvest. Baitfish diet composition changed for each group at 6 week intervals except for groups in cages 10 & 13 (Table 2.1). Values, mean $\pm$ SE (n=10) at each harvest not significantly different (P>0.05) to one another share superscript letters.

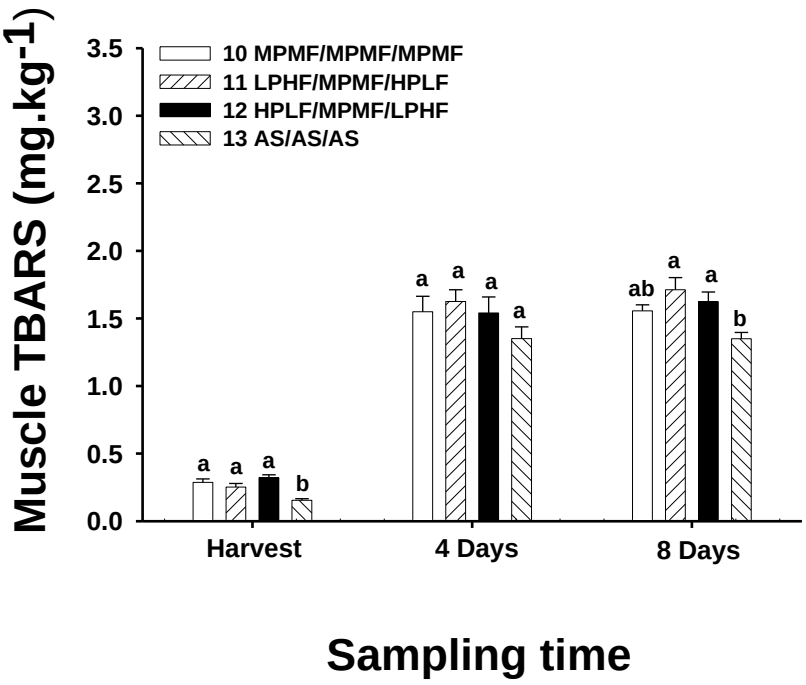

Figure 2.4c. Muscle TBARS concentration of SBT at the day of harvest (22/08/2005) and at 4 & 8 days post harvest. Baitfish diet composition changed for each group at 6 week intervals except for groups in cages 10 & 13 (Table 2.1). Values, mean ± SE (n=10) at each harvest not significantly different (P>0.05) to one another share superscript letters.

At each harvest the visually assessed colour shelf life consistently showed that the muscle of the AS diet group browned more slowly than those groups that had the high fat baitfish included in their diet during the treatment period. At the end of the first 6 week period on 30 May there were significantly higher frequencies of lower (redder) colour scores in the AS group than in the MPMF and LPHF groups on days 2 - 4 post harvest (Fig. 2.5a). The trend was more pronounced at the end of the second and final 6 week periods with significantly higher frequencies of lower colour scores in the AS muscle samples compared with all other groups from days 2 - 5 post harvest, 11 July (Fig. 2.5b) and days 1 – 4 post harvest, 22 August (Fig. 2.5c). Fewer colour differences were detected by colour meter but the results still supported the visual assessment trend.

After the 30 May harvest the AS group showed significantly lower (redder) muscle hue values and higher (more saturated) chroma values than the LPHF group on days 2 & 3 post harvest (Fig. 2.6a). Again the trend was more pronounced after the 11 July harvest with significantly lower hue values in the AS group compared with the MPMF/MPMF and LPHF/MPMF groups on day 2 and all other groups on days 3 & 4. The AS group chroma was higher than the MPMF/MPMF and LPHF/MPMF groups on days 2 & 3 (Fig. 2.6b). After the 22 August harvest, muscle hue in the AS/AS/AS group was significantly lower than the MPMF/MPMF/MPMF on days 1 & 2 post harvest. AS/AS/AS Chroma was significantly higher than all other groups on days 1 & 2 (Fig. 2.6c).

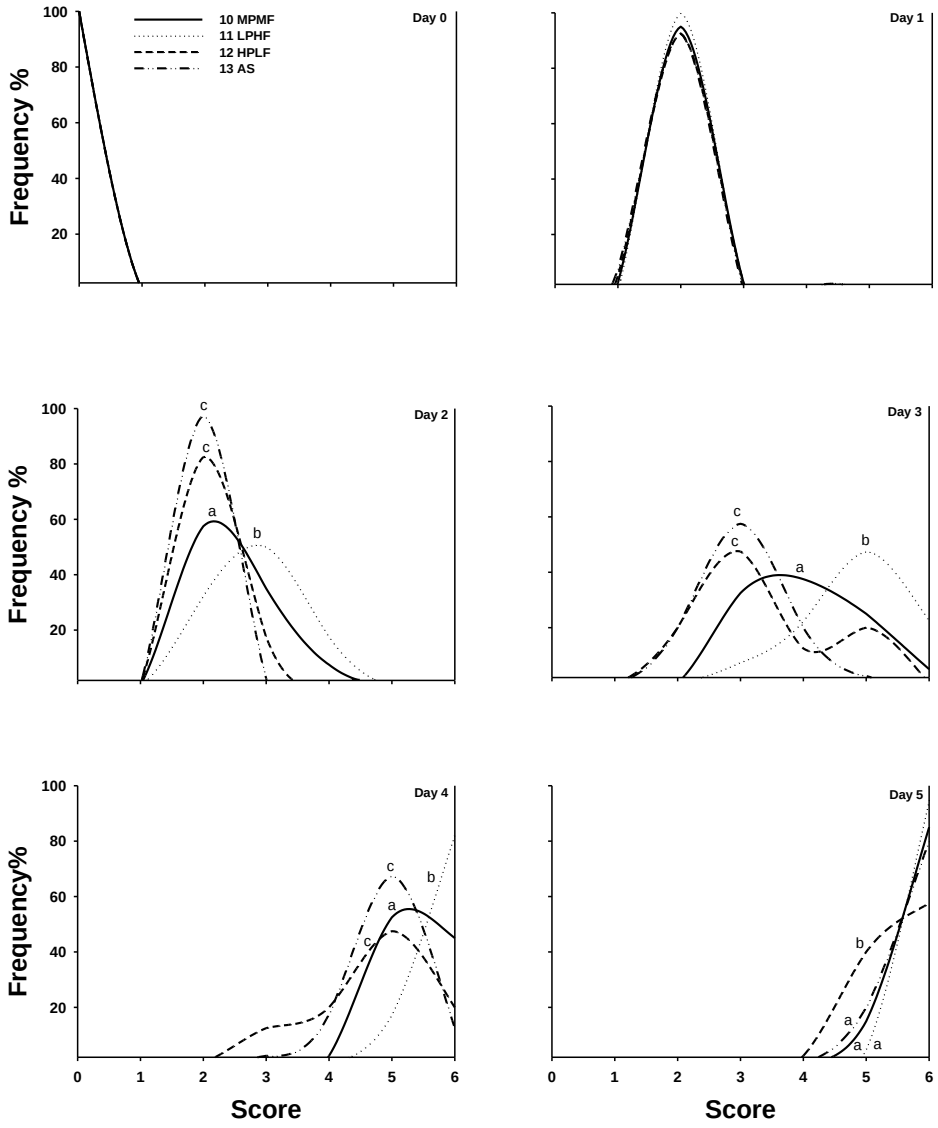

Figure 2.5a. SBT muscle mean (n=10) visual colour score distribution during 6 days of post harvest cold storage (Harvest: 30/05/2005) after exposure to diverse baitfish diets for the first 6 week experimental period (Table 2.1). Scores indicate colour change; 0 = red to 6 = brown. Scores that are not significantly different (P>0.05) to one another share superscript letters or have superscripts omitted.

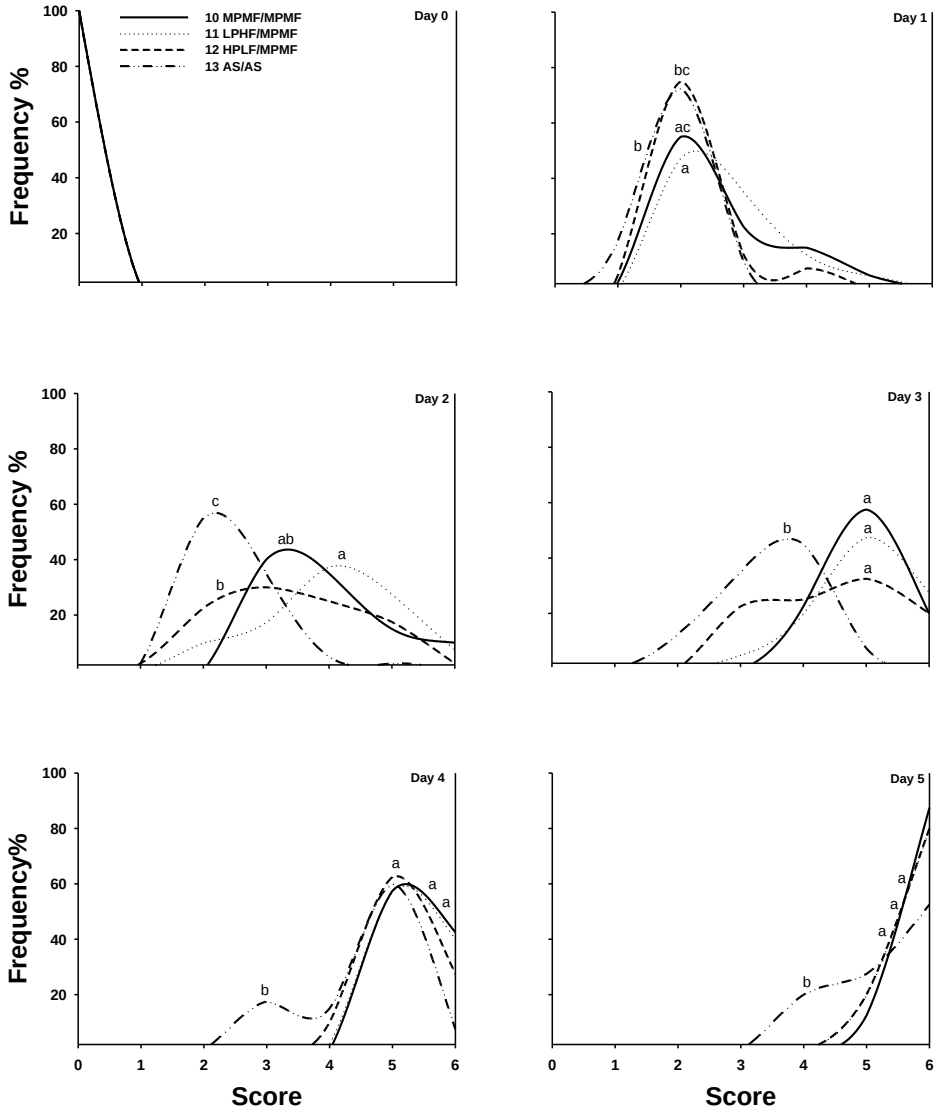

Figure 2.5b. SBT muscle mean (n=10) visual colour score distribution during 6 days of post harvest cold storage (Harvest: 11/07/2005) after exposure to diverse baitfish diets for the first and second 6 week experimental period (Table 2.1). Scores indicate colour change; 0 = red to 6 = brown. Scores that are not significantly different (P>0.05) to one another share superscript letters or have superscripts omitted.

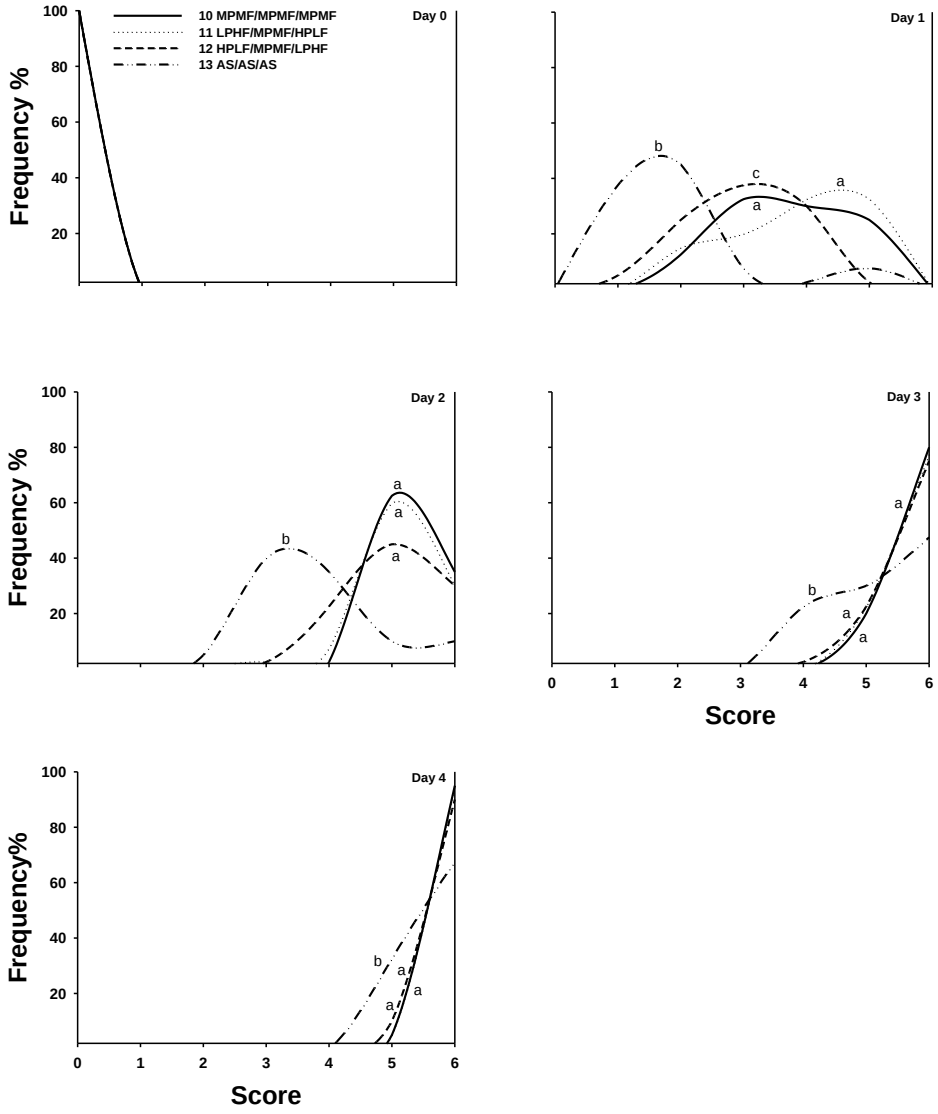

Figure 2.5c. SBT muscle mean (n=10) visual colour score distribution during 6 days of post harvest cold storage (Harvest: 22/08/2005) after exposure to diverse baitfish diets for the first, second and final 6 week experimental period (Table 2.1). Scores indicate colour change; 0 = red to 6 = brown. Scores that are not significantly different (P>0.05) to one another share superscript letters or have superscripts omitted.

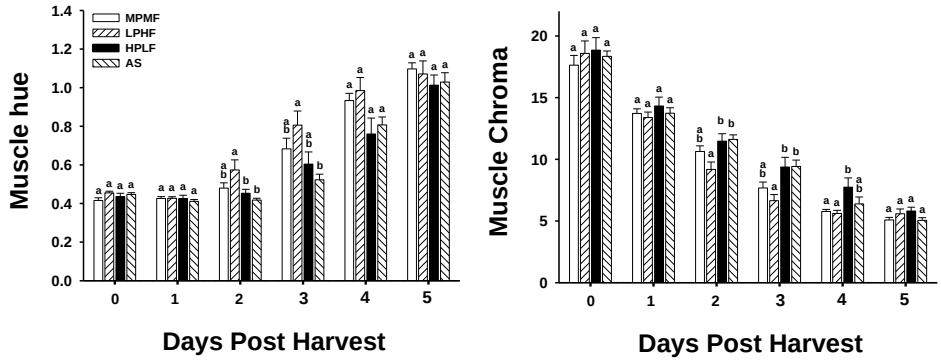

Figure 2.6a. SBT mean ± SE muscle hue and chroma values (n=10) during 6 days of post harvest cold storage (Harvest: 30/05/2005) after exposure to diverse baitfish diets for the first 6 week experimental period (Table 2.1). Values that are not significantly different (P>0.05) to one another share superscript letters.

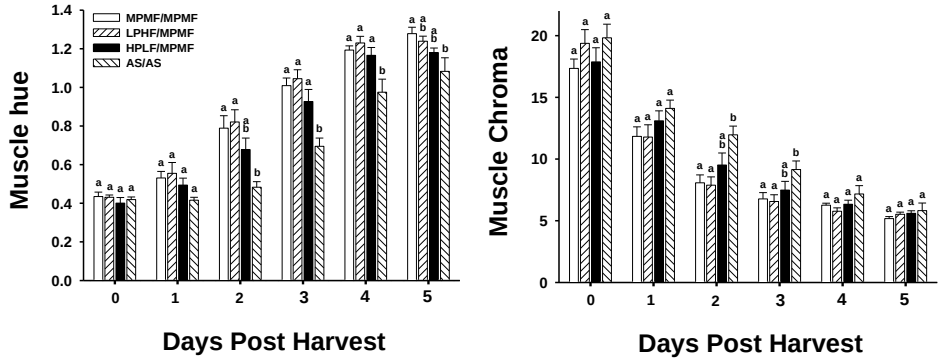

Figure 2.6b. SBT mean ± SE muscle hue and chroma values (n=10) during 6 days of post harvest cold storage (Harvest: 11/07/2005) after exposure to diverse baitfish diets for the first and second 6 week experimental period (Table 2.1). Scores that are not significantly different (P>0.05) to one another share superscript letters.

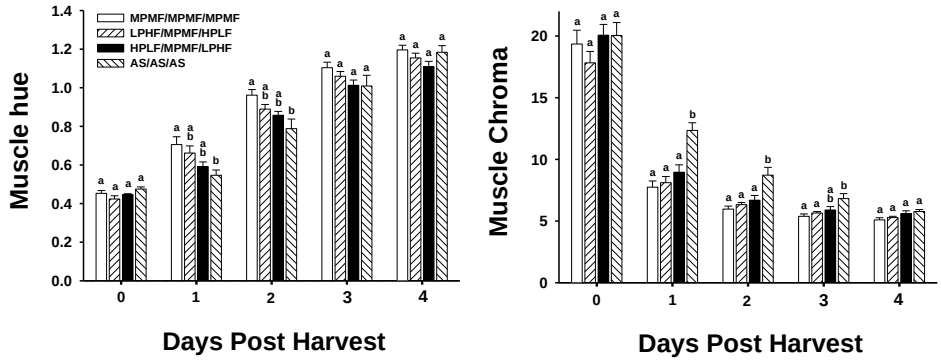

Figure 2.6c. SBT mean ± SE muscle hue and chroma values (n=10) during 5 days of post harvest cold storage (Harvest: 22/08/2005) after exposure to diverse baitfish diets for the first, second and final 6 week experimental period (Table 2.1). Scores that are not significantly different (P>0.05) to one another share superscript letters.

The results of this research indicate that, after 12 weeks of feeding, the muscle vitamin E and C concentration of SBT fed on Australian sardines only (and predominantly fresh Australian sardines) was higher than any of the other mixes of baitfish fed to the other groups. By the completion of 6 weeks of feeding there are indications that the colour shelf life is extended in the AS diet group compared to any other group and that this pattern continued throughout the treatment period. Also at each harvest time, the muscle TBARS concentration of the AS group tends to be lower at harvest and during 8 days cold storage than in SBT groups fed the other diets. These results should be interpreted with reference to the fish growth results of the nutrition group (van-Barneveld *et al*., 2009) and the results of the analysis of the composition and quality of the baitfish feeds fed during the experiment. The use of baitfish diets for the provision of nutrients for SBT will continue to result in unpredictable production and flesh quality. This is a result of the use of baitfish species and batches having variable chemical composition and the effect of unpredictable capture handling and storage conditions on the diet composition. Useful information that provides context for the results of the current report include Final reports (FRDC Project. 2000/221.20; 2000/221; 2000/221 and 2004/211).

2.1.4 Acknowledgements

The authors would like to acknowledge the contribution of DI Fishing Co Pty Ltd management and staff and the continued assistance and support of Research Manager, David Ellis, Aquaculture Management Consultants. We would also like to thank the management of Tony's Tuna International Pty Ltd for providing freezer space, without which this research could not have been completed.

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2.2 2005: Consumer testing of tuna sashimi from tuna fed three different mixed energy diets

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2.2.1 Introduction

A difference from control test was completed to establish whether or not a difference in odour, colour, texture and flavour could be perceived (at the 5% level of significance) in tuna sashimi from tuna fed local baitfish diet (Reference) and those fed mixed energy diets A, B or C. Thirty-five consumers (including 3 Japanese) recruited from the Port Lincoln area completed the difference from control test on 26 August 2005.

For all variables, there were no significant (P>0.05) differences between theSBT fed on local baitfish and those fed on mixed energy diets. The LSD's were similar to previous data on SBT sashimi so the measure of variability is similar. Therefore it would appear that SBT fed mixed energy diets are similar to those fed local baitfish in terms of their chutoro sashimi quality.

The same panel was also asked their opinion on the degree of liking or disliking for each of the SBT samples in terms of odour, colour, texture, flavour and overall. In terms of colour, flavour and overall liking there was a significant (P<0.05) difference in preference with tuna fed mixed energy diet C, which was the least preferred. There were no significant differences (P>0.05) in terms of odour and texture preference between the tuna samples.

2.2.2 Aims

- To establish whether or not a significant difference (P<0.05) in sensory attributes can be perceived in SBT sashimi from SBT fed three different mixed energy diets with those fed on a local baitfish diet.

- To establish whether significant differences (P<0.05) in odour, colour, flavour, texture and overall acceptability can be perceived in SBT sashimi from SBT fed on four different diets.

- To collect basic demographic data on the consumers assessing the SBT sashimi samples.

- To collect comments regarding the nature of any differences and degree of liking or disliking for each of the above products.

2.2.3 Methods

Consumers

Thirty-five consumers were recruited from the Port Lincoln area and included three local Japanese. It was assumed all were consumers of tuna sashimi, however one was allergic to seafood so did only the visual assessment.

Samples

The SBT sampled were fed three different mixed energy diets (A-C) and a control diet over an 18 week period (Table 2.5).

Table 2.5. Diet sequence and cage number of the tuna fed the mixed energy diets and the control. Diets were High Protein / Low Fat (HPLF), Medium Protein / Medium Fat (MPMF), Low Protein / High Fat (LPHF) and fresh Australian Sardines only (AS).

Mixed energy Diet	Diet sequence (6 week intervals)	Cage No.
A	MPMF/ MPMF / MPMF	10
B	LPHF/ MPMF/ HPLF	11
C	HPLF/ MPMF/ LPHF	12
Control	AS/ AS/ AS	13

Each test sample was given a treatment number and 3-digit blinding code (sample code) (Table 2.6).

Table 2.6. Description of sashimi test samples.

Mixed Energy Diet	Treatment No.	Sample Code
A	1	470
B	2	180
C	3	600
Control	4	545
Control	Reference	REF
C	Warm-up	768

The samples were prepared in Port Lincoln from fish harvested on 22 August 2005 and stored until Friday 26 August 2005. Four fish per treatment were “loined” and the chutoro section was chosen as the sample area.

Sample preparation and test procedure
Sashimi slices were prepared from the chutoro section of each fish and a fifth fish from the control group (treatment 4), was used for the reference sample. As the sashimi slices were large, they were cut down the middle so that more samples in the desired positions (see design below) for the reference sample were available to the panel. Samples were covered in cling-wrap and stored at 4°C until required for testing.

Two sessions were conducted at Port Lincoln TAFE on Friday 26 August 2005 at 11 am and 4 pm. Each consumer was given instructions on how to complete the assessments using the questionnaires provided (see Appendix 1). The test method used was a combination of the difference from control (reference) test and the rating test using hedonic scales.

Each consumer was given a reference sample that corresponded to the control group (treatment 4). For each test sample, consumers were asked to mark on a 15cm line scale what they thought was the size of the difference relative to the reference in terms of terms of odour, colour, texture and flavour. In addition they were asked to give their opinion on how much they liked or disliked each sample for odour, colour, texture, flavour and overall and to make comments on any of the samples. The reference sample was also presented as one of the test samples (treatment 4), as a blind control is required for the difference from control test. A warm-up sample was given to all consumers as their first sample and this was taken from a fish from treatment 3. Consumers were given rain-water and plain water crackers to cleanse their palate between samples. Sample size did vary among samples due to variations in the size of the fish, but this was minimised by using 2 slicers who trimmed and sliced the fish as consistently as possible.

Design and analysis
A tasting design was developed where each consumer assessed the warm-up sample first followed by four test samples in a specified order. Each consumer was given their sample slice from the same position in each fish but the sample order was randomised and balanced as much as possible. In session 1, the first 8 consumers (1A to 8A) assessed sashimi slices 1 to 8 from four fish from each treatment (treatment 1 to 4) and consumers 1B to 8B assessed slices 1 to 8 from another four fish from each treatment (treatment 1 to 4). As only one fish side was available for the reference sample, consumers 1A to 8A had the first 8 slices from the left hand side of the split sashimi slice, while slices from the right hand side were given to consumers 1B to 8B.

In session 2, consumers 1C to 8C assessed slices 11 to 18 from fish out of each treatment (treatment 1 to 4) and consumers 1D to 8D assessed slices 11 to 18 from another four fish from each treatment (treatment 1 to 4). Consumers in the J series had slices 9, 10, 19 or 20.

Each consumer also completed a demographics questionnaire (see Appendix 1). The Japanese consumers were given a translated copy of the questionnaires for them to use.

Data were transferred into Microsoft® Excel, scaled to 100 and then analysed using a randomised block analysis of variance where consumers were considered as a blocking factor. The warm-up sample data was not included in the analysis. Where significant differences ($P<0.05$) were identified, pair-wise comparisons were made using Fisher's least significant difference procedure (LSD).

2.2.4 Results and Discussion

The results from the difference from reference questions are presented in table 2.6.

Table 2.6. Difference from reference test averages (tuna fed control) for the tuna sashimi from tuna fed 3 different mixed energy diets (all consumers n = 35)

Attribute	Tuna fed mixed energy diet A	Tuna fed mixed energy diet B	Tuna fed mixed energy diet C	Tuna fed control (reference)	**LSD**
Odour	39	38	37	**34**	11.1 ns (p=0.8)
Colour	39	44	47	**42**	12.1 ns (p=0.6)
Texture	37	43	44	**40**	10.1 ns (p=0.5)
Flavour (n=34)	40	46	44	**48**	8.1 ns (p=0.2)

ns = not significant at probability level quoted

The figures in **bold** (table 2.6) represent 'placebo' effect for the tuna fed Australian sardines when assessed blind. This was a measure of the extent of the difference from the reference sample as expressed by the 35 consumers. It formed a check of internal validity of the panel and serves as a measure of the effect of asking the difference question. It also gives an indication of within sample variability. The 'placebo' levels were higher than that considered desirable, certainly higher than those given by the panel in 2004 (Thomas 2007) on tuna sashimi (most were in the 30's on the 100 pt scale). It should be considered that this panel was smaller (35 compared to 45) and included students who may not have been very experienced with tuna sashimi and this type of test. In addition here we had only one fish for the identified reference, whereas previously (Thomas 2007) there were two halves of a reference fish available as the identified and blind samples. This probably indicates that there

was more variability between fish than within fish. In addition, the identified reference sample was exposed for a longer period to the ambient conditions while the session was on and was being consumed as the panellists performed their assessments on the other fish. This reduced the amount of sample available for future comparisons, which could add some variability. In future evaluations, some form of insulation to maintain the coolness of the identified reference and making more of this sample available might be useful changes to the method.

For all variables (table 2.6) there were no significant (P>0.05) differences between the control and those fed on mixed energy diets. The LSD's were similar to the 2004 data so the measure of variability is similar. Therefore it would appear that tuna fed the mixed energy diets were similar to those fed local baitfish in terms of their chutoro sashimi quality.

The results from the hedonic scale questions are presented in table 2.7.

Table 2.7. Hedonic test results for the tuna sashimi (all consumers n = 35).

Attribute	SBT fed mixed energy diet A	SBT fed mixed energy diet B	SBT fed mixed energy diet C	SBT fed control (reference)	**LSD**
Odour	60	61	58	**62**	6.9 ns (p=0.6)
Colour	63 A	55 ab	52 b	**62 a**	8.9 (p=0.04)
Texture	71	65	62	**61**	9.0 ns (p=0.1)
Flavour (n=34)	67 a	66 a	55 b	**61 ab**	9.4 (p=0.04)
Overall	70 a	64 a	53 b	**63 a**	8.9 (p=0.003)

a, b means for each attribute not followed by a common letter are significantly different (P<0.05)
ns not significant (P>0.05)

In terms of odour and texture there was no significant (P>0.05) difference between any of the SBT fed different diets. However, for colour, flavour and overall there were significant differences in terms of preference. The SBT fed mixed energy diet C had the lower scores while those fed mixed energy diet A had the highest scores. In terms of significance, the colour of the control and the mixed energy diet A fed tuna were similar in their degree of liking and both were significantly (P<0.05) preferred to the tuna fed mixed energy diet C. For flavour, the tuna fed mixed energy diets A and B were significantly (P<0.05) preferred

over those fed mixed energy diet C but were similar in their degree of liking to the control. Overall, tuna fed mixed energy diet A or B or the control was all similarly "liked" and all were significantly preferred to the tuna fed mixed energy diet C. For all attributes and all treatments the average values were above 50 and ranged from 52 to 71, which would correspond to neither "like" nor "dislike" but to "like moderately" on the 100 pt scale.

In terms of colour and overall rating, there were no significant ($P>0.05$) differences between SBT fed mixed energy diet C and the control but there was a significant ($P<0.05$) preference for the control over the mixed energy C diet fed tuna. This may be explained by the fact that the panel did not rate the identified reference for acceptance and the acceptance scores for the control are based on 4 different fish. However if the panel had rated the identified reference, this would have compromised the validity of the sensory evaluation test and this complication was an artefact of running two types of tests in one session. Noting that, the acceptability testing results within the current testing may have more validity compared to the difference from reference results, because as noted earlier, only half of one reference fish was available. Previously, in 2004 (Thomas 2007), both sides of the reference fish were available.

Comments made by the consumers are presented in Appendix 1. It was notable that, with most consumers, there was a wide range of comments relating to similar samples.

2.2.5 Acknowledgments

The authors would like to thank the thirty-five consumers were recruited from the Port Lincoln community and the local SBT industry that assisted in the involvement of industry people and the visiting Japanese tuna grading professionals. We would also like to thank the TAFE South Australia and D I Fishing Co Pty Ltd for the use of their facilities. Without the cooperation of these participants this research would not have been possible

2.2.5 References

Thomas, P. (2007), Aquafin CRC - Southern Bluefin Tuna Aquaculture Subprogram: maximising the control of quality in farmed SBT, FRDC Final Report (FRDC Project No. 2001/248). SBT Aquaculture Subprogram, 216pp.

3.0 Antioxidant supplements

3.1 2006: High vitamin pellet supplement feeding of SBT

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3.1.1 Introduction

The Tuna Product Quality group has conducted on-going research in to the sashimi shelf life benefits of nutrient fortified southern bluefin tuna (SBT) (*Thunnus maccoyii*) diets and in developing practical delivery systems for those nutrients. It has been demonstrated that a commercial pelleted diet is a suitable nutrient supply for SBT (van-Barneveld *et al*. 2003) although attention to a weaning period and the timing of fat accretion were seen by the industry as commercially relevant problems and therefore baitfish diets are preferred by industry. Never the less research demonstrated significant muscle dose responses and extended colour shelf life in SBT fed high vitamin pellets exclusively (Thomas 2007). Therefore in the current research a feeding regime that included supplementing the standard baitfish diet with vitamin fortified pellets was trialled as a commercial nutrient delivery system. It was anticipated that by feeding a supplement diet throughout the farming season delivery of sufficient nutrients (antioxidants) could be achieved to result in a measurable muscle tissue dose response and a concurrent extension to the colour shelf life of the tuna sashimi product.

3.1.2 Methods

Fish Husbandry

On 8/3/06 1435 SBT were transferred from the tow pontoon to the control cage on the Ajka Pty Ltd tuna farm and then fed a standard commercial baitfish (SCB) diet at 1.8kg / SBT / day for the duration of the experiment. On 9/3/06 on the same farm another 1229 SBT were transferred from the same tow pontoon to the Pellet Supplement (PS) cage, fed as per the control fish and from 10/3/06 also supplemented with pellets fortified with added antioxidants dl – α – tocopherol and l-ascorbic acid 2-monophosphate (Table 3.1) at 5% (unless stated otherwise) by weight of the daily baitfish ration.

Table 3.1. Concentration of vitamins added to Standard Commercial Baitfish (SCB) and Pellet Supplement (PS) diets.

Diet	α-Tocopherol acetate[1] ($mg.kg^{-1}$)	Ascorbic acid[2] ($mg.kg^{-1}$)
SCB	undisclosed*	undisclosed*
PS	10000	10000

[1]Rovimix E–50
[2]Stay C 35
*Commercial application of vitamin premix powder

Feed pellets were made at The Australasian Experimental Stockfeed Extrusion Centre (AESEC), University of Adelaide, Roseworthy, and South Australia. Owing to circumstances beyond the control of the project a suitable weaning period was not possible and only a 2% supplement was available for the first 3 days of the experiment; the 5% supplement began on 13/3/06. The PS was presented to the fish across 31 days at the first feed of the day (SBT are typically fed twice daily) and usually before commencement of baitfish feeding. Supplement feeding days were missed twice due to adverse conditions at sea and once because of a pellet supply shortage. From 10/3/06 to 15/3/06 pellet intake by SBT was minimal and in some cases fish were observed rejecting pellets. Furthermore the sinking rate of the pellets was considered slower than a rate normally attractive to SBT (Lang, M. pers. comm.). Intake improved marginally when pellets were either soaked for a short period in seawater to increase the sinking rate or mixed in equal proportions with baitfish. From 17/3/06 until the end of the experiment a tighter bound batch of extruded pellet from AESEC was fed out. The sinking rate was faster than in the original batch and while intake was still generally low an improvement was observed. From 23/3/06 until the end of the experiment, in an effort to improve palatability, the PS was mixed in minced baitfish slurry (prepared immediately prior to feeding). Initially there was a greatly improved acceptance using this feeding strategy. Overall however, SBT showed a strong preference for baitfish over pellets for the duration of the experiment. By 28/3/06 pellet intake was estimated to be <10% and by 8/4/06 there was little if any response from SBT to the pellets. On 10/4/06 the PS was ceased and plans were made to sample experimental fish as soon as possible.

Sample collection

On 14/3/06, 6 days post transfer, 10 SBT with an average whole weight and condition index of 18.46kg and 18.46 respectively were hook harvested from the control cage to obtain muscle core samples for baseline biochemical information. The first grow-out harvests were conducted on 20/4/06 and 21/4/06 at 10 and 11 days (harvest was split because fish stopped striking the hook before the harvest was completed on the first day) after the cessation of the PS. Muscle core for

biochemical and colour assessment were sampled from 10 SBT each from the SCB and PS cages using methods previously described (FRDC Final Report 2001/248). In order to investigate residual tissue response of the PS, liver only was sampled from ~60 fish net harvested from each experimental cage at the second grow-out harvests (split over 2 days owing to the large scale of the harvests), ~80 days after the cessation of the PS.

Sample preparation
The muscle core samples, anterior chutoro and akami combined, were homogenised to a fine mince. Several grams of sub sample were retained at 4°C for colour shelf life determination; the remainder was stored at –80°C for biochemical analysis. Liver was also stored at –80°C for biochemical analysis.

Analysis
The biochemical and physical characteristics (listed below) of the PS and SCB groups were compared to determine SBT responses to the PS diet. Analytical methods are described in section 2.1.2 of this report except where additional description is given in this section.

Biochemical measures

- Muscle core crude fat

The crude fat content (CFC) of the muscle was determined gravimetrically by a method based on the Norwegian Standard method (NS 9402 E). Approximately 10 g of macerated tissue, 40 g of anhydrous sodium sulphate and 80 ml of ethyl acetate was agitated in the stomacher mixer (IUL Instruments) for 3 min. The resulting homogenate was filtered (Watmans GF/C filter papers). The filtrate was decanted into weighed plastic beakers then evaporated in a fume hood until no solvent remained. The beakers were then placed in an oven at 60^{o}C for approximately 1 hr to evaporate remaining water and then weighed to determine the fat weight (g), which was expressed as a percentage of the muscle wet weight (g) using the following formula:

$$CFC = \frac{(Evaporated\ Homogenate\ wt / Solvent\ \ Dilution)}{(Extracted\ Muscle\ wwt\ \ g)} \times 100$$

- Muscle oxidation (TBARS)
- Muscle vitamins E and C
- Liver vitamin E

Physical measures:

- Whole fish weight
- Condition Index (CI)
- Muscle colour shelf life assessed daily (visual and instrumental) for up to 5 days post harvest.

Statistical analysis.
Results were reported as PS and SCB group means $\pm$ SE (Harvest 1: n=10, Harvest 2: n=20) and tested for differences with a t-test using statistical software Sigma Stat 3.1. Data were log transformed where necessary to satisfy assumptions of normality and homogeneity of variance. Differences were significant where P<0.5.

3.1.3 Results

Fish Condition

Both the SCB and PS fed SBT showed substantial increases after Transfer in all condition parameters at the first grow-out harvests. However there were no significant differences between groups with muscle core crude fat levels of ~8.6%, whole weight of ~45.0kg and CI of ~21.0 (Table 3.2). At the second grow-out harvests, whole weight and CI of 36.5kg and 24.2 respectively in the PS group were significantly higher than 28.4kg and 23.1 respectively in the SCB group (Table 3.3).

Table 3.2. Mean ± SE muscle core crude fat, whole weight and Condition Index in SBT following 31 days of Standard Commercial Baitfish (SCB) diet and fortified pellet diet

Diet	Crude fat (% wet wt)	Whole weight (kg)	Condition Index
Transfer	0.35 ± 0.11	18.5 ± 0.6	18.5 ± 0.7
SCB (Control)	8.71 ± 1.26	41.9 ± 6.6	22.3 ± 0.7
PS	8.56 ± 1.44	49.8 ± 4.0	20.9 ± 0.9

supplemented at ~5% of daily baitfish ration (PS).
* Values (n=10) significantly different (P<0.05).
Transfer values are not included in the comparison.

Table 3.3. Mean ± SE whole weight and Condition Index in Standard Commercial baitfish (SCB) and Pellet Supplement (PS) fed SBT 80 days after cessation of pelleted diet, supplemented at ~5% of daily baitfish ration for 31 days.

Diet	Whole weight (kg)	Condition Index
SCB (Control)	28.4 ± 1.6	23.1 ± 0.3
PS	36.5 ± 2.3*	24.2 ± 0.3*

* Values (n=20) significantly different (P<0.05).

Muscle Dose Response

At the first grow-out harvests α-tocopherol values were significantly higher in PS fish than in SCB fish at 9.93 and 6.31 $mg.kg^{-1}$wet wt respectively (Table 3.4). Ascorbic acid levels were similar in SBT for both dietary treatments at ~9.00 $mg.kg^{-1}$wet wt. Of the ~60 liver samples collected from each group at the second grow-out harvest 20 were initially assayed for α-tocopherol. There was no significant difference between PS and SCB with values of ~30.00 $mg.kg^{-1}$wet wt (Table 3.5).

Table 3.4. Mean ± SE muscle tissue vitamin levels in SBT following 31 days of Standard Commercial Baitfish (SCB) diet and fortified pellet diet supplemented at ~5% of daily baitfish ration (PS).

Diet	α-tocopherol ($mg.kg^{-1}$wet wt)	Ascorbic acid ($mg.kg^{-1}$wet wt)
Transfer (0 weeks)	5.94 ± 0.32	4.00 ± 0.35
SCB (Control)	6.31 ± 0.52	7.63 ± 1.10
PS	9.93 ± 0.97*	10.23 ± 1.44

* Values (n=10) significantly different (P<0.05).
Transfer values are not included in the comparison

Table 3.5. Mean ± SE liver tissue level of α-tocopherol in Standard Commercial baitfish (SCB) and Pellet Supplement (PS) fed SBT 80 days after cessation of pelleted diet, supplemented at ~5% of daily baitfish ration for 31 days.

Diet	α-tocopherol ($mg.kg^{-1}$wet wt)
SCB (Control)	33.90 ± 2.78
PS	27.87 ± 2.23

* Values (n=20) significantly different (P<0.05).

Muscle Post Mortem Change - biochemical

On the day of the first grow-out harvest TBARS levels (expressed as malonaldehyde concentration in muscle) were significantly lower in the PS group than in the SCB group at 0.18 and 0.25 $mg.kg^{-1}$wet wt respectively (Table 3.6). Muscle samples from these groups were stored to assess colour shelf life at $4^{o}C$ until the tissue reached a colour change end point. At 5 days post mortem (colour end point) the TBARS trend had reversed and values were significantly different at 1.88 and 1.41 $mg.kg^{-1}$ wet wt in the PS and SCB tissue respectively.

Table 3.6. Mean ±SE muscle tissue TBARS in SBT following 31 days of Standard Commercial Baitfish (SCB) diet and fortified pellet diet supplemented at ~5% of daily baitfish ration (PS). * Values (n=10) significantly different ($P<0.05$).

Diet		TBARS Day 0 ($mg.kg^{-1}$wet wt)	TBARS Day 5 ($mg.kg^{-1}$wet wt)
Transfer	(0 weeks)	0.09 ± 0.01	no extended storage
SCB		0.25 ± 0.03	1.41 ± 0.13
PS		0.18 ± 0.01*	1.88 ± 0.17*

Transfer values are not included in the comparison.
TBARS defined as malonaldehyde concentration in muscle.

Muscle Post Mortem Change - colour

After the first grow-out harvest muscle tissue stored at $4^{o}C$ from both dietary treatments retained a reasonably constant hue of ~0.5 for 2 days (Fig. 3.1). From Day 2 to Day 5 post mortem hue increased steadily up to ~1.2. There were no significant differences in hue between the PS and SCB fish on any day. Muscle chroma declined over 5 days post mortem and was not significantly different between groups except at Day 0 where values were 22.0 and 19.2 for PS and SCB respectively (Fig. 3.2). The samples were visually assessed for post mortem colour change at the same time as the instrumental analysis. According to subjectively applied colour scores (Table 3.3) the PS and SCB groups both browned at a similar rate (Fig. 3.3). There were no significant differences between the frequencies of scores given to each group on any of the 5 days post mortem.

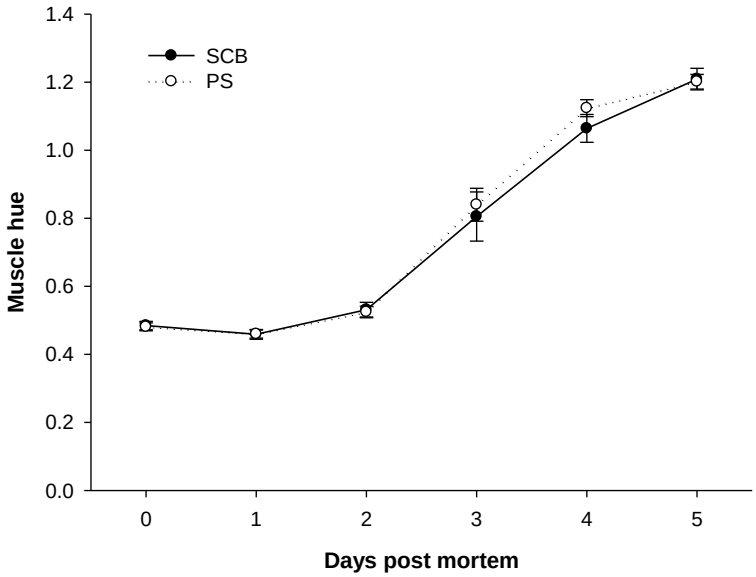

Figure 3.1. Mean ± SE hue in SBT muscle stored at 4°C for 5 days post mortem following 31 days of Standard Commercial Baitfish (SCB) diet and fortified pellet diet supplemented at ~5% of daily baitfish ration (PS). * Values (n=10) significantly different (P<0.05).

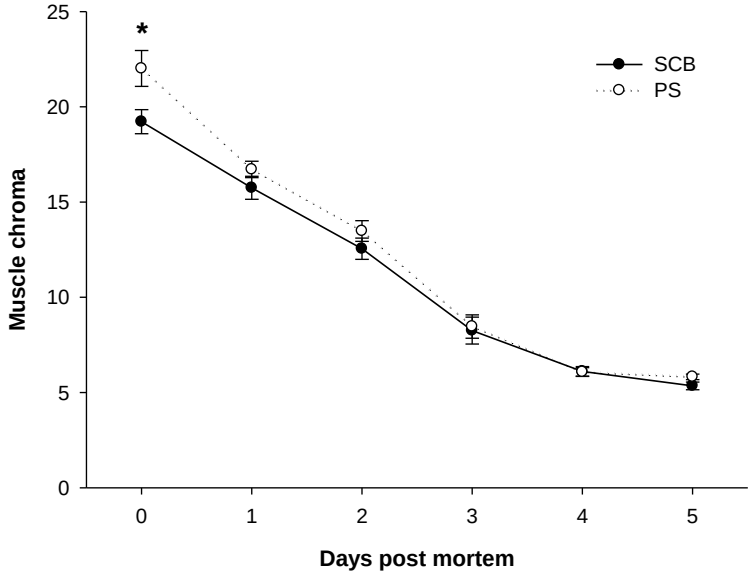

Figure 3.2. Mean ± SE chroma in SBT muscle stored at 4°C for 5 days post mortem following 31 days of Standard Commercial Baitfish (SCB) diet and fortified pellet diet supplemented at ~5% of daily baitfish ration (PS). * Values (n=10) significantly different (P<0.05).

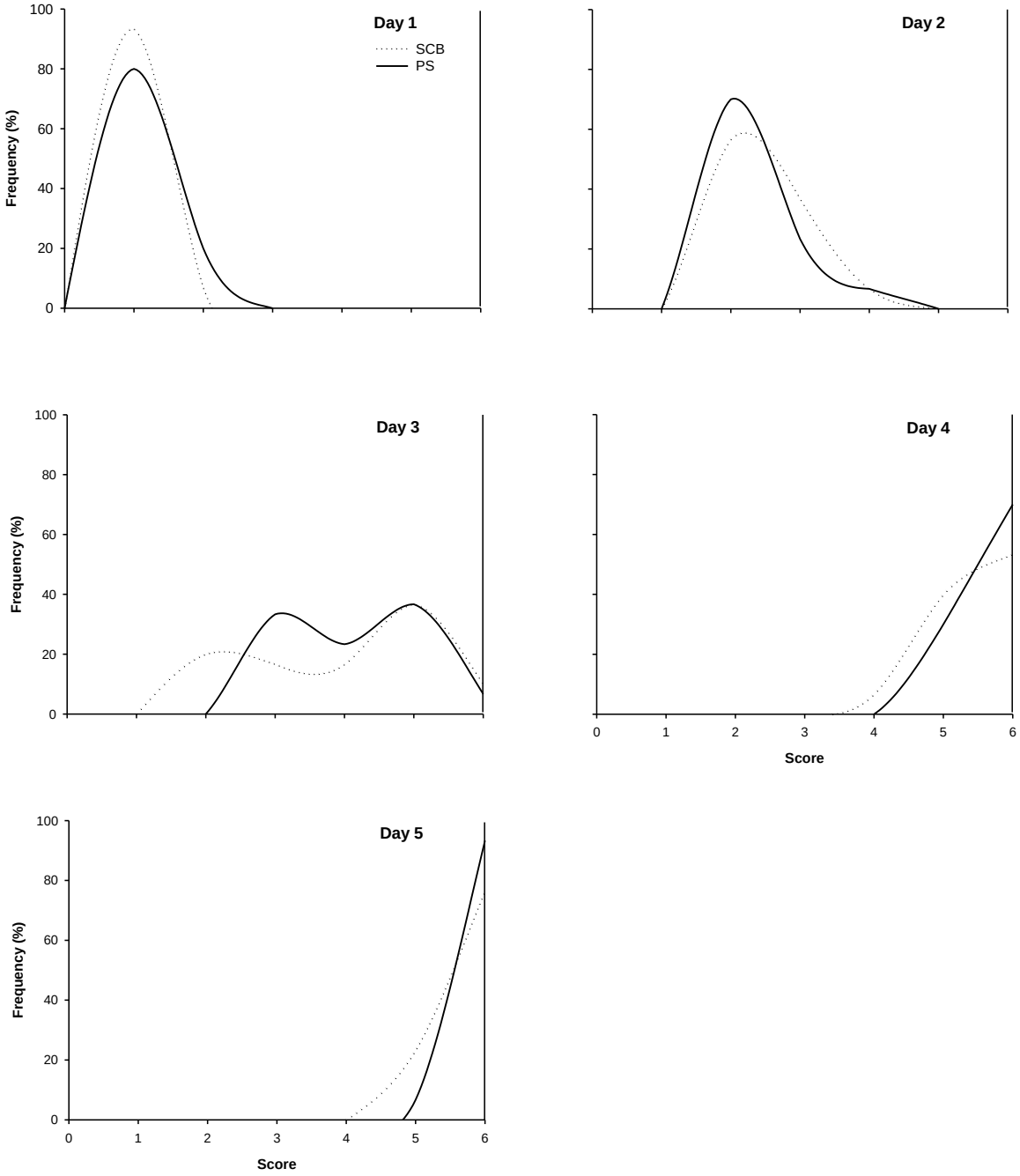

Fig. 3.3. Colour score frequency distribution of SBT muscle stored at 4^{o}C for 5 days post mortem following 31 days of Standard Commercial Baitfish (SCB) diet and fortified pellet diet supplemented at ~5% of daily baitfish ration (PS). * Values (n=10) significantly different (P<0.05).

3.1.4 Discussion

Vitamin tissue dose response was either marginal, relative to the greatly elevated levels achieved in the previous high vitamin pellet exclusive trial (Thomas 2007) or not apparent in SBT fed the pellet supplement diet. Extended colour shelf life has previously been achieved with a vitamin fortified diet (Buchanan and Thomas, 2008) yet was absent in this trial except for a more saturated redness detected by chroma meter on the day of harvest; but this was not different from the control group during the following 5 days of assessment. This result at harvest remains unexplained but is probably unimportant in commercial terms, given that the difference between groups was not detected visually. It is most likely that the absence of any measurable overall colour benefit from the pellet supplement was a result of the poor vitamin dose response which was in turn due to the low to variable pellet intakes observed throughout the trial. During this trail observations from research staff and feed boat crew indicated that when presented a mixed diet of standard baitfish and vitamin fortified pellets, SBT showed a strong preference for baitfish. This preference has been noted by the authors in almost all of the trial where a pelleted diet has been fed along with a baitfish diet. It was noted that the time and effort required endeavour to make pellets more attractive to SBT (mixing with minced bait or alternating with whole bait as “chum”) did not warrant the small increase in intake that was observed and that the small increase did not result in SBT flesh quality improvements. In this trial it was found that there was no advantage supplementing the standard baitfish diet with vitamin fortified pellets when compared to the industry practice of “sprinkling” bait with a nutrient premix. Although the levels applied to the baitfish diet in this trail were undisclosed the SBT tissue levels resulting from both diets were close to that measured in previous studies where no nutrient was added or commercial sprinkling practices were employed.

However it should be noted that the practice of sprinkling nutrients has been found to have some effect on colour shelf life of SBT flesh (Douglas 2009) and in some cases increased tissue levels of vitamin C have been detected in fish fed baitfish sprinkled with antioxidant nutrients (D’Antignana 2008 and Thomas 2009) but the tissue response recorded were very dependent on the application rate of the nutrient (Thomas 2008 and Thomas and Buchanan 2006) with the stabilised form of vitamin C (l-ascorbic acid 2-monophosphate) being the taken up by SBT more readily than the vitamin E added to the diets.

When considering the results of this trial it should be noted that in terms of improved pellet intake, the benefits of a suitable weaning period are unknown. This is because there was no weaning period opportunity at the beginning of this trial and therefore no opportunity to establish the pellet supplement as a recognised food with the SBT prior to the commencement of feeding baitfish. As weaning SBT onto pelleted diets has been recognised to be important to the eventual acceptance of pellet diets, a suitable allocation of time dedicated to adapting the SBT to the supplement diet may have resulted in higher consumption of the supplement by the SBT in this trial.

3.1.5 References

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3.1.6 Acknowledgments

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3.2 2007: Gel encapsulated pellet supplement feeding of SBT

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3.2.1 Introduction

The attempts to deliver vitamins to SBT in pellets as a supplement with baitfish were met with limited success in a 2006 Aquafin CRC trial (section 3.1, this report). Independent of that trial, a contract research trial was undertaken with Skretting Australia in the same year. Over two months the trial evaluated SBT feeding response to a novel form of tuna pellet, a composite of small feed pellets within a gelatine binder matrix (Australian patent no. 2003273194) developed by Skretting Australia. Apparent intake and feeding behaviour of the composite product in SBT was measured. Results indicated excellent consumption. Furthermore, surface observation along with underwater video footage showed prolonged vigorous feeding behaviour. These encouraging results prompted the trial reported in this chapter which was designed to evaluate aspects of SBT muscle shelf life and vitamin dose response to a vitamin supplement delivered in gelatine encapsulated pellets on a commercial tuna farm.

3.2.2 Methods

Diet preparation

The gelatine encapsulated pellets were prepared on the day prior to feeding SBT as follows: 120 litres of fresh water was heated with a *Grimwood* OTS36TLD over-the-side immersion heater in a standard 44 gallon steel drum. When the water reached ~60^0C the heater was replaced with an airlift rig custom made from PVC pipe and driven by a *Resun* heavy duty air pump. The airlift was balanced (2 outlets on opposite sides of the drum to create a mixing vortex) and set to run vigorously. 6kg of fish gelatine crystals were gradually added to the water and stirred in manually to prevent aggregation of crystals. The drum was then covered to retain heat and the airlift was left to run to dissolve the crystals. While the gelatine was dissolving, 100kg of antioxidant fortified tuna feed pellets were removed from frozen storage and spread evenly among 4 x 1000mm x 1000mm x 100mm stainless steel trays. The airlift rig was removed and the gelatine solution poured equally among the 4 pellet trays. The gelatine / pellet mix was then left to set in ambient temperature overnight. Short term cold storage was used to promote setting during warm weather. The bulk gelatine encapsulated pellets were broken into consumable ~40mm^3 portions immediately prior to feeding out.

Fish husbandry

On 15 and 16 February 2007, SBT were transferred from cages towed direct from the fishery to two commercial sea cages near Port Lincoln, South Australia. One cage (Cage 3) was stocked with 1601 SBT and fed standard commercial baitfish, supplemented at the first feed each day with vitamin fortified fish gelatine encapsulated pellets at 5% by weight of total feed / day for 37 days (Table 3.7). The gelatine / pellet matrix was fanned in to the cage one shovel load at a time. Frozen blocks of standard bait were withheld until the supplement was dispensed.

The control cage (Cage 4) was stocked with 2041 SBT and fed standard commercial baitfish periodically applied with powdered vitamin pre-mix over 40 days (Table 3.7). The experimental diets began on 17 February 2007. Feeding days were occasionally missed owing to adverse conditions at sea and an aborted harvest (conventionally, feeding is withheld on the day prior to harvest).

Table 3.7. Total vitamins added to feed and total expected dose for SBT presented with either standard commercial baitfish* or standard commercial baitfish supplemented with gelatine encapsulated pellets.

Antioxidant	Applied to commercial baitfish (g)*+	SBT dose (mg / fish)*	Added to pellet (mg/kg)**	Measured in pellet (mg/kg)	Target SBT dose (mg / fish)**
Vitamin E	13800	6761	10000	9772	23111
Vitamin C	20700	10142	10000	n/a***	23111

** Amounts calculated using stocking rates, feeding period and 100kg pellets presented / day. + = Total of 20 daily applications regularly across 40 day period. ***= l ascorbate 2 monophosphate assay not developed at time of experiment.

Feeding behaviour

A log of SBT surface feeding observations was maintained by research staff and boat crew (sub surface behaviour was not monitored). Intakes of the gelatine pellet supplement were commonly estimated at 0 – 10% with a slow response to the diet landing in the water compared with high intake and vigorous response to shovelled bait. Several strategies were employed with the intention of improving the attractiveness of the diet to SBT:

- adding food colouring to matrix;
- adding krill meal to matrix;
- adding bait to matrix;
- “chumming” with bait while feeding supplement.

These strategies were observed and estimated to increase intakes to ~20 – 30% on some occasions and up to ~50% on rare occasions. Overall SBT showed a strong preference for bait over the gelatine pellet supplement.

Sample collection
For commercial reasons the 2 experimental cages were harvested a week apart. ~80 SBT were harvested from Cage 3 on 10 April 2007 and ~80 SBT were harvested from Cage 4 on 16 April 2007. On each occasion left and right anterior akami / chutoro muscle cores (~10g / fish), liver (~5g) and blood were sampled from 10 fish and stored on ice. The blood was collected in heparinised 10ml centrifuge tubes. Liver samples only were collected from a further 60 fish and also stored on ice. Lengths and weights (gilled and gutted) from all sampled fish were recorded at a commercial tuna processing facility later in the day. Upon returning from harvest to the laboratory, muscle core samples were homogenised to a fine mince. Several grams were stored at 4^0C for colour shelf life determination; the remainder was stored at -70^0C for biochemical analysis. Liver and blood plasma were also stored at -70^0C for biochemical analysis.

Analysis
The biochemical and physical characteristics (listed below) of the gelatine pellet supplement and commercial baitfish groups were compared to determine the effects of the supplement on farmed SBT. Analytical methods are described in section 2.1.2 of this report.

Biochemical measures
- Muscle oxidation (TBARS)
- Muscle vitamins E and C
- Liver vitamin E

Physical measures
- Whole fish weight
- Condition Index (CI)
- Muscle colour shelf life assessed daily (visual and instrumental) for 4 days post harvest.

Statistical analysis
Results were reported as gelatine pellet supplement and standard commercial baitfish group means ± SE and tested for differences with t-test using statistical software Sigma Stat 3.1. Data were log transformed where necessary to meet assumptions of normality and homogeneity of variance. Differences were significant where $P<0.5$.

3.2.3 Results
Fish condition
After 37 days exposure to the experimental diets there were no significant condition differences between SBT fed the pellet supplement and those fed commercial baitfish only. Whole weight and condition index were ~32kg and ~26 respectively for both groups (Table 3.8)

Table 3.8. Mean Condition ± SE of SBT fed either commercial baitfish or commercial baitfish supplemented with antioxidant fortified gelatine encapsulated pellet. *n* = 68, except for gelatine pellet CI where *n* = 64. Absence of superscripts = no significant difference.

Diet treatment	Whole weight (kg)	Condition Index (CI)
Commercial baitfish	31.4 ± 0.9	25.8 ± 0.2
Gelatine pellet supplement	33.1 ± 0.9	25.6 ± 0.2

Tissue biochemistry

Mean muscle and liver vitamin E levels were higher in the SBT fed the pellet supplement than in the commercial diet group but not significantly so (Table 3.9.). Muscle vitamin C was similar in both groups at ~11 $mg.kg^{-1}$. Owing to the lack of difference in liver vitamin E response it was not necessary to assay liver vitamin C as a marker of pellet uptake. The treatment similarities were also reflected in the muscle TBARS levels of ~0.05 $mg.kg^{-1}$ on the day of harvest increasing to ~0.65 $mg.kg^{-1}$ after 4 days cold storage for both dietary groups (Table 3.10)

Table 3.9. Mean tissue vitamin levels ± SE in SBT fed either commercial baitfish or commercial baitfish supplemented with antioxidant fortified gelatine encapsulated pellet. *n* = 10 except for commercial baitfish liver, n = 26 and gelatine pellet liver, *n* = 34. * = alpha tocopherol acetate. ** = ascorbic acid. Absence of superscripts = no significant difference.

Diet treatment	Muscle vitamin E* ($mg.kg^{-1}$)	Liver vitamin E* ($mg.kg^{-1}$)	Muscle vitamin C** ($mg.kg^{-1}$)
Commercial baitfish	6.70 ± 0.55	29.7 ± 1.2	11.97 ± 1.25
Gelatine pellet supplement	8.64 ± 1.67	35.4 ± 4.0	10.27 ± 0.96

Table 3.10. Mean muscle TBARS levels ± SE in SBT fed either commercial baitfish or commercial baitfish supplemented with antioxidant fortified gelatine encapsulated pellet. *n* = 10. * = measured as malonaldehyde. Absence of superscripts = no significant difference.

Diet treatment	Muscle TBARS* Day 0 ($mg.kg^{-1}$)	Muscle TBARS* Day 4 ($mg.kg^{-1}$)
Commercial baitfish	0.050 ± 0.005	0.639 ± 0.044
Gelatine pellet supplement	0.053 ± 0.006	0.693 ± 0.054

Muscle colour shelf life

Subjective colour scores were applied to muscle samples from each dietary treatment (Table 3.3). There was an overall higher frequency of lower (redder) visual colour scores given to samples from the commercial diet group compared with those from the pellet supplement treatment. However, the difference was significant only on day 2 (Fig. 3.4). All samples were either close to or at the colour endpoint (no remaining red) by day 4. Instrumentally, muscle hue increased (red to brown) at a similar rate with similar values in both dietary groups over 4 days (Fig. 3.5). Muscle chroma also decreased (less saturation) at a similar rate in both groups but was significantly higher in the commercial diet group than in the pellet supplement treatment on days 1 and 2 (Fig. 3.6).

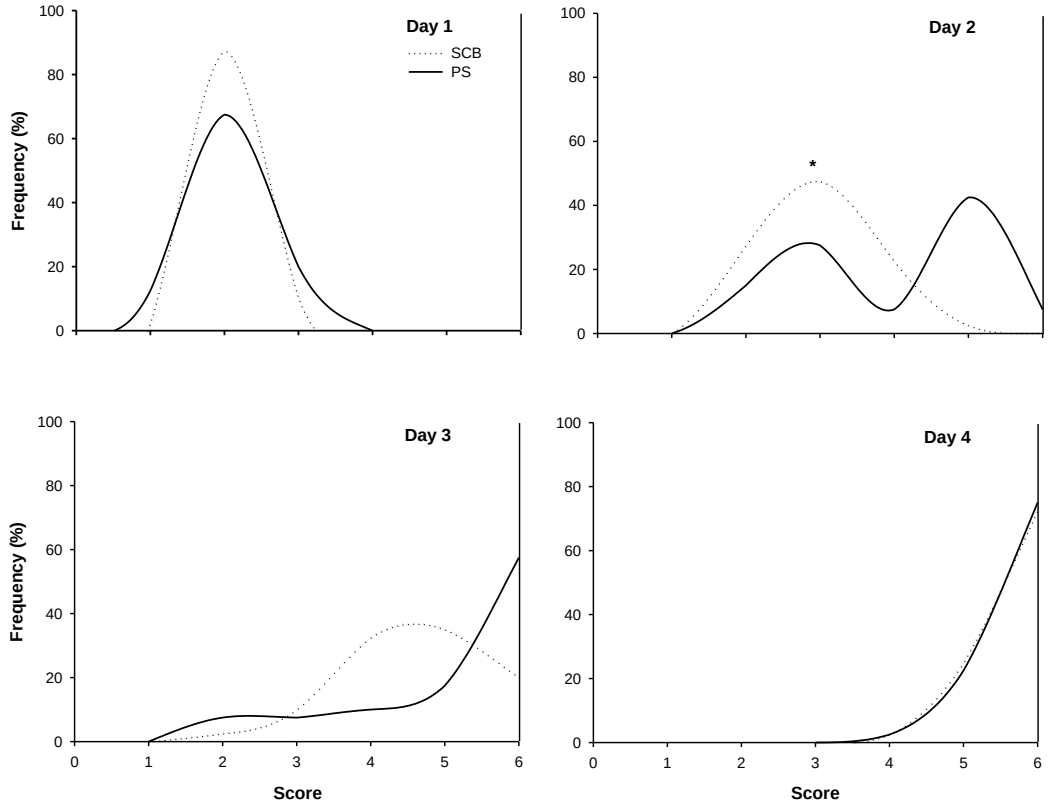

Figure 3.4. Frequency of visual colour score given to muscle stored at 4^0C for 4 days post harvest from SBT fed either commercial baitfish (SCB) or commercial baitfish supplemented with antioxidant fortified gelatine encapsulated pellet (PS). *n* = 10. * = significant difference.

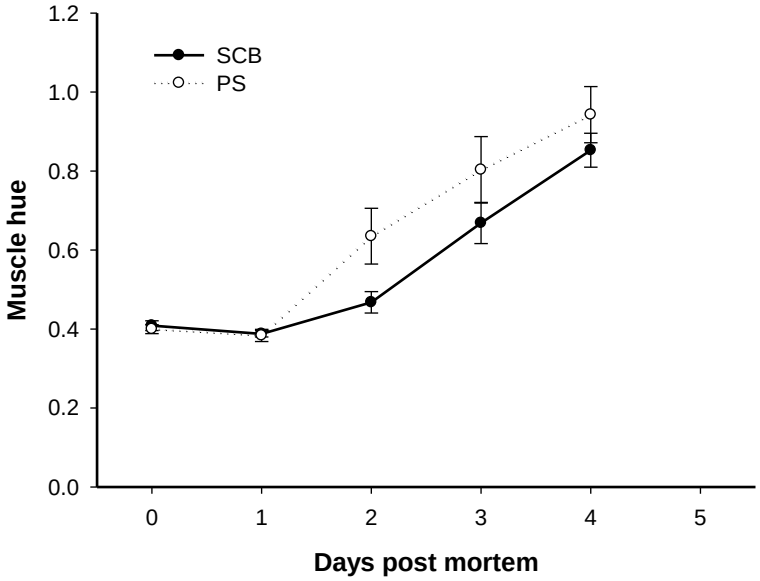

Figure 3.5. Mean hue ±se of muscle stored at 4^0C for 4 days from SBT fed either commercial baitfish (SCB) or commercial baitfish supplemented with antioxidant fortified gelatine encapsulated pellet (PS). *n* = 10. Absence of superscripts = no significant difference.

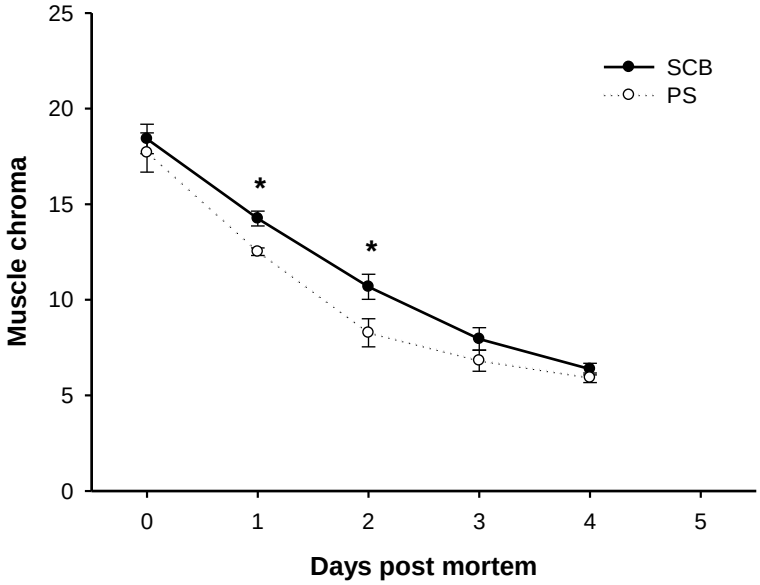

Figure 3.6. Mean chroma ±se of muscle stored at 4^0C for 4 days from SBT fed either commercial baitfish (SCB) or commercial baitfish supplemented with antioxidant fortified gelatine encapsulated pellet (PS). *n* = 10. * = significant difference.

3.2.4 Discussion

Despite previous success in improving sashimi quality in SBT by feeding vitamin supplements (Buchanan and Thomas, 2008) the results of this research indicate that there was no colour shelf life benefit gained from feeding a gelatin encapsulated vitamin fortified pellet as a supplement to SBT. That fish fed the supplement pellet did not have a higher muscle or liver concentration of vitamin E or muscle concentration of vitamin C, is consistent with the colour shelf life observed, except that there was evidence of a slightly extended shelf life in the commercially fed group. That result remains unexplained.

It was noted intake of the gelatin encapsulated pellet was not as high as the intake observed with SBT fed (as a trial with Skretting) at the end of the 2006 season. The lower intake during the current experiment was most likely due to the mode of feeding. That is, in 2007 SBT were fed frozen blocks, where the bait was available to the SBT as it thawed and fell through the mesh feeding cages suspended in the holding pontoons. This feeding method results in subsurface and "passive" feeding behaviour that is less likely to result in consumption of a supplement diet than the frenetic feeding behaviour of shovel fed SBT, such as those in the trial in 2006. It would be ideal to trial this gelatin encapsulated diet with a farming company that shovel feeds; however in these farm based experiments, embedded in commercial production, researchers are constrained by the operations of the collaborating farming company at the time.

3.2.5 Acknowledgements

The authors would like to acknowledge the contribution of Con Karaberidis of SEKOL and SEKOL Farmed Tuna Pty for supplying equipment to drive the airlift gelatin mixing system.

3.2.6 References

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3.3 2007: Baitfish LVP injection

2004/209 SBT Aquaculture Subprogram- Aquafin CRC/FRDC: Provide a baitfish supplement diet for the effective delivery of specific nutrient requirements to juvenile farmed SBT (*Thunnus maccoyii*)

This project was an extension of the original project that has been reported above as despite the convincing evidence that the addition of specific nutrients added to SBT diet resulted in a muscle dose response and improved product shelf life, a suitable nutrient delivery system had not been found. As reported above our research with SBT had examined the use of increased levels of dietary antioxidants to slow the browning process of tuna meat using fortified pellets and coated baitfish as a nutrient delivery system. We established that feeding SBT pellets fortified with boosted levels of vitamins E and C and selenium will raise the level of these natural antioxidants in the fish muscle, and also that higher levels of these antioxidants in the muscle consistently result in an extension of the colour shelf life of sashimi grade tuna meat. We showed that pellet diets fortified with antioxidants are by far the most effective delivery system for these nutrients and therefore the most effective way to improve the shelf life of farmed SBT flesh. However for commercial reasons the industry prefers to use baitfish diet for on growing wild caught juvenile SBT. Although attending to baitfish storage, the use of local caught fish and the addition of powdered antioxidants have been shown to increase SBT muscle antioxidant nutrient levels, an effective nutrient delivery system was still required. Preliminary results carried out in 2006 demonstrated that combining meat marinating technology with commercially available vitamins could provide a useful nutrient delivery system through injection of baitfish.

Included in the current report are the Outcomes Achieved and the Technical Summary from the report on the research conducted within the project extension. If more detail is required the full report should be referred to directly.

OUTCOMES ACHIEVED

This project demonstrated that meat marination technology can be successfully used to inject dietary supplements into baitfish fed to Southern bluefin tuna (SBT) (*Thunnus maccoyii*). The project identified that the technologies filtration system was inadequate for injecting pilchards, however an alternative filtration system was developed, which could be adopted by the industry. Using this technology a prescribed dose of ascorbic acid (AA) and α-tocopherol acetate (α-toc ace) was delivered directly into baitfish, producing injected baitfish with considerable quantities of added vitamins which could be fed either fresh or as frozen blocks. The added vitamins in the LVP injected baitfish were quite stable during two months frozen storage, and a substantial quantity of the added vitamins was present in the LVP baitfish after defrosting in seawater. SBT fed LVP baitfish had substantially higher levels of tissue AA and α-tocopherol when compared to SBT fed a standard baitfish diet. This technology offers a practical method of delivering supplements to SBT which was previously unavailable using the predominant diet, baitfish. It is expected

that this technology will be used to improve the health, growth and flesh quality characteristics of farmed SBT.

NON TECHNICAL SUMMARY

The SBT farming industry in Port Lincoln, SA identified the need to develop a supplement diet for SBT that had the capacity to deliver specific nutrients to the fish at any time during the production cycle. Various techniques have been examined by the Aquafin CRC Tuna Product Quality (TPQ) project in an attempt to satisfy the industry requirements for an effective delivery system of dietary supplements to SBT. This has included; experimental vitamin powder and / or emulsion coating baitfish; industry vitamin powder sprinkle on baitfish; high vitamin pelleted diets; and gelatine coated pellets as a supplement for SBT. The research has generally been successful within the experimental framework and established that farmed SBT fed diets fortified with the micro nutrients, vitamins E & C, exhibit a tissue dose response, extended meat colour shelf life and reduced post-mortem lipid oxidation (Buchanan and Thomas in press; Thomas 2007; D'Antignana, 2007). However, the delivery systems examined were not practical in a commercial environment. Consequently, to date development of a suitable technique to deliver specific nutrient supplements to SBT has been unsuccessful.

The predominant and preferred food source for commercially farmed SBT is baitfish. In order to assimilate a nutrient delivery system with current feeding practices, the TPQ group took a novel approach to delivering supplements into baitfish, by exploring the potential to adapt high through put meat marination technology to baitfish. This technology is commonly used in the food processing industry to flavour meat ranging in size from small fish fillets to whole chickens and roasts. Utilising the Convenience Food Systems (CFS) *AccuJector 450*, the primary objective of the study was to develop a method of injecting baitfish with a Liquid Vitamin Premix (LVP) containing α-tocopherol acetate (α-toc ace) and Ascorbic acid (AA).This entailed testing retention of the LVP over time in various injected baitfish including: LVP injected fresh Australian sardines (*Sardinops sagax*); fresh Australian sardines that had been injected, then frozen and thawed; and frozen imported species thawed then injected. Additionally, the effect of freezing method, duration of frozen storage and sea water immersion on LVP injected baitfish retention was examined. The second objective of the study was to compare the muscle nutrient concentration in SBT fed the LVP baitfish supplement diet with a commercially accepted feed.

The results and recommendations of this study are as follows:

- Meat injection technology was successfully adapted and delivered known doses of supplements into sardines destined for tuna feed.

- The filtration system on the *AccuJector 450* was inadequate at handling the large volumes of loose particles such as scales and spines of the baitfish. Simple modifications to the filtration system prevented blockages and enabled the machine to process four tonnes of sardines per hour.

- Baitfish quality influenced retention of the added supplements, with high physical integrity found to be necessary for maximum LVP retention. It is recommended that

fresh Australian sardines for injection be handled as if they were for human consumption.

- An injection volume of 4-6% produced sardines with high physical integrity and LVP retention. Exceeding this injection volume reduced LVP retention in soft skinned baitfish species. Conversely, species with tough skin, such as Indian oilfish (*Sardinops longiceps*) and red bait (*Emmelichthys nitidus nitidus*) may be able to with stand larger injection volumes without any detrimental effect on physical integrity and supplement retention.

- Storing injected baitfish in ambient air for up to 48h had little effect on the concentration of α-toc ace, but AA generally decreased with time. Despite a considerable quantity of the supplements remaining in the baitfish after 48h of ambient air storage, it is recommended that the baitfish be fed to SBT as soon as possible after being injected.

- Thawing baitfish prior to injection is not recommended unless the species have a tough skin. Indian oilfish is one such fish. This species may be ideal for injecting and shovel feeding as its integrity and nutrient retention was maintained after 48h of ambient air storage unlike fresh Australian sardines and defrosted Monterey sardine (*Sardinops sagax*).

- In a commercial environment, freezing the injected baitfish is logistically more feasible than feeding post-injection and maximises the nutritional and supplement content in the baitfish. Frozen storage appeared to have little effect on the concentration of α-toc ace, but AA did decrease with time. Nevertheless, the dietary supplements were highly elevated in the Australian sardines after 8 weeks of frozen storage. Comparing freezing methods it was apparent that the basket frozen product retained higher concentrations of α-toc ace and AA when compared to product which had been plate frozen. Whether this result holds when large volumes (30-40 tonnes) of product is basket frozen remains to be determined, but the results suggest that injected product could be frozen using baskets or plates and still retain considerable quantities of added supplements. It is recommended that product be frozen as soon as possible after injection in order to minimise leaching of the added supplements.

- Simulating block feeding at sea demonstrated that sea water immersion time had no influence on the concentration of the supplements in the injected baitfish. Consequently, considerable quantities of the added supplements would have been available to SBT by feeding frozen/thawed LVP injected baitfish. As defrosting blocks in bins was not investigated, it is recommended that block feeding at sea be the preferred method of feeding the frozen injected baitfish to SBT.

- Block feeding LVP injected frozen baitfish for 5-7 weeks resulted in a two and three fold increase in the tissue α-toc and AA concentrations respectively when compared to SBT which received a standard commercial diet. This result provides clear and conclusive evidence that meat marination technology can be successfully used to deliver dietary antioxidants to SBT.

In summary, these results demonstrate that meat marination technology can be adapted to the SBT industry and is successful in delivering supplements into local and imported baitfish, with substantial concentrations of the added supplements remaining in the baitfish after defrosting and long term frozen storage. This has resulted in the successful supplementation of dietary antioxidants to SBT. Consequently, the industry now has an effective nutrient supplement delivery system to administer known doses of dietary supplements or pharmaceuticals to SBT using the preferred diet, baitfish. Once the appropriate supplements and doses are identified, it is anticipated that this technology will result in production benefits which stem from improved weight gain and health, reduced stress (handling and oxidative) and lower mortalities.

4.0 Tuna surrogate

4.1 2006: The antioxidant defence systems and post mortem quality of an SBT surrogate yellowtail kingfish *(Seriola lalandi)* fed diets containing different levels of oxidised oil, vitamin E or astaxanthin.

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4.1.1 Introduction

Astaxanthin is a carotenoid belonging to a class of phytochemicals known as terpenes. It is a fat-soluble pigment found in microalgae, yeast, salmon, trout, krill, and other crustaceans. Astaxanthin is a powerful antioxidant; and is reported to be 10 times more capable than other carotenoids. While astaxanthin is a natural nutritional component, it is a supplement in human, animal, and aquaculture food and is specifically added to feeds in the salmonid industry as a flesh colorant (Sommer, *et al*. 1991). The commercial production of astaxanthin comes from both natural and synthetic sources (Astaxanthin for Aquaculture, a DSM Product). Astaxanthin has also been found to benefit animal production and animal health in addition to its pigmentation properties.

Despite variable results from experiments, astaxanthin supplementation has been shown to be associated with increased accretion of vitamins A, C, and E in Atlantic salmon (Christiansen *et al*. 1995) and in rainbow trout fed oxidized oil; it was reported to increase defences against oxidative stress (Nakano *et al*. 1999).

Although the mechanisms of action are still not well understood, (Sigurgisladottir *et al*. 1994), astaxanthin is a powerful antioxidant and might be considered an essential vitamin for fish and crustaceans (Torrissen and Christiansen 1995). In order to improve our understanding of the oxidation protection processes in SBT the surrogate yellowtail kingfish (YTK) identified by Herbert & Poortenaar (2002), was the subject of this experiment. Although the use of vitamin E has been previously explored with SBT, investigating some of the other possible antioxidants for use on this species had not been investigated. The use of the surrogate for SBT was necessary here as the cost logistics and risk of financial losses associated with running robust experiment designs with SBT are prohibitive.

In the current experiment astaxanthin and vitamin E were added separately and in combination to diets that had unoxidised or oxidised oil components. The aim of this experiment was to compare fish growth flesh chemical properties and product quality characteristics of the YTK fed a diet with a level of oxidised products and vitamin E similar to that recorded in baitfish routinely fed to SBT, with fish fed that same diet fortified with antioxidants. In so doing establish if the

effect of oxidised feeds on the YTK surrogate can be ameliorated by the fortifying the diet with vitamin E or astaxanthin or both.

Design

YTK were fed diets according to a 2 way factorial design. Factor 1 was the level of oxidation in the fish oil added to the experimental pellets, either fresh (unoxidised) or highly oxidised (oxidised). Factor 2 was the level of antioxidants added to the pellets, either basal, elevated vitamin E, elevated astaxanthin or elevated vitamin E with elevated astaxanthin (Table 4.1). This equated to 8 distinct dietary treatments that were each assigned randomly to 3 replicate tanks of fish.

Table 4.1. Added antioxidant and unoxidised or oxidised oil factorial feed pellet design for YTK experiment. 3 tanks / dietary treatment. 15 fish / tank. Total, 24 tanks. Fish fed to satiation daily. * = alpha tocopherol acetate as Rovimix E-50 Adsorbate and Rovimix E40. ** = Carophyll Red 10%.

Antioxidant	Unoxidised oil	Oxidised oil
Basal	30 $mg.kg^{-1}$ Vit. E 0 $mg.kg^{-1}$ Astax.	30$mg.kg^{-1}$ Vit. E 0 $mg.kg^{-1}$ Astax.
Vitamin E*	300 $mg.kg^{-1}$ Vit. E 0 $mg.kg^{-1}$ Astax.	300 $mg.kg^{-1}$ Vit. E 0 $mg.kg^{-1}$ Astax.
Astaxanthin**	30 $mg.kg^{-1}$ Vit. E 1.4 $mg.kg^{-1}$ Astax.	30 $mg.kg^{-1}$ Vit. E 1.4 $mg.kg^{-1}$ Astax.
Vitamin E + Astaxanthin	300 $mg.kg^{-1}$ Vit. E 1.4 $mg.kg^{-1}$ Astax.	300 $mg.kg^{-1}$ Vit. E 1.4 $mg.kg^{-1}$ Astax.

Diet preparation

Oil used in the preparation of the feed pellets was fresh SBT oil from South Australian Marine Product Industries (SAMPI), Port Lincoln, South Australia. To induce peroxidation in the oil for the oxidised pellets, oil was aerated at ~30^{o}C for 126 hours (after Tocher *et al*., 2003). Ethoxyquin was then added at 500mg/l to arrest oxidation. Fresh oil was used for the unoxidised pellets (Table 4.2). The experimental pellets were manufactured by SARDI at AESEC, Roseworthy, South Australia by extrusion and subsequent vacuum infusion of the oils premixed with antioxidants. There was ~12% crude fat in the pellets which is a level typical of that found in SBT baitfish diets. All pellets contained at least basal levels of vitamins and micronutrients (Table 4.3).

Table 4.2. Peroxide value (PV) of oil added to YTK pellet diets and TBARS levels in same pellet diets. * = malonaldehyde. Significant differences indicated by different superscripts. Significance does not apply to added oil PV. Oil from same batch in each group.

Oil in feed	Diet antioxidant	Added oil PV ($meq.kg^{-1}$)	Pellet TBARS* ($mg.kg^{-1}$)
Unoxidised	Basal	17.1	0.39 ± 0.02^{a}
(fresh)	Vitamin E	17.1	0.54 ± 0.02^{b}
	Astaxanthin	17.1	0.53 ± 0.01^{b}
	Vitamin E* & Astaxanthin	17.1	0.52 ± 0.02^{b}
Oxidised	Basal	306.9	0.50 ± 0.02^{b}
(126 hours	Vitamin E	306.9	0.55 ± 0.04^{b}
at 30^{0}C with	Astaxanthin	306.9	0.53 ± 0.01^{b}
aeration)	Vitamin E & Astaxanthin	306.9	0.55 ± 0.02^{b}

Table 4.3. Tuna vitamin/mineral premix with decreased vitamin E & C to match levels found in Australian sardines for this experiment. Amounts quoted as final feed values then increased by five hundred times for a premix which was added to the basal feed at a rate of 2g/kg. Choline chloride was added separately.

Component	Final feed	Premix added at 2g/kg	
Fat-soluble vitamins			
Vitamin A, IU/kg	3 000	1 500 000	Vitamin A, IU/kg
Vitamin D, IU/kg	500	250 000	Vitamin D, IU/kg
Vitamin E, mg/kg	30	15	g/kg
Vitamin K, mg/kg	7	3.5	g/kg
Water-soluble vitamin, mg/kg			
Riboflavin	20	10	g/kg
Pantothenic acid	30	15	g/kg
Niacin	65	32.5	g/kg
Vitamin B12	0.03	0.015	g/kg
Biotin	0.3	0.15	g/kg
Folate	4	2	g/kg
Thiamin	2	1	g/kg
Vitamin B6	15	7.5	g/kg
Vitamin C	45	22.5	g/kg
myo-Inositol	50	25	g/kg
Minerals, mg/kg			
Magnesium (as magnesium sulphate)	5	2.5	g/kg
Iron (as iron sulphate)	40	20	g/kg
Zinc (as zinc sulphate)	30	15	g/kg
Manganese (as manganese sulphate)	20	10	g/kg
Copper (as copper sulphate)	3	1.5	g/kg
Iodine (as potassium iodide)	1.1	0.55	g/kg
Selenium (as sodium selenite)	0	0	g/kg
Cobalt (as cobalt sulphate)	1	0.5	g/kg
Ethoxyquin	75	37.5	g/kg

Fish husbandry

On 28 June 2006 YTK juveniles, average weight ~4g, were transferred from the South Australia Aquaculture Management hatchery, Port Augusta, SA to a 5000 L holding tank at SARDI Aquatic Sciences, West Beach, SA and fed to satiation daily on a Skretting Nova ME pellet diet. On 29 September 2006 24 (8 treatments x 3 replicates) x 5000 L experimental tanks (outdoors, under shade) were each stocked with 15 ~110g YTK from the holding tank. An additional tank was stocked with tagged ~110g fish to use as replacements for mortalities to maintain even stocking densities in the experimental tanks (tagged fish were not sampled or used in analysis). Tanks were continuously supplied with filtered (biological & mechanical) seawater at 37ppt salinity, ~17^{0}C, ~100% dissolved oxygen saturation and a flow rate of ~58 L.minute^{-1}. The fish were fed exclusively on experimental diets to satiation daily, 6 days out of every 7 for 8 weeks and the daily feed intake was recorded. Water quality parameters were measured thus: Daily – pH, dissolved oxygen, temperature; Weekly – ammonia. Growth and survival parameters were also measured.

Sampling

At the end of the 8 week pellet trial YTK were starved for 24 hours to allow for digestion of all food in the gut. Each tank was then completely harvested by gradual draining and netting. The fish were killed in ice slurry and 6 fish were randomly sampled immediately. Length (tail fork to head) and whole wet weight was recorded. Blood was drawn from the caudal vein using a 3ml heparinised syringe and 18 gauge needle. The blood was transferred into 1.5ml eppendorf tubes and centrifuged at 5000 rpm for 10 minutes. The resulting plasma was transferred to 2ml cryo-vials and held on dry ice. Whole fillets were then taken from both sides of the fish; one side frozen on dry ice for Day 0 post harvest analysis, the other held on ice for aged muscle analysis and sensory evaluation. Whole liver was weighed and then frozen on dry ice. 3 extra YTK were randomly selected from each tank and held on ice for whole fish proximate analysis.

All samples were freighted overnight to the LMSC for analysis. At LMSC on Day 1post harvest, the frozen fillets were pooled from each tank, partially thawed and then homogenised to a fine mince in a domestic food processor. ~200g mince was stored at -70^{o}C until needed for biochemical analysis. The frozen liver was pooled from each tank and ground to a powder by mortar and pestle and stored at -70^{o}C. Individual blood plasma was stored at -70^{o}C and pooled by tank when required for analysis. The fillets for sensory evaluation were transferred to 4^{o}C refrigeration and aged until Day 6 post harvest. The fillets were then stored at -70^{o}C to preserve their Day 6 condition. On the day of sensory evaluation fillets were pooled from each tank and homogenised as per Day 0 fillets. In addition to the requirements of this project, small sections of muscle and liver were sampled for enzyme analysis for the research of PhD candidate Alex Korte (chapter 5.2).

Physical and biochemical analysis
Specific Growth Rate (SGR) was expressed as % weight gain.day^{-1} and calculated:

SGR (% weight gain/day) = (100[ln(final wt)-ln(initial wt)]/days) (Wooten, 1990).

Muscle and liver vitamin E (α tocopherol) was determined by methanol extraction and HPLC by a method modified from Huo *et al*.(1999). Astaxanthin in the feed, muscle and liver was analysed by DSM Nutrition, Kaiseraugst, Switzerland. Thiobarbituric reactive substances (TBARS) in the muscle, liver, blood and feed were determined as malonaldehyde extracted in perchloric acid and measured spectrophotometrically. Peroxide Value (PV) in the SBT oil added to the feed pellets was measured following the American Oil Chemists Society titration method (AOCS, 1998).

Whole YTK were prepared for proximate analysis by pooling fish from each tank and passing twice through an industrial meat mincer fitted with a 5mm Ø die. Crude fat was then determined by a modification of the Norwegian ethyl acetate gravimetric method. Mince was oven dried to completeness to determine moisture content. Crude protein content was calculated by applying the Jones conversion factor to total nitrogen content determined by LECO analysis:

Crude protein % = Total nitrogen % x 6.25 (Jones factor, recommended by FAO).

Sensory analysis
A panel of 9 volunteers were trained in the sensory evaluation of cooked YTK under the supervision of a Food and Consumer scientist from the Queensland Department of Primary Industries and Fisheries. For the training sessions, commercially sourced market size YTK was used to prepare cooked products with extremes in ageing meat qualities. For the sensory evaluation of the experimental YTK, ~10g of freshly prepared pooled Day 6 mince was cooked in aluminium foil “cup cake” pans covered in foil wrap at 200^{o}C for 3 minutes (cooked throughout but still moist). The sample was presented immediately to the assessor to score on a continuous line scale, where 0 = none to 50 = extremely strong, the presence of the following attributes: aroma intensity, acidity / sourness, rancidity, umami (Japanese term meaning, “meaty’’ flavour). 1 replicate blind sample from each treatment was presented to each assessor in random order. The remaining replicates were presented on each of 2 consecutive days.

Statistical analysis
Physical, biochemical and sensory attribute dietary treatment means were tested for significant differences ($P < 0.05$) by 2 way factorial analysis (Oil oxidation level and added antioxidant interaction) in SPSS 14. Residual plots were examined to assess assumptions of normality and homogeneity of variance.

4.1.3 Results

Diet

Although the PV of the oxidised oil added to the feed pellets was 306.9 compared with 17.1 for the unoxidised pellets, there were no differences in TBARS levels of the diets between oil treatments (Table 4.2). The unoxidised basal diet did have significantly lower TBARS than all other pellets.

Physical and biochemical

For all YTK parameters tested in this experiment, 2 way factorial analysis found no interaction (P >0.05) between level of oil oxidation and the addition of elevated antioxidants in the pellet diet. However there were some tissue responses to vitamin E addition irrespective of the oxidised state of the oil. Muscle vitamin E levels were similar in all groups with added vitamin E in their diets and significantly higher than those groups without (Table 4.4). Liver vitamin E showed the same pattern of significance. There were no differences in muscle or liver vitamin C levels between dietary groups.

Table 4.4. Mean YTK tissue vitamin levels ± SE (*n* =3) after 8 weeks of experimental pellet diet. ** = alpha tocopherol. *** = ascorbic acid. Significant differences indicated by different superscripts. Absence of superscripts = no significant differences.

Oil in feed	Diet antioxidant	Muscle vitamin E** ($mg.kg^{-1}$)	Liver vitamin E ($mg.kg^{-1}$)	Muscle vitamin C*** ($mg.kg^{-1}$)	Liver vitamin C ($mg.kg^{-1}$)
Unoxidised	Basal	3.0 ± 0.2^{a}	25.6 ± 0.6^{a}	1.8 ± 0.3	6.0 ± 0.7
	Vitamin E	7.5 ± 0.5^{b}	92.0 ± 4.5^{b}	2.4 ± 0.4	7.3 ± 0.5
	Astaxanthin	3.0 ± 0.3^{a}	23.4 ± 2.8^{a}	2.3 ± 0.4	6.7 ± 0.6
	Vitamin E & Astaxanthin	8.2 ± 0.3^{b}	109.1 ± 17.8^{b}	2.6 ± 0.4	7.5 ± 0.2
Oxidised	Basal	2.5 ± 0.3^{a}	17.9 ± 1.5^{a}	2.0 ± 0.1	7.4 ± 0.8
	Vitamin E	8.7 ± 0.9^{b}	124.0 ± 13.3^{b}	1.8 ± 0.1	7.2 ± 0.4
	Astaxanthin	2.6 ± 0.1^{a}	18.2 ± 0.7^{a}	2.0 ± 0.1	7.0 ± 0.4
	Vitamin E & Astaxanthin	7.9 ± 1.1^{b}	110.3 ± 7.9^{b}	1.9 ± 0.1	6.6 ± 0.2

All feed pellets were assayed for astaxanthin content. The diets with added astaxanthin contained ~0.70$mg.kg^{-1}$ which was slightly higher than in the pellets without added astaxanthin but considerably lower than the expected inclusion rate (Table 4.5). Astaxanthin levels in YTK muscle and liver were mostly below either the assay limit of detection or lower limit of quantification. There was very little tissue astaxanthin response to any of the experimental diets.

Table 4.5. Mean YTK tissue astaxanthin levels ± SE after 8 weeks of experimental pellet diet and actual astaxanthin levels in feed pellets. * = Below limit of assay detection. ** = Below limit of assay quantification. *n* = 1 except, $^{+}$ *n* = 2 or $^{++}$ *n* =3 (because of LOD or LLQ or insufficient sample for assay). Absence of superscripts = no significant differences.

Oil in feed	Diet antioxidant	Pellet astaxanthin ($mg.kg^{-1}$)	Muscle astaxanthin ($mg.kg^{-1}$)	Liver astaxanthin ($mg.kg^{-1}$)
Unoxidised	Basal	0.12	LOD*	LLQ**
	Vitamin E	0.11	0.018	$0.011 \pm 0.002^{+}$
	Astaxanthin	0.71	LOD	0.008
	Vitamin E & Astaxanthin	0.73	LOD	$0.013 \pm 0.001^{++}$
Oxidised	Basal	0.18	LOD	LLQ
	Vitamin E	0.12	LOD	$0.010 \pm 0.000^{+}$
	Astaxanthin	0.81	LOD	$0.009 \pm 0.000^{+}$
	Vitamin E & Astaxanthin	0.66	LOD	$0.014 \pm 0.002^{++}$

The group that was fed the unoxidised pellet with added vitamin E and astaxanthin showed the greatest mean total weight gain and mean Specific Growth Rate but was not significantly different to any other treatment (Table 4.6). Proximate analysis showed no differences in crude fat, moisture or crude protein in whole fish between all pellet groups (Table 4.7).

Table 4.6. Mean YTK growth ± SE (*n* = 3) after 8 weeks of experimental pellet diet. * % weight gain / day. Absence of superscripts = no significant differences.

Oil in feed	Diet antioxidant	Total weight gain (%)	Specific Growth Rate*
Unoxidised	Basal	126.2 ± 9.1	1.45 ± 0.07
	Vitamin E	120.4 ± 3.1	1.41 ± 0.02
	Astaxanthin	125.0 ± 4.2	1.45 ± 0.03
	Vitamin E & Astaxanthin	140.4 ± 10.4	1.56 ± 0.08
Oxidised	Basal	131.0 ± 7.8	1.49 ± 0.06
	Vitamin E	123.3 ± 4.0	1.43 ± 0.03
	Astaxanthin	139.8 ± 4.1	1.56 ± 0.03
	Vitamin E & Astaxanthin	120.0 ± 4.5	1.41 ± 0.04

Table 4.7. Proximate analysis of whole YTK. Mean crude fat, moisture and crude protein percentages ± SE (*n* = 3) after 8 weeks of experimental pellet diet. Absence of superscripts = no significant differences.

Oil in feed	Diet supplement*	Crude fat (% w/w)	Moisture (% w/w)	Protein (% w/w)
Unoxidised	Basal	6.2 ± 0.6	71.5 ± 0.2	18.2 ± 0.3
	Vitamin E	5.8 ± 0.4	70.8 ± 0.1	17.9 ± 0.3
	Astaxanthin	5.5 ± 0.2	71.3 ± 0.3	19.2 ± 0.4
	Vitamin E & Astaxanthin	5.7 ± 0.3	71.1 ± 0.5	18.0 ± 0.6
Oxidised	Basal	5.9 ± 0.4	71.2 ± 0.2	17.4 ± 0.5
	Vitamin E	5.9 ± 0.6	70.8 ± 0.6	18.1 ± 0.4
	Astaxanthin	5.9 ± 0.2	71.5 ± 0.3	18.4 ± 0.4
	Vitamin E & Astaxanthin	6.0 ± 0.3	71.0 ± 0.4	18.5 ± 0.7

Post mortem changes

For muscle sampled at Day 0 post harvest there were no significant treatment differences in TBARS. All groups were ~0.06 $mg.kg^{-1}$ malonaldehyde (Table 4.8). TBARS increased to ~0.60 $mg.kg^{-1}$ in all pellet groups with no significant differences after 6 days stored as fillets at 4^{o}C. Fresh blood TBARS also showed no treatment differences. There were fresh liver TBARS responses to all diets with added vitamin E. These had significantly lower TBARS than both the oxidised and unoxidised basal diets but similar liver TBARS to the groups fed the pellet with added astaxanthin only.

Table 4.8. Mean YTK tissue and blood plasma TBARS levels ± SE (*n*=3) after 8 weeks of experimental pellet diet. ** = measured as malonaldehyde. Significant differences indicated by different superscripts. Absence of superscripts = no significant differences.

Oil in feed	Diet antioxidant	Muscle Day 0 TBARS** ($mg.kg^{-1}$)	Muscle Day 6 TBARS ($mg.kg^{-1}$)	Liver TBARS ($mg.kg^{-1}$)	Blood plasma TBARS ($mg.kg^{-1}$)
Unoxidised	Basal	0.04 ± 0.00	0.57 ± 0.08	0.07 ± 0.01^{a}	0.03 ± 0.01
	Vitamin E	0.04 ± 0.00	0.60 ± 0.05	0.04 ± 0.00^{b}	0.06 ± 0.00
	Astaxanthin	0.05 ± 0.00	0.61 ± 0.04	0.05 ± 0.01^{ab}	0.03 ± 0.01
	Vitamin E & Astaxanthin	0.06 ± 0.02	0.59 ± 0.03	0.04 ± 0.00^{b}	0.04 ± 0.01
Oxidised	Basal	0.06 ± 0.01	0.76 ± 0.04	0.07 ± 0.01^{a}	0.07 ± 0.01
	Vitamin E	0.04 ± 0.01	0.55 ± 0.09	0.05 ± 0.00^{b}	0.06 ± 0.01
	Astaxanthin	0.11 ± 0.04	0.59 ± 0.02	0.06 ± 0.00^{ab}	0.02 ± 0.01
	Vitamin E & Astaxanthin	0.03 ± 0.01	0.51 ± 0.11	0.05 ± 0.00^{b}	0.06 ± 0.03

Sensory evaluation detected no differences between dietary treatments for any of the 4 sensory attributes in YTK muscle aged for 6 days post harvest at 4°C then cooked (Table 4.9). Level of oxidation in the diet or antioxidant supplement did not have an impact on the scores given to the samples presented. Scores given to aroma intensity, acidity / sourness and rancidity ranged from ~15 - 26 overall but were ~17 or less for umami, suggesting that umami was either weak or more difficult to identify by the panel compared with the other attributes.

Table 4.9. Mean scores ± SE (9 participants in 3 repeat evaluation sessions: *n* = 27) applied to presence of YTK cooked muscle sensory attributes. Score range: 0 – 50 (continuous), where 0 = none, 50 = extremely strong. Absence of superscripts = no significant differences.

Oil in feed	Diet antioxidant	Aroma intensity	Acidity / sourness	Rancidity	Umami
Unoxidised	Basal	23.33 ± 1.95	23.37 ± 2.67	22.67 ± 2.48	11.04 ± 1.85
	Vitamin E	22.48 ± 2.29	20.81 ± 2.85	21.19 ± 2.78	13.11 ± 1.90
	Astaxanthin	21.41 ± 2.59	20.59 ± 2.62	18.15 ± 2.68	13.33 ± 2.11
	Vitamin E & Astaxanthin	23.74 ± 2.24	22.00 ± 2.80	22.48 ± 2.81	15.15 ± 2.67
Oxidised	Basal	26.04 ± 1.77	19.19 ± 2.06	20.85 ± 2.17	13.93 ± 2.26
	Vitamin E	21.85 ± 1.90	20.67 ± 2.58	15.44 ± 2.34	17.26 ± 2.40
	Astaxanthin	23.81 ± 1.73	18.44 ± 2.81	20.00 ± 3.24	14.56 ± 2.52
	Vitamin E & Astaxanthin	21.37 ± 2.01	21.37 ± 2.54	18.89 ± 2.60	15.07 ± 1.77

4.1.4 Discussion

The purpose of this experiment was largely to examine the effect of astaxanthin and vitamin E on the oxidation process in the SBT surrogate YTK. This experiment was seen as a way of providing an insight into the effects of oxidised oil on oxidative processes in fish. This is relevant to tuna farming because it is recognised that oxidised feeds and are likely having an effect on SBT production and the rate oxidation processes that occur in the product post mortem that change attributes that are used to measure quality and shelf life. That said the methods to evaluate the effect of antioxidants are not easily interpreted and will depend on what methods are used to measure lipid oxidation. In the current study the methods used to measure the oxidation processes and the quality attributes of the fish products are well accepted and widely used within the literature. The current study did show that vitamin E added to the diet in a synthetic form was able to be taken up by the fish. Although this resulted in treatment differences for flesh vitamin E levels there was no measurable effect on growth oxidation indicators or post mortem quality indicators (chemical or sensory). This is surprising as a simple increase in the amount of lipid added to a diet can have an impact on in-vivo oxidation processes, growth and post mortem flesh quality attributes Chaiyapechara *et al*. (2003). However in the current research there was no measurable effect of the oxidized oil or the antioxidants added on any of the parameters of interest. This result may have been due to a diet composition that otherwise adequately compensated for the addition of the oxidized oils, that is without the additional antioxidants. Within the current experiment, it may also have been useful to use various levels of oil in the diets rather than manipulating the level of oxidation of the oil added. The added problem with the current research is the level of astaxanthin added to the diets was much lower than that stated in the experimental design. This was due to an error in the making of the diet. It is difficult to speculate as to the effect that a higher level of dietary astaxanthin may have had on the parameters measured in the current research, however it remains highly possible that astaxantin included in tuna diets at a level that is below that expected to cause flesh pigmentation may be cost effective means of ameliorating the effects of autoxidation processes in SBT. Should fingerling (<1kg) SBT become available, they would be a much more appropriate substitute for juvenile (~20kg) SBT for this type of investigation.

4.1.5 Acknowledgments

The authors would like to thank the staff at SARDI for their hard work and cooperation in running this experiment at West Beach. We would also like to thank DSM Nutritional Products for the analysis of the astaxanthin and the donation of the astaxanthin product used in this experiment**.**

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4.2 2007: The effect of diets containing different levels of selenium, vitamin E or vitamin C on the composition and post mortem oxidation of a surrogate for the southern bluefin tuna, the yellowtail kingfish (*Seriola lalandi*)

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4.2.1 Introduction

Research by this group on the quality of farmed southern bluefin tuna (SBT) has been focused on improving the colour shelf life of the sashimi product. Previous outputs of our research group and supporting data from associated project groups, has led to the current interest in evaluating the use of a supplementary feed for tuna farming that can be used to extend shelf life. As with terrestrial red meats shelf life is an important product quality feature of farmed SBT. Also, in common with beef, the bright red colour of SBT is due to the myoglobin content of the meat. During storage, the myoglobin is oxidised to met-myoglobin and goes brown. In addition, and unlike beef, the high levels of highly unsaturated fatty acids found in SBT meat provide a strong oxidative pressure that can increase the rate of post mortem browning.

Increasing the tissue level of α-tocopherol (vitamin E), Ascorbic acid (vitamin C) or selenium, alone or in combinations, has been shown to successfully extend the shelf life of beef (Sanders, *et al*., 1997), pork (Houben, *et al*., 1998) and fish (Baker, 1997, Bell and Cowey, 1985, Hemre, *et al*., 1997). These nutrients have natural anti-oxidant properties, which aid in the inhibition of the oxidation of fatty acids, development of off flavours and browning. By slowing the oxidation process in SBT meat it is possible to extend the window of sale opportunity at the market and reduce the losses that are currently experienced through the practice of trimming browned meat during cold storage of portioned carcasses. These improvements obviously have the capacity to improve the reputation of this valuable product in what is becoming an increasingly competitive export market.

From the 2001 farming season and up to and including the 2004 season our research group ran experiments that were designed to investigate and evaluate the use of feeds (baitfish and pelleted) fortified with the natural antioxidants *dl*-α-tocopherol acetate, l - ascorbic acid phosphate and selenium (as sodium selenate or selenomethionine as Sel Plex ®). The aims of the research were first, to determine if feeding diets fortified with these antioxidants would result in a muscle dose response and second, that the colour shelf life of the product could be extended.

The research up to that point did indeed provide strong and highly repeatable evidence that diets fortified with *dl*-α-tocopherol acetate, l - ascorbic acid

phosphate and selenium (selenomethionine as Sel Plex ®) will result in a muscle dose response that occurs quickly enough to be compatible with industry husbandry practice constraints and has a clear benefit to the retention of colour stability of the SBT flesh post mortem. However the experiments carried out have been of a "crude" nature in that they have been designed principally to provide results for industry that can be converted immediately into improved industry outcomes. This has not allowed a more detailed investigation of the contribution that individual component antioxidants or combinations of specific components might make to the improvement to SBT colour shelf life that has been repeatedly observed. With this in mind the current research was carried out using the surrogate YTK in an attempt to tease out the contribution that the individual nutrient components, and combinations of those components, have on the overall antioxidant protection that result in extended colour shelf life in SBT flesh.

4.2.2 Methods

Fish husbandry and experimental design

Juvenile yellowtail kingfish (YTK) (*Seriola lalandi*) (mean weight 3.3g) were obtained for the experiment from a commercial hatchery (Cleanseas Pty Ltd, Arno Bay, South Australia) and maintained in tanks at SARDI for 3 months prior to the experiment. During this time they were fed a commercial YTK diet (Skretting), once a day to satiation.

The experiment commenced on the 28th March 2007 and ran for approximately 6 weeks, ending on the 9th of May 2007. At the beginning of the experiment the YTK were randomly allocated to 8 groups of 3 replicate1000 litre tanks. From a pool of 240 YTK (mean weight 336.04g), 10 YTK were allocated to each of the 24 experimental tanks.

The experiment was conducted at the South Australian Research and Development Institute - Aquatic Sciences, West Beach, South Australia. Twenty four 1000 L tanks were continuously supplied with sea water at a flow rate of x l.min^{-1} in a recirculating/flow through system in which mechanical and biological filtration was used. Water quality variables (dissolved oxygen and temperature) were monitored weekly (see Table 1 in Appendix 2). Oxygen was supplied by air stone aeration with an average dissolved oxygen level of 93.4 % saturation over the duration of the experiment. Average water temperature was 20^{o}C. The tanks were situated outside, under cover, and therefore subjected to ambient photoperiod lighting conditions.

Within the experimental design, eight different diets were randomly assigned to each tank (Table 4.10). During the experiment, any dead fish were replaced with marked individuals taken from a pool of tagged fish that was maintained in an additional separate tank. In this way, the stocking density of the tanks was maintained, but the marked replacement fish that were not sampled at the conclusion of the experiment. The different diets were hand fed to fish, within the

respective tanks, to satiation once a day, 6 days a week. Survival and feed intake was monitored daily.

Diet preparation

The 8 diets, with different inclusion levels of vitamin C, E and selenium, were made at the Australasian Experimental Stockfeed Extrusion Centre (AESEC), Roseworthy, South Australia. The Basal diet (4 mm diameter pellets) was made using a Wenger X85 Extruder (Wenger Manufacturing Inc., Sabetha, Kansas, USA). The additional selenium and vitamin C and E, for the other 7 diets were added to the Basal diet via a UAS vacuum infusion system (UAS Industries, Canada). Prior to vacuum infusion, the additional selenium and vitamin C and E were suspended in oil that was blended at high speed for 30 seconds. The level of additional nutrients in the oil varied according to the diet that was being made (Table 4.10). The oil was then added to the basal diet via vacuum infusion.

Table 4.10. Combinations and inclusion levels of vitamin C, vitamin E and selenium ($mg.kg^{-1}$) in YTK pellet diets.

Diet code	Diet treatment	Vit C inclusion[1] ($mg.kg^{-1}$)	Vit E inclusion[2] ($mg.kg^{-1}$)	Se inclusion[3] ($mg.kg^{-1}$)
1	Basal	45*	30	-
2	+ Vit C	300	30	-
3	+ Vit E	45*	300	-
4	+ Vit C + Vit E	300	300	-
5	+ Se	45*	30	0.5
6	+ Vit C + Se	300	30	0.5
7	+ Vit E + Se	45*	300	0.5
8	+ Vit C + Vit E + Se	300	300	0.5

[1] Stay C35, L-ascorbic acid 2-monophosphate
[2] Rovimix E50, dl-α tocopherol acetate
[3] Selplex ®, selenomethionine
* Represents minimum recommended inclusion or level of vitamin C in baitfish diets (dry weight basis)

Fish sampling

At the conclusion of the experiment, in order to purge the gut of feed, the fish were starved 24 hours prior to sampling. Each tank was then completely harvested by gradual draining and netting. The fish were killed in ice slurry and 5 fish per tank were randomly sampled immediately. The fork length (mm) and total wet weight (g) was measured. Blood was drawn from each fish from the caudal vein using a 3ml heparinised syringe and 18 gauge needle. The blood was transferred into 1.5ml eppendorf tubes and centrifuged at 5000 rpm for 10 minutes. The resulting plasma was transferred to 2ml cryo-vials and held on dry ice before being placed into -70^{o}C freezer for storage.

Whole fillets were then taken from both sides of the fish; one side frozen on dry ice for Day 0 post harvest analysis, the other held on ice for aged muscle analysis (held at 4°C for 4 days). The bones and skin were removed from the muscle fillets. The whole liver was removed, weighed then frozen on dry ice. From 3 of the sampled fish, small sections of the liver, muscle and intestine (approximately 0.5 cm^3) were put into histology cassettes and preserved in a formal buffered saline solution for histopathological examination. 2 extra fish were randomly selected from each tank and held on ice for whole fish proximate analysis.

The muscle fillets, aged muscle fillets and livers for each tank were pooled for biochemical analysis. The muscle fillets were pooled by slightly thawing them at room temperature, chopping them roughly into small pieces and mincing them together using a domestic food processor at maximum speed. The liver samples were pooled using a chilled mortar and pestle. The frozen sample was crushed into a fine powder. The pooled samples were transferred back into -70°C storage prior to biochemical analysis. 2 fish from each replicate tank (6 fish per diet treatment) were pooled for proximate analysis (crude fat, water content and protein); minced twice with a TC22 Meat Mincer (Guangzhou Youjia Machinery Co., Ltd., China) and placed back into -20^{o}C storage prior to proximate analysis.

Physical and biochemical analysis

Specific growth rate (SGR) was calculated following Wooten (1990), as:

$$\text{SGR(\% weight gain/day)} = \frac{100\ [\text{ln(final weight)} - \text{ln(initial weight)}]}{\text{days}}$$

The hepatosomatic index (HSI) was calculated as:

$$\text{HSI} = \left(\frac{\text{liver weight}}{\text{body weight}} \right) \times 100$$

TBARS as malonaldehyde was determined in the muscle and aged muscle fillets, vitamin E and C and selenium concentration was determined in the muscle fillet and liver for each tank. The measurement of TBARS was performed using a method adapted from (Wong, Fletcher *et al*. 1991). The vitamin E (α-tocopherol) concentration was determined by a HPLC method based on the method of (Huo, Nelis *et al*. 1999). Vitamin C was determined by a technique based on the HPLC fluorescence detection method of (Brown and Miller 1992). For selenium analysis, frozen muscle samples were sent frozen, to Regional Laboratory Services, Benalla, Victoria, Australia. Selenium concentration was determined using a method based on the fluorometric technique of (Watkinson 1966) modified as per (Paynter, Halpin *et al*. 1993).

The Crude Fat Content (CFC) of the fish was determined gravimetrically by a method based on the Norwegian Standard method (NS 9402 E). Approximately 10g of the minced tissue, 40 g of anhydrous sodium sulphate and 80 mL of ethyl acetate was agitated in a stomacher mixer (IUL Instruments) for 3 min, and the resulting homogenate was filtered (Whatmans GF/C filter papers). The filtrate was decanted into plastic beakers and evaporated in a fume hood until no solvent was evident. The beakers were then placed in an oven at 60^{o}C for approximately 1h and then weighed to determine the fat weight (g), which was expressed as a percentage of the muscle wet weight (g) using the following formula:

$$CFC = \frac{(\text{Evaporated Homogenate wt/Solvent Dilution})}{(\text{Extracted Muscle wwt g})} \times 100$$

Water content (% moisture) was obtained by placing approximately 5 g of finely chopped minced tissue in aluminium foil trays and drying at 100^{o}C for 24 h. Water content was calculated using the following formulae:

$$\%\ \text{water} = \frac{\text{Muscle dry wt (g)}}{\text{Muscle wet wt (g)}} \times 100$$

Elementary combustion technique (University of Adelaide Waite Institute) was used to determine total nitrogen (%), and then multiplied by Jones factor of 6.25 to determine crude protein (%).

Statistical analysis

A three-way between-groups analysis of variance (ANOVA) was conducted to explore the effect of added vitamin E, C and selenium on fish growth and biochemical parameters. This was carried out within a 3 way factorial design (Table 4.11) using the statistical software SPSS 14. A significance level of *P* = 0.05 was used. Data were log transformed where necessary to meet assumptions of equal variance and homogeneity. Survival was assessed by Chi-Squared.

Table 4.11. Factorial design to test for between subjects effects (interaction) among individual dietary antioxidant components added to YTK feed pellets in various combinations.

	Elevated antioxidant inclusion		
Diet code	Vitamin C	Vitamin E	Selenium
1	No	No	No
2	Yes	No	No
3	No	Yes	No
4	Yes	Yes	No
5	No	No	Yes
6	Yes	No	Yes
7	No	Yes	Yes
8	Yes	Yes	Yes

4.2.3 Results

Proximate analysis

Table 4.12 shows the proximate analysis of YTK for each diet. There was a significant interaction effect for vitamin E and C on moisture (mean 71.6 ± 0.3 % w/w, P=0.049). The main effects for vitamin E (mean 72.63 ± 0.5 % w/w) and C (mean 71.9 ± 0.3 % w/w) contributed equally to the interaction. There were no significant main or interaction effects for added vitamins and selenium on fat levels.

There was a significant main effect for vitamin E on crude protein (mean 18.4 ± 0.3 %, P=0.029) compared to no added vitamin E (mean 17.5 ± 0.3%). There was also a significant main effect for vitamin C on crude protein (mean 18.5 ± 0.3 %, P=0.007) compared to no added vitamin C (mean 17.4 ± 0.2%). However, there was no significant interaction effect.

Table 4.12. Proximate analysis (mean ± SE) of YTK (whole fish) for each diet (n=3).

Diet code	Diet treatment	Moisture (%) ± SE	Fat (% w/w) ± SE	Crude protein (%)± SE
1	Basal	74.30 ± 0.84	4.23 ± 0.20	17.41 ± 0.97
2	+ Vit C	71.26 ± 0.18	6.03 ± 0.70	18.27 ± 0.06
3	+ Vit E	72.38 ± 0.85	5.31 ± 0.53	18.00 ± 0.37
4	+ Vit C + Vit E	71.73 ± 1.01	5.61 ± 0.99	19.17 ± 2.08
5	+ Se	75.31 ± 1.97	5.22 ± 2.50	16.46 ± 0.50
6	+ Vit C + Se	72.5 ± 0.35	4.78 ± 0.21	18.00 ± 0.43
7	+ Vit E + Se	72.88 ± 1.52	4.75 ± 1.13	17.78 ± 0.60
8	+ Vit C + Vit E + Se	71.39 ± 0.53	5.55 ± 0.91	18.67 ± 0.35

Table 4.13. Total mortality of YTK for each tank replicate and per diet (excluding the fish that died due to jumping out of the tanks)

Diet code	Diet treatment	Tank replicate	Total mortality per tank replicate	Total mortality per diet
1	Basal	1 2 3	2 2 2	6
2	+ Vit C	1 2 3	0 0 1	1
3	+ Vit E	1 2 3	1 1 0	2
4	+ Vit C + Vit E	1 2 3	0 0 0	0
5	+ Se	1 2 3	2 3 4	9
6	+ Vit C + Se	1 2 3	0 3 1	4
7	+ Vit E + Se	1 2 3	1 0 0	1
8	+ Vit C + Vit E + Se	1 2 3	0 0 1	1

Fish growth and survival

For SGR there was a significant interaction effect for vitamin E and C (mean 1.4 ± 0.1, P = 0.002). The main effects for vitamin E (mean 1.5 ± 0.1) and C (mean 1.5 ± 0.1) contributed equally to the interaction (Figure 4.1). There was also a significant interaction effect for vitamin E and C on HSI (mean 0.89 ± 0.02, P=0.006) and similar to SGR the main effects for vitamin E and C contributed to the interaction (Figure 4.2). Table 4.13 shows the total mortality of YTK per tank and diet. There was a significant difference in the number of mortalities for the diets (P=0.010).

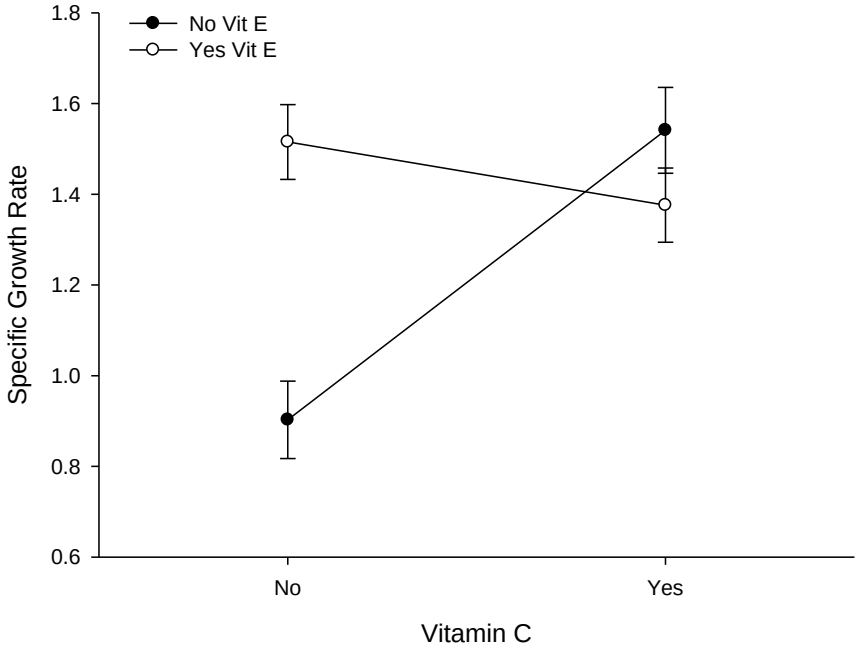

Figure 4.1. Interaction effects of added vitamin E and C in the diet on mean specific growth rate of YTK (n=6)

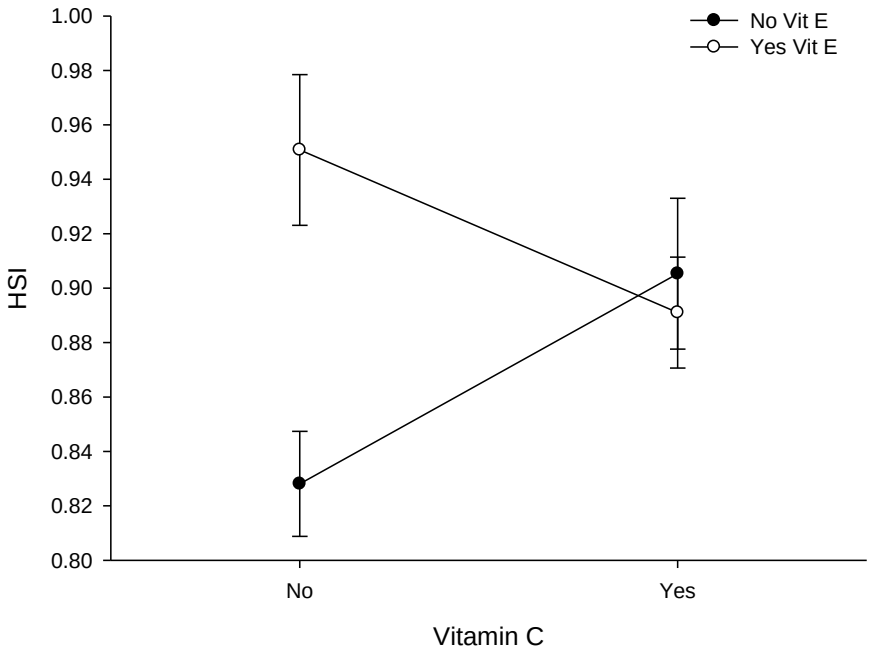

Figure 4.2. Interaction effects of added vitamin E and C in the diet on mean HSI of YTK (n=6)

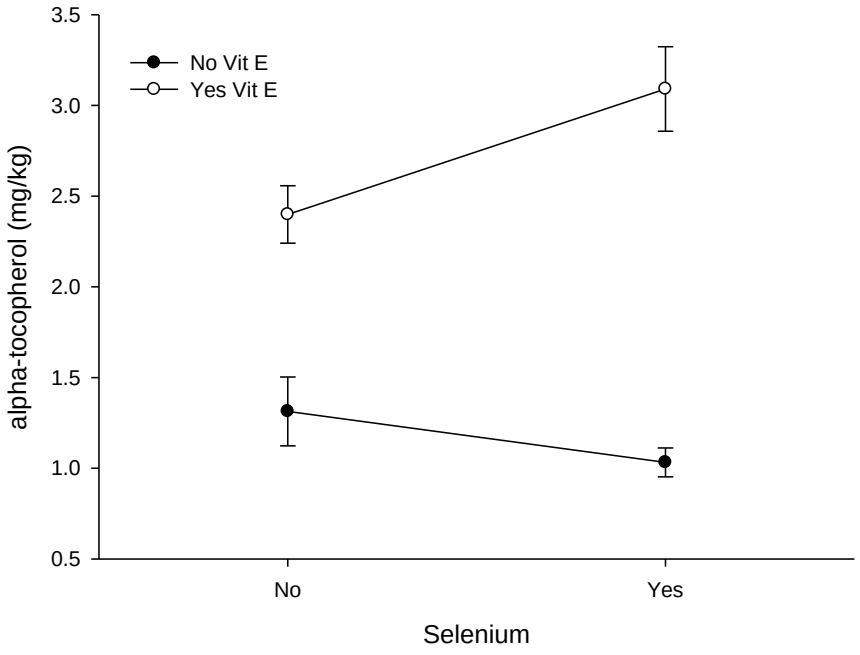

Figure 4.3. Interaction effects of added vitamin E and selenium in the diet on mean vitamin C (alpha-tocopherol) concentration ($mg.kg^{-1}$) in the muscle of YTK (n=6)

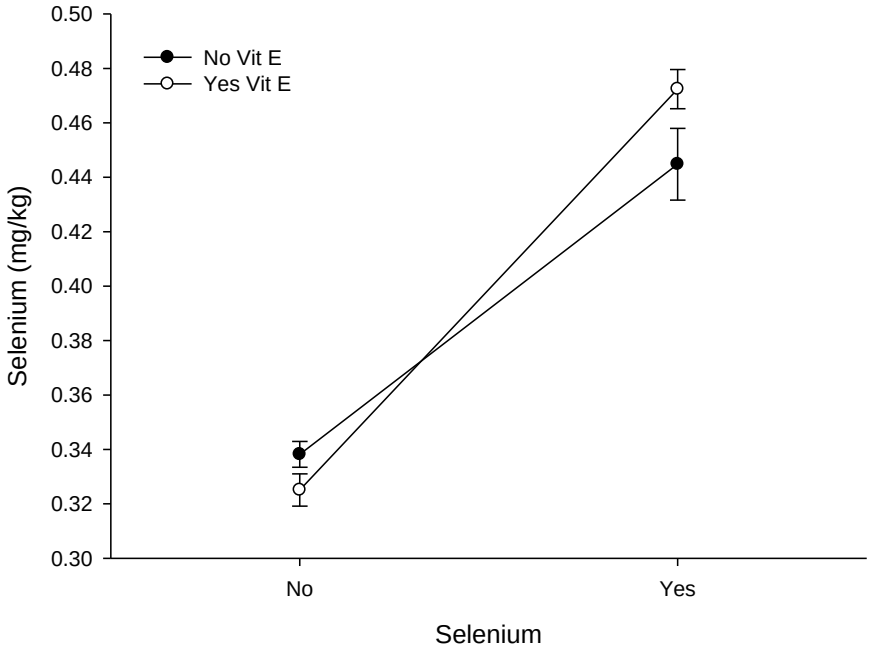

Figure 4.4a Interaction effect of added vitamin E and selenium in the diet on mean selenium concentration ($mg.kg^{-1}$) in the muscle of YTK (n=6)

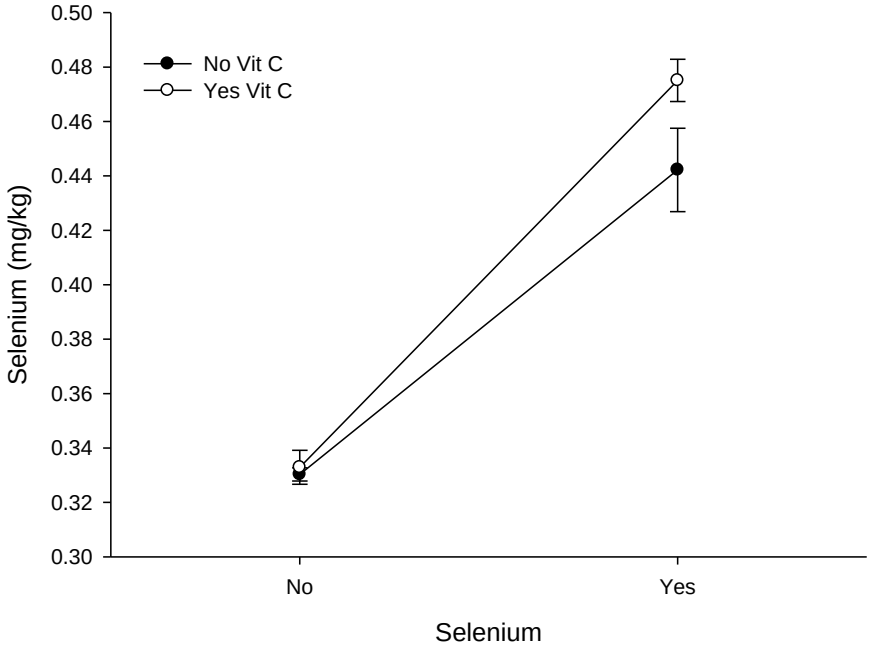

Figure 4.4b Interaction effect of added vitamin C and selenium in the diet on mean selenium concentration ($mg.kg^{-1}$) in the muscle of YTK (n=6)

Muscle parameters - Vitamins and selenium
There was a significant main effect for vitamin C on fillet vitamin C concentration (mean 14.2 ± 0.5 $mg.kg^{-1}$, $P < 0.001$) compared to the no added vitamin C fillet concentration (mean 2.1 ± 0.2 $mg.kg^{-1}$). There was a significant interaction effect for vitamin E and selenium on muscle vitamin E concentration (mean 3.1 ± 0.2 $mg.kg^{-1}$, P=0.009). With the main effect vitamin E (mean 2.4 ± 0.2 $mg.kg^{-1}$) contributing strongly to the interaction with an added effect for selenium (Figure 4.3).

There was a significant interaction effect for selenium and vitamin E (mean 0.47 ± 0.01 $mg.kg^{-1}$, P = 0.003), and selenium and vitamin C (mean 0.48 ± 0.01 $mg.kg^{-1}$, P= 0.021) on muscle selenium concentration. The main effect of selenium was strong within these interactions (Figure 4.4a, b respectively).

Liver parameters - Vitamins and selenium
There was a significant main effect for vitamin C on liver concentration of vitamin C (mean 57.1 ± 2.0 $mg.kg^{-1}$, P<0.001) compared to no added vitamin C (mean 7.5 ± 0.4 $mg.kg^{-1}$). However, there were no significant interaction effects.

There was a significant main effect for vitamin E on liver concentrations of vitamin E (mean 20.5 ± 1.6 $mg.kg^{-1}$, P<0.001) compared to no added vitamin E (mean 5.3 ± 0.8 $mg.kg^{-1}$). There was also a significant main effect for vitamin C on liver concentrations of vitamin E (mean 14.9 ± 2.8 $mg.kg^{-1}$, P=0.023) compared to no added vitamin C (mean 10.9 ± 2.3 $mg.kg^{-1}$). However, there were no significant interaction effects.

There was a significant main effect for selenium on liver concentrations of selenium (mean 2.4 ± 0.1 $mg.kg^{-1}$, P<0.001) compared to no added selenium (mean 2.0 ± 0.1 $mg.kg^{-1}$). There was also a significant main effect for vitamin C on liver concentrations of selenium (mean 2.5 ± 0.1 $mg.kg^{-1}$, P<0.001) compared to no added vitamin C (mean 2.0 ± 0.1 $mg.kg^{-1}$). However, there were no significant interaction effects.

Post mortem change
There was a significant main effect for selenium on malonaldehyde concentration on fish muscle at harvest (mean 0.12 ± 0.02 $mg.kg^{-1}$, P=0.049) compared to no added selenium (mean 0.09 ± 0.06 $mg.kg^{-1}$). There were no significant interaction effects. The data was not normally distributed however the test was still run and therefore should be interpreted with caution.

There were no significant main or interaction effects for added vitamins and selenium on liver malonaldehyde concentration at harvest. There was a significant main effect for vitamin C on malonaldehyde concentration on fish muscle stored at 4°C for 5 days post harvest (mean 1.3 ± 0.1 $mg.kg^{-1}$, P=0.001) compared to no added vitamin C (mean 1.0 ± 0.0 $mg.kg^{-1}$).

Figures showing significant main effects and the statistic tables appear in Appendix 2.

4.2.4 Discussion

It is not known why there was an interaction effect on proximate analysis. The result implies that the additional vitamin E and C influenced the composition of the fish flesh; however the difference in the fillet composition was extremely small and unlikely to be of any commercial significance.

SGR was also influenced by the level of vitamin E and C however due to the interaction there is not a clear picture of how this might practically be useful to diet formulation. Nevertheless the higher of vitamin C and or vitamin E appear to have had a small positive effect on SGR and HSI. This is not surprising as there a dearth of information supporting the inclusion of these nutrients, for optimal growth and health, at similar or higher levels than used in the high treatments of the current study recently including Sau, *et al*. (2004). There was also a significant difference in the mortality of fish from treatments that did not receive the higher level of vitamin E or C. Higher deaths in the treatment that received only selenium are unlikely to have resulted from selenium toxicity. The selenium inclusion was an organic form of selenium that is recognised for low toxicity - even at very high doses (Surai 2002). It is more likely that the mortality the occurred was related to the low levels of vitamin E or C of the treatments.

Not surprisingly, fish that received higher vitamin C diets had higher levels in the flesh and the liver. This was also the case for liver selenium and vitamin E concentrations. Interestingly dietary selenium had had some effect on muscle vitamin E and vitamin C inclusion directly affected liver vitamin E concentration. This is evidence supporting a sparing effect of these nutrients on each other however the mechanism is not well understood. There was also evidence suggesting that the inclusion of higher vitamin C results in higher liver selenium, but again if a sparing mechanism is occurring here it is not well understood.

Post mortem there was little evidence that the treatment diets had any effect on oxidation processes. Although selenium added to the diet did show some indication of influencing malonaldyhde fillet concentration post mortem, the effect was small and difficult to interpret clearly.

Overall this experiment did show that there is some interaction between the nutrient components that researchers within this project have been adding to the diets of SBT. Although the evidence does not directly indicate a synergistic effect on in-vivo activity of these nutrients on oxidative processes there is evident that additions of more than one of the components can influence the tissue level of another. The real benefit of these nutrients to the production and post mortem quality attributes of this surrogate would probably be best realised in a situation where the diet was high in fat which has oxidised. This is a difficult scenario to create experimentally (with a surrogate) and with the vast improvements made

within the tuna farming industry over the past 10 years, is unlikely to occur (chronically) within industry practices.

4.2.5 Acknowledgements

The authors would like to thank the staff at SARDI for their hard work and cooperation in running this experiment at West Beach. We would also like to thank DSM Nutritional Products for the donation of the nutrients used in this experiment**.**

4.2.6 References

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5.0 Cell culture

The research reported in section 5.1 was carried out by PhD candidate Alex Korte as part of her postgraduate studies. The research reported in section 5.2 was carried out by Josephine Nocillado as part of FRDC Project. 2007/221.

5.1 2004-2007: Development of a southern bluefin tuna cell line and its use to screen dietary antioxidants to improve fish flesh quality

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Introduction

The DCFH-DA assay has been adapted for use with the SBTG-1 cell line and a broad range of antioxidants has been screened using the DCFH-DA assay and the SBTG-1 cell line. Two commercially available products, NatuRose® (a carotenoid extract from *Haematococcus* microalgae) and Vin*life*® (a grape seed extract) were both identified as very effective antioxidants in the *in vitro* system.
Unfortunately, during the course of this work, it was discovered that the SBTG-1 cell line actually originates from fathead minnow (*Pimephales promelas*) and not from southern bluefin Tuna (SBT). The designation SBTG-1 has been retained to avoid confusion. The species of origin of the SBTG-1 cell line was discovered using PCR and karyotyping. We suspect that the SBTG-1 cell line became contaminated with an EPC cell line supplied to us by the Australian Animal Health Laboratory, CSIRO, Geelong (we refer to this cell line as EPC-Geelong). The original EPC cell line was derived from carp (*Cyprinus carpio*). However, our testing (PCR and karyotyping) has shown that EPC-Geelong originates from fathead minnow. Interestingly, EPC cell line CRL-2872™ held by the ATCC (an internationally-recognized repository of cell lines for research) also originates from fathead minnow. Thus, it appears that contamination of supposed EPC cell lines by Fathead minnow cell lines has occurred several times and even in internationally-recognized laboratories. Recently, during August 2008, we PCR-tested several very early passages of the cell cultures derived from SBT gonad and kidney tissues. Unfortunately, these were also contaminated with fathead minnow cells.

This report is divided into the following subsections:

(a) Adaptation of the DCFH-DA assay for use with the SBTG-1 cell line
(b) Adaptation of the Neutral Red cell viability assay for use with the SBTG-1 cell line
(c) Screening of a broad range of antioxidants using the DCFH-DA and Neutral Red assays with the SBTG-1 cell line
(d) Identification of the species of origin of the SBTG-1, SBTK and EPC-Geelong cell lines

Although this work has not been done with SBT, we nevertheless believe it has made a valuable contribution towards the development of fish cell lines as a platform for testing the effectiveness of antioxidants in preserving flesh quality. The only previous work done in this area was with an EPC cell line investigating the effects of pro-oxidants and antioxidants on cell viability using the Neutral Red assay (George *et al*., 2000; Wright *et al*., 2000). Here we report the use of the DCFH-DA assay in tandem with the Neutral Red assay and, in addition, we have screened a broader range of antioxidants than the earlier researchers. The Neutral Red assay is a simple end-point assay indicating whether the cells are alive or dead whereas the DCFH-DA assay measures the production of oxygen radicals and their quenching by antioxidants inside living cells. In the DCFH-DA assay, only antioxidants that are taken up by cells give a result. Thus, the DCFH-DA assay is a better indicator of antioxidant activity than the Neutral Red assay. In addition, our work to date has highlighted two commercially available antioxidant preparations (NatuRose® and Vin*life*®) that are worthy of further testing as dietary antioxidant supplements in fish feeding trials.

5.1.1 Adaptation of the DCFH-DA assay for use with the SBTG-1 cell line

5.1.1.2 Introduction

The DCFH-DA assay was adapted from the method described by Takamatsu *et al*. (2003). DCFH-DA (dichlorofluorescein-diacetate) is a non-polar compound that readily enters cells. Inside cells, it is converted by esterase enzymes to DCFH which is polar and therefore unable to leak out of the cells. Cells treated with pro-oxidants produce reactive oxygen species (ROS) such as the highly reactive but short-lived hydroxyl radical which initiates lipid peroxidation and the consequent oxidative deterioration of fish flesh. In the presence of the hydroxyl radical and other ROS, DCFH which is non-fluorescent is converted to DCF which is fluorescent. The fluorescence intensity can be measured using a fluorometer. Fluorescence should be proportional to the concentration of the pro-oxidant and antioxidants should cause fluorescence quenching. In the DCFH-DA assay, only antioxidants that are taken up by cells give a result.

5.1.1.3 Methods

For routine maintenance, the SBTG-1 cell line was cultured in 20ml of Leibovitz's L-15 growth medium supplemented with 10% (v/v) foetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin in 75 cm^2 cell culture flasks at 25^oC. The cells grew as a monolayer attached to the surface of the flask. Prior to experiments, the growth medium was decanted, the surface of the cell monolayer was rinsed with phosphate buffered saline (PBS) and the cells were detached from the flask by incubation with 0.25% trypsin-EDTA (Invitrogen catalogue no. 25200-056). The detached cells were harvested by centrifugation and the cell pellet was resuspended in the appropriate volume of fresh growth medium. The resuspended cells were then seeded into the required number of 96-well plates at a density of 1.5 x 10^4 cells per well in 200 μl of growth medium. After 4 days incubation at 25^oC, the growth medium was removed and replaced with 100μl of

20μM DCFH-DA (Invitrogen catalogue no. D-399) diluted in Hanks Balanced Salt Solution (HBSS; Invitrogen catalogue no. 14185-052). Working solutions of DCFH-DA in HBSS were prepared fresh immediately before experiments from a stock solution of 10 mM DCFH-DA dissolved in DMSO. All experiments with DCFH-DA were performed in a darkened room. The cells were incubated with DCFH-DA for 15 minutes to allow time for the probe to be taken up and then 100 μl of various concentrations of hydrogen peroxide was added and fluorescence was measured immediately, after 30 minutes and after 60 minutes. The fluorescence measurements were done using a fluorometer (Fluoroskan Ascent® from Labsystems) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

5.1.1.4 Results

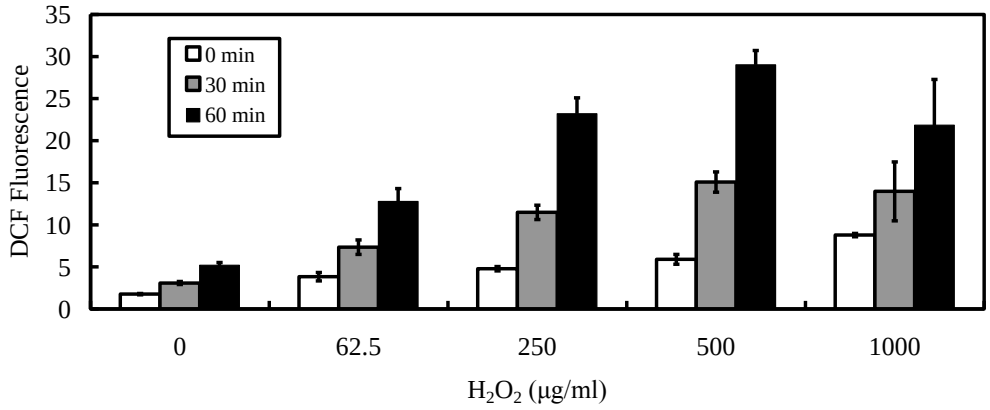

Figure 5.1. Effect of hydrogen concentration and time of exposure on DCF fluorescence in the SBTG-1 cells. The columns represent the mean of three independent experiments and the vertical bars represent the standard error of the mean. SBTG-1 cells at passages 19, 25 and 30 were used.

5.1.1.5 Discussion

For the DCFH-DA assay to be valid, fluorescence should increase with increasing concentrations of hydrogen peroxide and increasing time of exposure to hydrogen peroxide. Figure 5.1 shows that both of these conditions were met. Based on these results, the 60 minute exposure was chosen for all subsequent experiments.

5.1.2 Adaptation of the Neutral Red assay for use with the SBTG-1 cell line

5.1.2.1 Introduction

The Neutral Red cell viability assay was adapted from the method of Borenfreund *et al*. (1990) and Babich and Borenfreund (1990). The Neutral Red dye is taken up by living cells but not by dead cells. Thus, living cells stain red and the intensity, which is proportional to the number of living cells, can be measured at a wavelength of 550nm using a microplate reader. A decrease in

fluorescence measured using the DCFH-DA assay could be due to fluorescence quenching by an antioxidant or it could be due to cell death. The Neutral Red assay distinguishes between these two different possibilities.

5.1.2.2 Methods

The SBTG-1 cells were prepared as described for the DCFH-DA assay and the required number of 96-well plates was seeded with the required number of SBTG-1 cells in 200 μl of growth medium. After the appropriate amount of time (depending on the experiment), the growth medium was removed and 200 μl of Neutral Red dye (50 μg ml^{-1}) was added to each well. After 3 h incubation at 25^{o}C, the Neutral red dye was removed and the cells were fixed in 0.5% (v/v) formaldehyde:1% (w/v) $CaCl_2$. After 1 min, the fixative was removed and 200 μl of 1% (v/v) acetic acid:50% (v/v) ethanol was added to extract the Neutral red dye from the cells. After 20 min incubation, the absorbance was read at 550 nm using a microplate reader.

5.1.2.3 Results

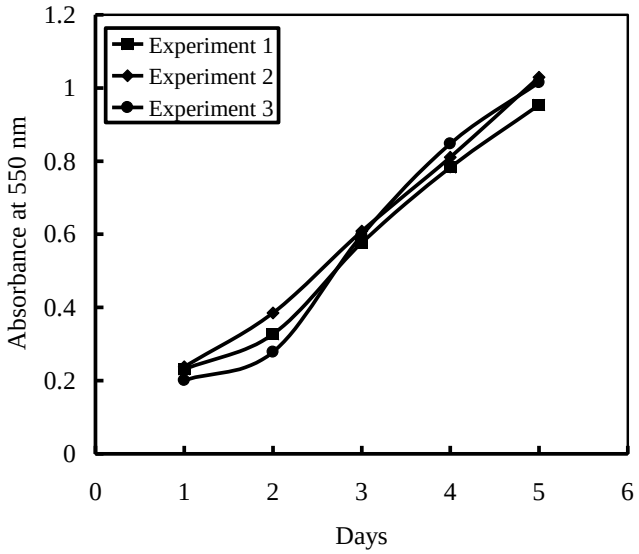

Figure 5.2. Proliferation of the SBTG-1 cells determined using the Neutral Red assay. The required number of 96-well plates was seeded with SBTG-1 cells at a density of 1.5 x 10^4 cells per well in 200 μl of growth medium. Each day, one of the 96-well plates was used to determine cell viability using the Neutral Red assay. The experiment was repeated 3 times with SBTG-1 cells at passages 19, 25 and 30.

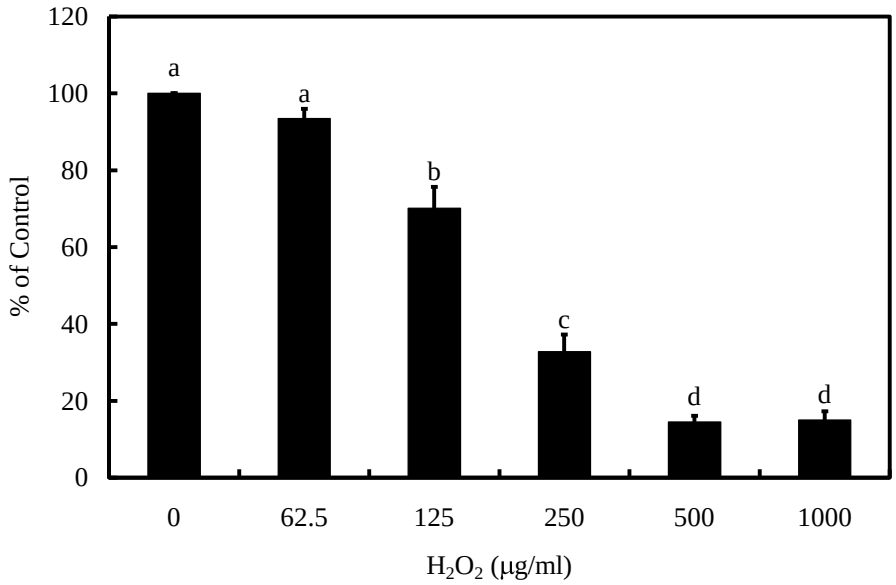

Figure 5.3. Effect of hydrogen peroxide concentration on the viability of the SBTG-1 cells determined using the Neutral Red assay. The required number of 96-well plates was seeded with SBTG-1 cells at a density of 1.5 x 10^4 cells per well in 200 μl of growth medium. After 4 days, the original growth medium was replaced with fresh growth medium containing various concentrations of hydrogen peroxide. After 1 hour exposure to hydrogen peroxide, cell viability was determined using the Neutral Red assay. The data are expressed as % of control where the control is the untreated cells. Each value is the mean of 3 experiments. The vertical bars represent the standard error of the mean. Values that are significantly different ($P<0.05$) have different superscripts. The SBTG-1 cells used in the three separate experiments were at passages 19, 25 and 30.

5.1.2.3 Discussion

Absorbance at 550 nm due to the Neutral Red dye increased over time as the SBTG-1 cells proliferated (Figure 5.2). Between days 2 and 5, there was rapid proliferation of the cells following a short lag between days 1 and 2. These results suggested that day 4 would be the best day to treat the cells with hydrogen peroxide to determine its effect on cell viability. The results of the hydrogen peroxide treatment are shown in Figure 5.3. Increasing concentrations of hydrogen peroxide decreased cell viability. The $LD_{50}(H_2O_2)$ or the concentration of hydrogen peroxide causing death of approximately 50% of the cells was in the range 125 – 250 μg ml^{-1}. Thus, 250 μg ml^{-1} was the hydrogen peroxide chosen for testing the protective effects of the antioxidants (see below). The decrease in cell viability with increasing hydrogen peroxide concentration measured using the Neutral Red assay (Figure 5.3) was inversely related to the increase in ROS production (DCF fluorescence) measured using the DCFH-DA assay (Figure 5.1). This provides evidence that cell death was due to ROS production initiated by hydrogen peroxide treatment.

Prior to our work, the effects of hydrogen peroxide and t-butyl hydroperoxide (an organic peroxide) on cell viability had previously been investigated in EPC cells

by Wright *et al*. (2000) and George and Wright (2000). In their work, the $LD_{50}(H_2O_2)$ was 50 – 100 μg ml^{-1} when the cells were grown in L-15 growth medium but only 5 – 10 μg ml^{-1} when the cells were grown in MEM growth medium. In addition, their exposure time to hydrogen peroxide was 48 hours whereas ours was only 1 hour. Since their $LD_{50}(H_2O_2)$ in L-15 growth medium was similar to ours, this suggests that a 1 hour exposure to hydrogen peroxide is sufficient and no extra effect is seen with a 48 hour exposure. It is also apparent that the growth medium makes a difference to the susceptibility of cells to hydrogen peroxide. Wright *et al*. (2000) found that EPC cells grown in L-15 growth medium had higher concentrations of glutathione and metallothionein and higher activities of glutathione-dependent enzymes than cells grown in MEM growth medium and George *et al*. (2000) found that cells grown in MEM growth medium were vitamin E deficient and that adding vitamin E to the growth medium counteracted the cytotoxic effect of t-butyl hydroperoxide. It could be worthwhile to investigate the effects of different growth media on the hydrogen peroxide sensitivity of the SBTG-1 cell line.

Rau *et al*. (2004) compared a fish cell line (PLHC-1, topminnow hepatocellular carcinoma) with a mammalian cell line (II-E, rat hepatoma) and concluded that the fish cell line was more susceptible than the mammalian cell line to oxidative stress induced by either copper sulphate or Fenton reagents. Fenton reagents are mixtures of Fe^{2+} and H_2O_2 which generate the highly reactive but short-lived hydroxyl radical, the most damaging of all ROS. Rau *et al*. (2004) concluded that the greater sensitivity of the fish cell line might be due to the higher proportion of long-chain polyunsaturated fatty acids (LC-PUFA) in fish lipids as compared with mammalian lipids. However, in other studies it has been observed that the fatty acid composition of fish cells in culture changes to reflect the fatty acid composition of the foetal bovine serum (FBS) in the cell culture growth medium (e.g. Tocher *et al*., 1989; Tocher and Dick, 1999). Thus, fish cells in culture have a lower proportion of LC-PUFA in their lipids than the fish tissues they originate from. In a related project, we are investigating the impact of the growth medium PUFA composition on the cellular PUFA composition of SBTG-1 cells.

5.1.3 Screening of a broad range of antioxidants using the DCFH-DA and Neutral Red assays with the SBTG-1 cell line

5.1.3.1 Introduction

Nine different antioxidants were tested to determine whether or not they could protect the SBTG-1 cells against the damaging effects of hydrogen peroxide treatment. Both the DCFH-DA assay and the Neutral Red assay were used. The antioxidants tested were NatuRose® (a carotenoid extract from *Haematococcus* microalgae supplied by Cyanotech Corporation, Kona, Hawaii), astaxanthin (Sigma-Aldrich catalogue no. A9335), Vin*life*® (a grape seed extract supplied by Tarac Technologies Pty. Ltd., Nuriootpa, South Australia), Trolox™ (a water soluble analogue of vitamin E, Sigma-Aldrich catalogue no. 238813), sodium L-ascorbate (the sodium salt of vitamin C, Sigma-Aldrich catalogue no. A7631),

butylated hydroxytoluene (BHT, Sigma-Aldrich catalogue no. B1378), ethoxyquin (Sigma-Aldrich catalogue no. E8260), sodium selenite (a source of selenium, Sigma-Aldrich catalogue no. S5261) and Herbalox® (a rosemary oleoresin supplied by Kalsec® (Kalamazoo, MI, United States of America).

5.1.3.2 Methods

The required number of 96-well plates was seeded with SBTG-1 cells at a density of 1.5 x 10^4 cells per well in 200 μl of growth medium as described above. After 3 days incubation at 25°C, the growth medium was removed and replaced with fresh growth medium containing the antioxidant to be tested. The cells were incubated with the antioxidant for 24 hours. At the end of the 24 hour incubation period, half of the 96-well plates were used for the DCFH-DA assay and the other half were used for the Neutral Red assay as described above. The Neutral Red assay was used to measure the number of viable cells relative to the control which was the no hydrogen peroxide, no antioxidant treatment. The DCFH-DA assay was used to measure the abundance of intracellular reactive oxygen species (ROS) relative to the control which was the 250 μg ml^{-1} H_2O_2, no antioxidant treatment. ROS include the lipid peroxyl radicals involved in lipid peroxidation as well as other oxygen-derived radicals such as the superoxide, hydroxyl and hydroperoxyl radicals. All ROS have the potential to damage cellular lipids, proteins and DNA.

5.1.3.3 Results

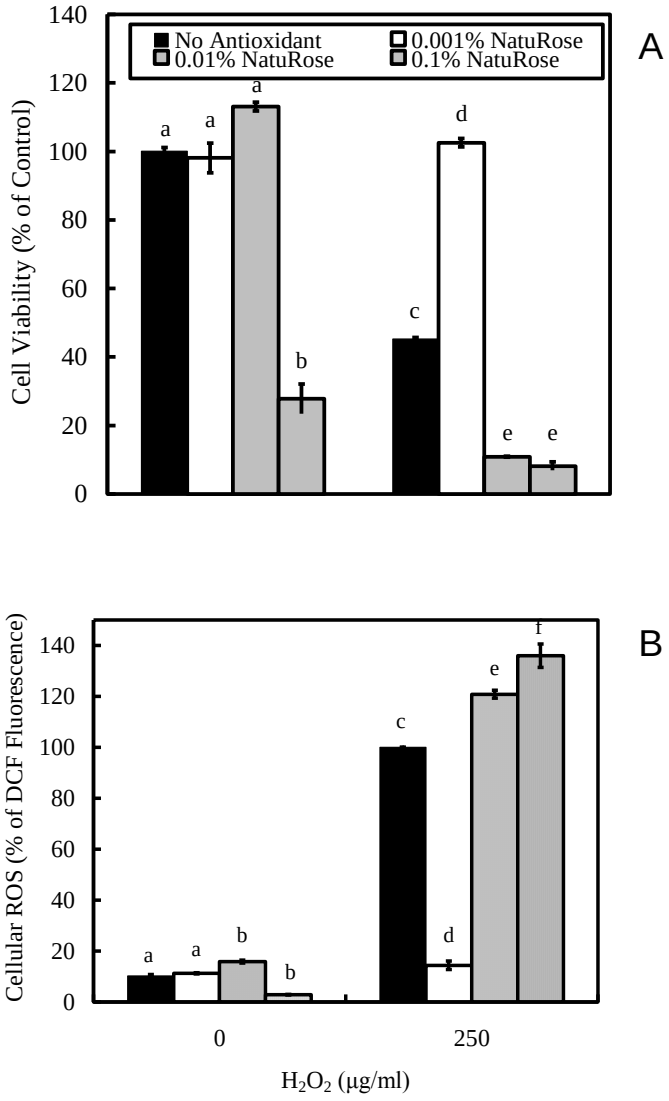

Figure 5.4. Effect of NatuRose® on cell viability (A) and intracellular ROS abundance (B) in the absence and presence of hydrogen peroxide. In panel A, the control was the untreated SBTG-1 cells. In panel B, the control was the SBTG-1 cells exposed to 250 μg/ml hydrogen peroxide without any added antioxidant. The columns represent the mean of three independent experiments. The vertical bars represent the standard error of the mean. Values that have different superscripts are significantly different ($P<0.05$). The SBTG-1 cells used in the three separate experiments were at passages 19, 25 and 30.

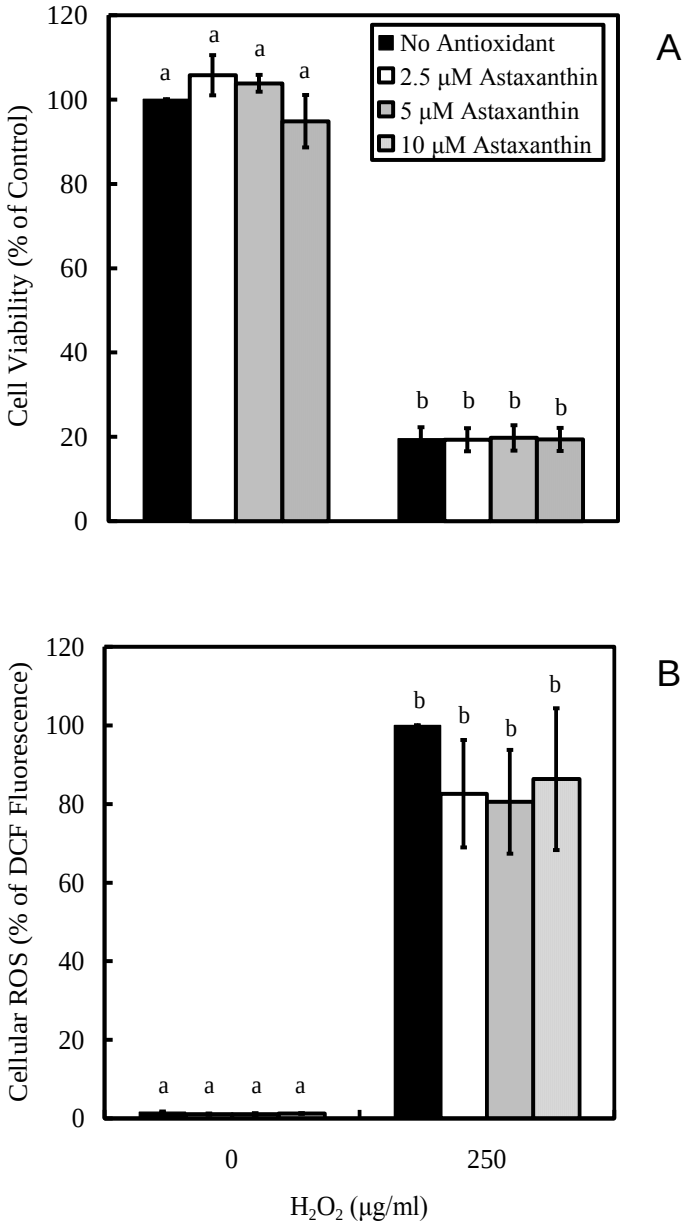

Figure 5.5. Effect of Astaxanthin on cell viability (A) and ROS abundance (B) in the absence and presence of hydrogen peroxide. In panel A, the control was the untreated SBTG-1 cells. In panel B, the control was the SBTG cells exposed to 250 μg/ml hydrogen peroxide without any added antioxidant. The columns represent the mean of three independent experiments. The vertical bars represent the standard error of the mean. Values that have different superscripts are significantly different ($P<0.05$). The SBTG-1 cells used in the three separate experiments were at passages 19, 25 and 30.

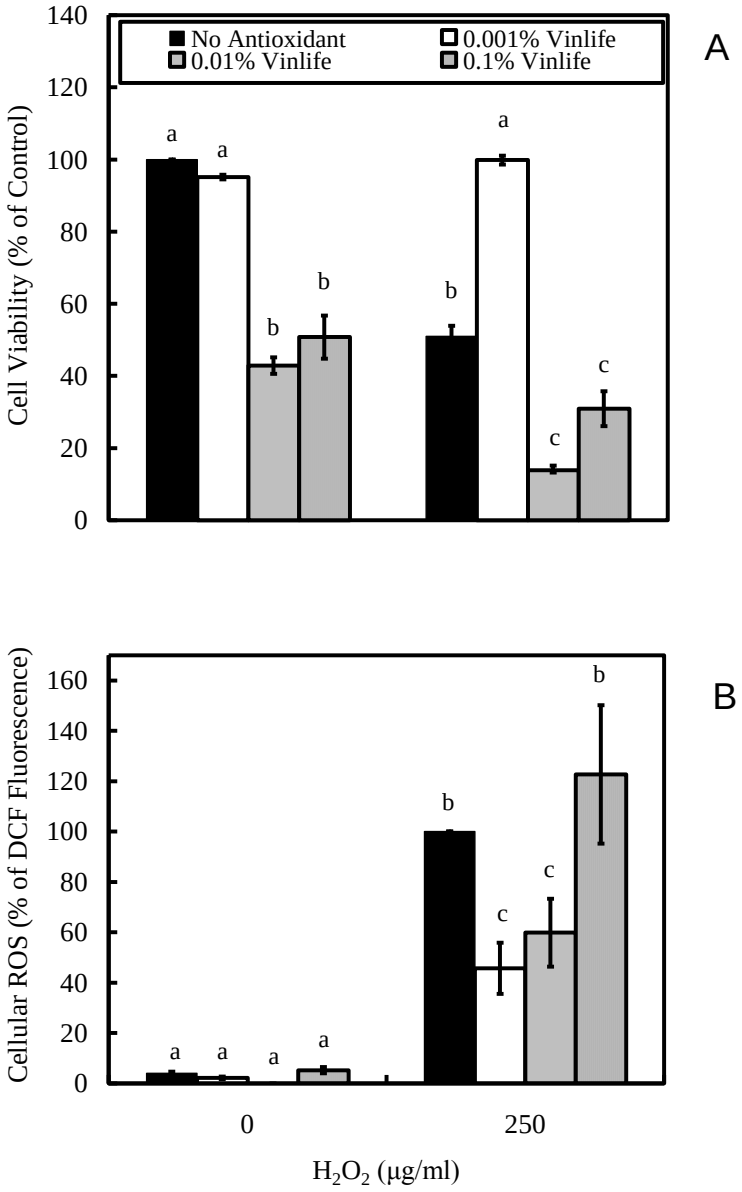

Figure 5.6. Effect of Vin*life*® on cell viability (A) and ROS abundance (B) in the absence and presence of hydrogen peroxide. In panel A, the control was the untreated SBTG-1 cells. In panel B, the control was the SBTG-1 cells exposed to 250 μg/ml hydrogen peroxide without any added antioxidant. The columns represent the mean of three independent experiments. The vertical bars represent the standard error of the mean. Values that have different superscripts are significantly different ($P<0.05$). The SBTG-1 cells used in the three separate experiments were at passages 19, 25 and 30.

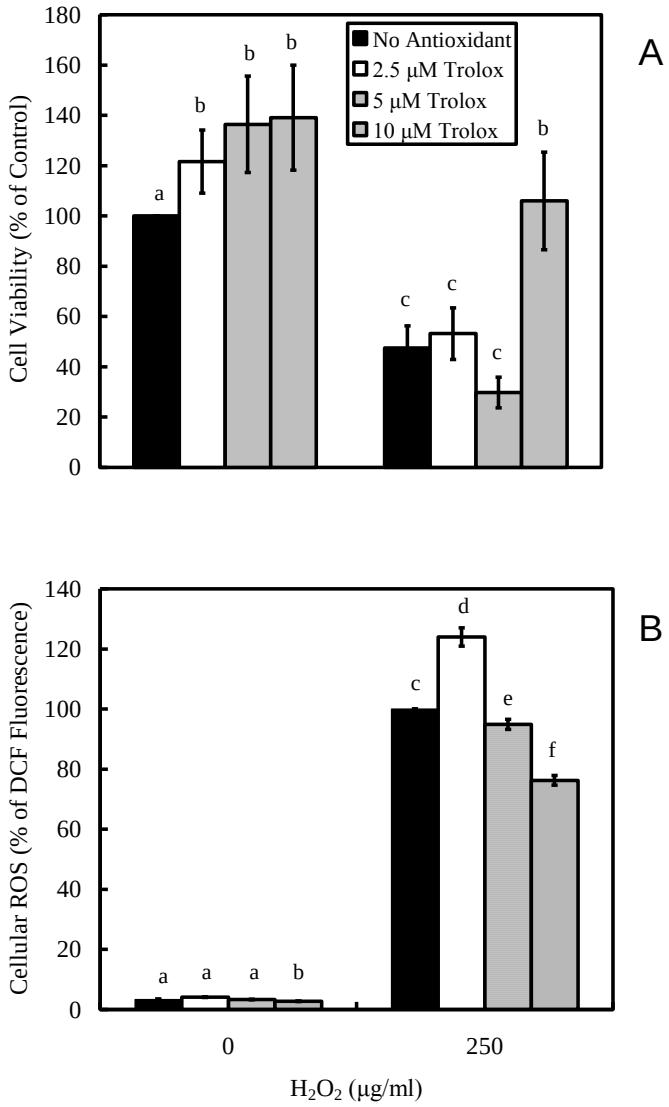

Figure 5.7. Effect of Trolox™ on cell viability (A) and ROS abundance (B) in the absence and presence of hydrogen peroxide. In panel A, the control was the untreated SBTG-1 cells. In panel B, the control was the SBTG-1 cells exposed to 250 μg/ml hydrogen peroxide without any added antioxidant. The columns represent the mean of three independent experiments. The vertical bars represent the standard error of the mean. Values that have different superscripts are significantly different ($P<0.05$). The SBTG-1 cells used in the three separate experiments were at passages 19, 25 and 30.

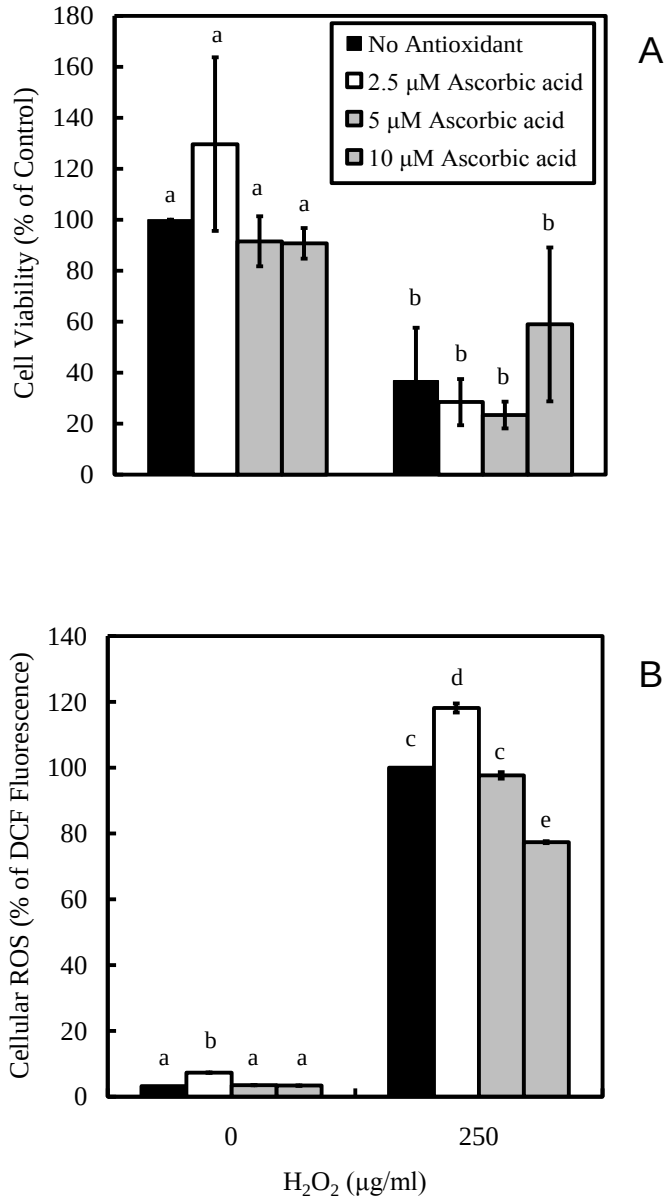

Figure 5.8. Effect of ascorbic acid on cell viability (A) and ROS abundance (B) in the absence and presence of hydrogen peroxide. In panel A, the control was the untreated SBTG-1 cells. In panel B, the control was the SBTG-1 cells exposed to 250 μg/ml hydrogen peroxide without any added antioxidant. The columns represent the mean of three independent experiments. The vertical bars represent the standard error of the mean. Values that have different superscripts are significantly different ($P<0.05$). The SBTG-1 cells used in the three separate experiments were at passages 19, 25 and 30.

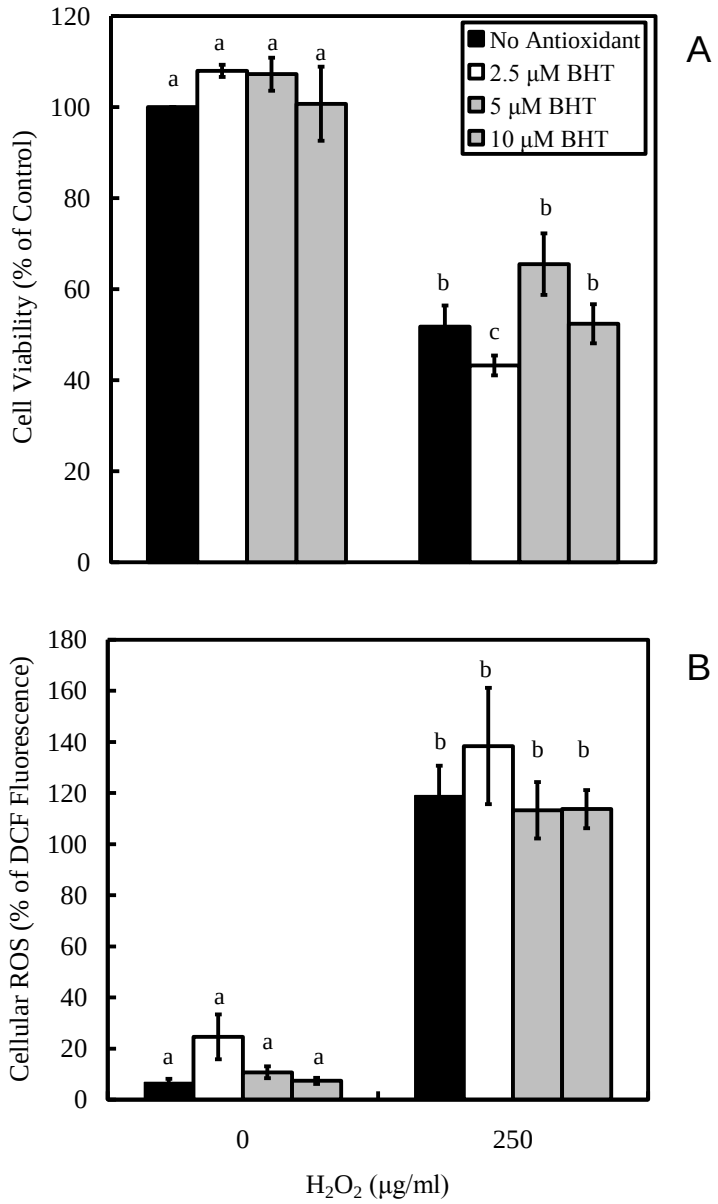

Figure 5.9. Effect of BHT on cell viability (A) and ROS abundance (B) in the absence and presence of hydrogen peroxide. In panel A, the control was the untreated SBTG-1 cells. In panel B, the control was the SBTG-1 cells exposed to 250 μg/ml hydrogen peroxide without any added antioxidant. The columns represent the mean of three independent experiments. The vertical bars represent the standard error of the mean. Values that have different superscripts are significantly different ($P<0.05$). The SBTG-1 cells used in the three separate experiments were at passages 19, 25 and 30.

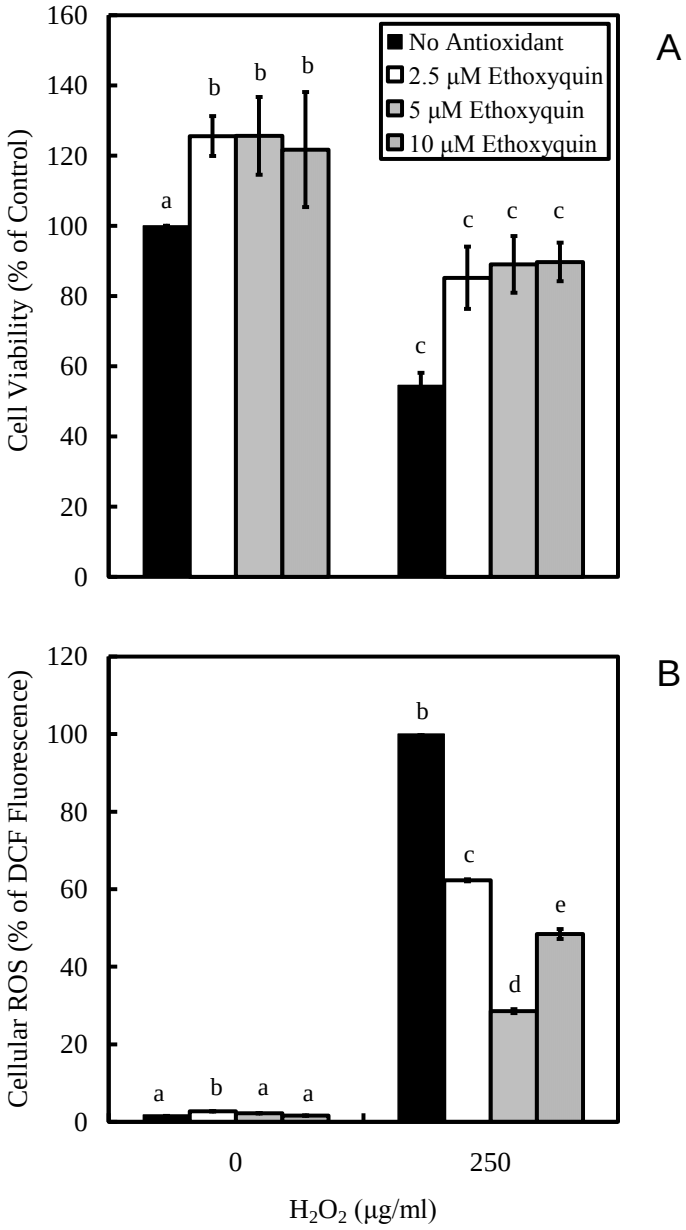

Figure 5.10. Effect of ethoxyquin on cell viability (A) and ROS abundance (B) in the absence and presence of hydrogen peroxide. In panel A, the control was the untreated SBTG-1 cells. In panel B, the control was the SBTG-1 cells exposed to 250 μg/ml hydrogen peroxide without any added antioxidant. The columns represent the mean of three independent experiments. The vertical bars represent the standard error of the mean. Values that have different superscripts are significantly different ($P<0.05$). The SBTG-1 cells used in the three separate experiments were at passages 19, 25 and 30.

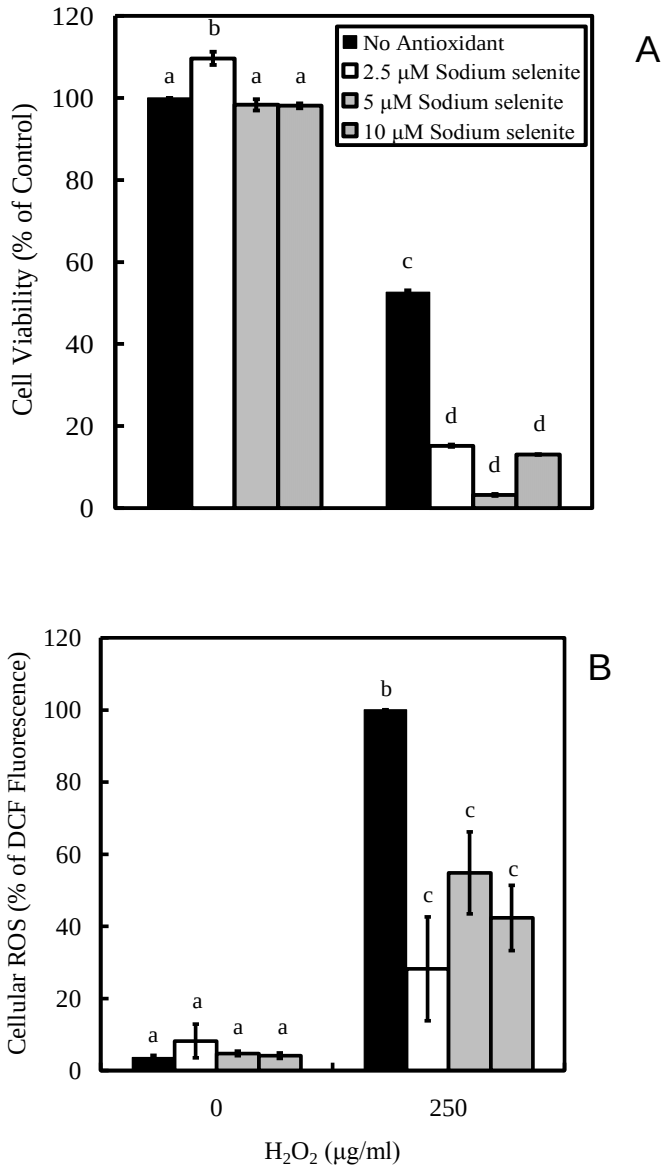

Figure 5.11. Effect of sodium selenite on cell viability (A) and ROS abundance (B) in the absence and presence of hydrogen peroxide. In panel A, the control was the untreated SBTG-1 cells. In panel B, the control was the SBTG-1 cells exposed to 250 μg/ml hydrogen peroxide without any added antioxidant. The columns represent the mean of three independent experiments. The vertical bars represent the standard error of the mean. Values that have different superscripts are significantly different ($P<0.05$). The SBTG-1 cells used in the three separate experiments were at passages 19, 25 and 30.

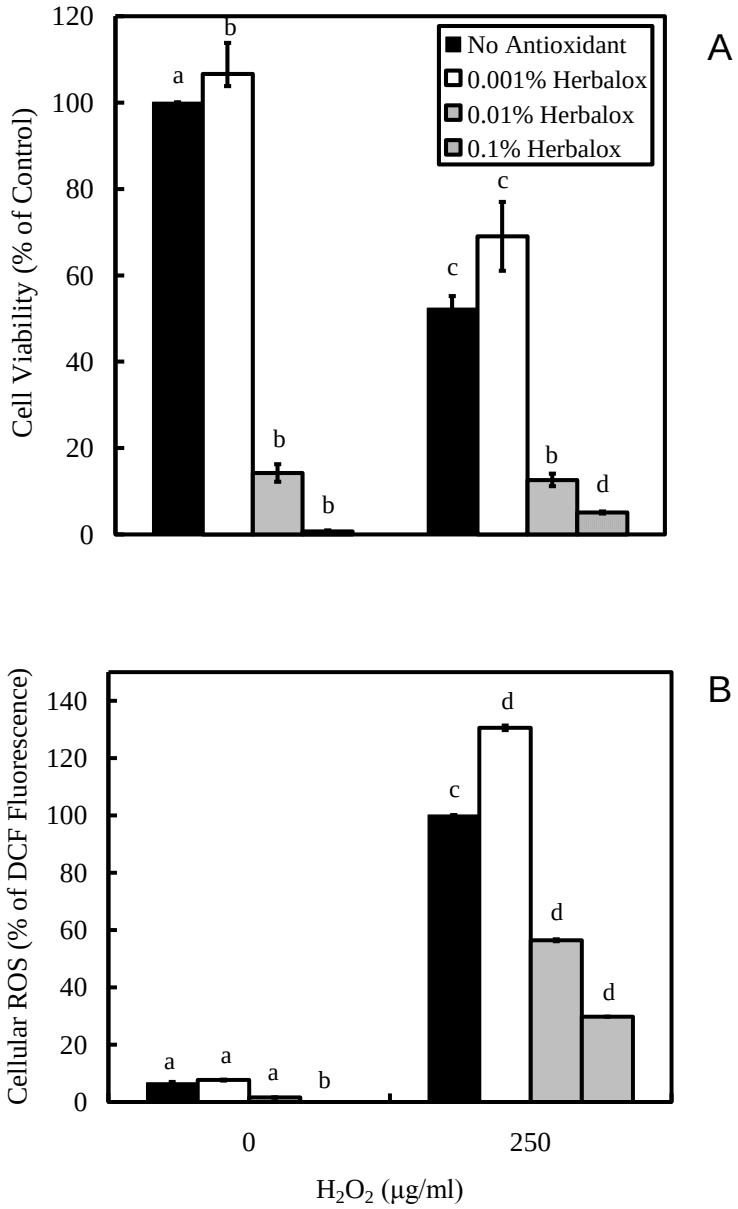

Figure 5.12. Effect of Herbalox® on cell viability (A) and ROS abundance (B) in the absence and presence of hydrogen peroxide. In panel A, the control was the untreated SBTG-1 cells. In panel B, the control was the SBTG-1 cells exposed to 250 μg/ml hydrogen peroxide without any added antioxidant. The columns represent the mean of three independent experiments. The vertical bars represent the standard error of the mean. Values that have different superscripts are significantly different ($P<0.05$). The SBTG-1 cells used in the three separate experiments were at passages 19, 25 and 30.

5.1.3.4 Discussion

Figure 5.4 shows the effects of NatuRose® on cell viability and intracellular ROS abundance. NatuRose® was a very effective antioxidant. At a concentration of only 0.001% (w/v), it significantly increased cell viability and at the same time significantly decreased intracellular ROS abundance in the presence of 250 μg ml^{-1} hydrogen peroxide. NatuRose® is a carotenoid extract from *Haematococcus* microalgae produced by Cyanotech Corporation (Kona, Hawaii). It contains a mixture of beta-carotene (6%), canthaxanthin (5%), lutein (4%), free astaxanthin (5%), astaxanthin diester (10%) and astaxanthin monoester (70%). NatuRose® is marketed for enhancing the pigmentation of salmonids and ornamental fish. It has not previously been investigated as an antioxidant supplement for fish feeds. Our results indicate that it should be investigated in this way.

Figure 5.5 shows the effects of astaxanthin on cell viability and intracellular ROS abundance. Astaxanthin had no significant effect on either of these parameters. This was surprising given the striking effect of NatuRose®. This suggests that there is something about the complex mixture of carotenoids in NatuRose® that is important and necessary for the antioxidant effect. Many authors have argued that "whole foods" are more effective as antioxidants than any of their individual chemical constituents. For example, there is evidence that the mixture of carotenoids in tomatoes is more effective than lycopene alone in reducing the risk of prostate cancer (Gann and Khachik, 2003; Boileau *et al*., 2003).

However, there could also be other explanations. Free astaxanthin and the other carotenoids in NatuRose® are only sparingly water-soluble. Thus, it is surprising that NatuRose® had any effect at all on the SBTG-1 cells, i.e., astaxanthin and the other carotenoids in NatuRose® should not have dissolved in the cell culture medium and therefore they should not have been able to interact with the cells. In mammalian cell culture research, it is usual to dissolve lipophilic antioxidants in organic solvents before diluting them in the cell culture medium. This ensures that they dissolve in the cell culture medium and interact with the cells. For example, in a recent study of the effects of carotenoids on a human breast cancer cell line, astaxanthin, β-carotene and lycopene were dissolved in tetrahydrofuran (THF) containing 0.025% butylated hydroxytoluene as an antioxidant (Hirsch *et al*., 2007) and this seems to be a common practice. Interestingly, in an earlier study, this same group of researchers showed that spontaneously formed hydrophilic oxidation products of lycopene were just as effective in eliciting an antioxidant response as lycopene itself (Ben-Dor *et al*., 2005). Nara et al. (2001) specifically focussed on the hydrophilic oxidation products of lycopene and other tomato carotenoids and their effects on cells in culture. They found that when they deliberately oxidized tomato carotenoids before adding them to cell culture media, the anti-cancer effect on HL-60 human promyelocytic leukaemia cells was greater than it was for the intact carotenoids before oxidation. The important point here is that these highly effective spontaneous oxidation products are more hydrophilic than their parent carotenoids. Thus, since in our study we dissolved NatuRose® directly in the cell

culture medium, it is likely that the antioxidant effects we observed were due to the hydrophilic oxidation products of the NatuRose® carotenoids and not the intact carotenoids themselves. It will be interesting to identify these oxidation products. They should be more stable than their parent compounds and a better understanding of the storage conditions leading to their formation may have practical applications. It will also be worthwhile to compare the effectiveness of NatuRose® dissolved in organic (lipophilic) solvents with NatuRose® dissolved in aqueous (hydrophilic) solvents. This should differentiate between the lipophilic and hydrophilic constituents of the NatuRose® carotenoids and their spontaneously oxidized derivatives.

Figure 5.6 shows the effects of Vin*life*® on cell viability and intracellular ROS abundance. Vin*life*®, like NatuRose®, was a very effective antioxidant. At a concentration of only 0.001% (w/v), Vin*life*® significantly increased cell viability and at the same time significantly decreased intracellular ROS abundance in the presence of 250 μg ml^{-1} hydrogen peroxide. The antioxidants in Vin*life*® are condensed tannins, also known as oligomeric proanthocyanins (OPCs) (Tarac Technologies product information, www.tarac.com.au). OPCs are polymers of flavonoids (Ververidis *et al*., 2007). Important flavonoids with significant antioxidant activity include quercetin and various catechins. In contrast to carotenoids, OPCs and flavonoids are highly water soluble. Thus, they are directly compatible with the aqueous media used in cell culture systems. Recent studies have shown that grape skin and seed preparations can significantly improve the oxidative stability of lipids in processed meat and fish products. For example, grape antioxidant dietary fibre (GADF, processed red grape skins and seeds) mixed with minced horse mackerel (*Trachurus trachurus*) muscle tissue was shown to significantly reduce lipid oxidation during frozen storage at -20^{o}C over a period of 90 days (Sánchez-Alonzo *et al*., 2006). Similarly, a grape seed extract added to minced turkey meat prior to cooking and subsequent cold storage, significantly reduced lipid oxidation over a period of 13 days at 4^{o}C (Mielnik *et al*., 2006). In another study, a grape seed extract added to cooked ground beef significantly reduced lipid oxidation over a period of 9 days at 4^{o}C (Ahn *et al*., 2007). In this last study, the grape seed extract which was a commercial product called ActiVin™ was shown to be equally as effective as a commercial pine bark extract (Pycnogenol®) and a commercial rosemary oleoresin (Herbalox®) and more effective at a concentration of 1.0% (w/v) than a mixture of 0.01% (w/v) butylated hydroxyanisole (BHA) with 0.01% (w/v) butylated hydroxytoluene (BHT).

It is widely accepted that moderate consumption of red wine as a rich source of OPCs or dietary supplementation with grape seed extracts have positive effects on human health, particularly with respect to improved cardiovascular health and reduced risk of cancer (Bagchi *et al*., 2000; Cos *et al*., 2003; Kar *et al*., 2006; Ververidis *et al*., 2007). However, supplementation of animal diets with grape seed extracts to investigate the impact on meat quality has not received nearly as much attention except for the work of Lau and King (2003) with poultry and

O'Grady *et al*. (2008) with pork. In their work with poultry, Lau and King (2003) found that the grape seed extract dietary supplement strongly reduced the growth of the birds and as a result they did not continue with their investigations of meat quality. In their work with pork, O'Grady *et al*. (2008) found no effect of dietary grape seed extract supplementation on the lipid stability of pork steaks. They attributed this to the propensity of condensed tannins to form indigestible complexes with proteins in formulated feeds and in animal diets in general. Thus, it will be important to investigate the impact of Vin*life*® on protein digestibility in fish.

Figure 5.7 shows the effects of Trolox™ on cell viability and intracellular ROS abundance. Trolox™ is a water-soluble, cell permeable derivative of vitamin E with similar antioxidant properties. At a concentration of 10 μM, Trolox™ significantly increased cell viability and significantly decreased intracellular ROS production in the presence of 250 μg ml^{-1} hydrogen peroxide. However, these results were not reproducible because at higher Trolox™ concentrations, there were no protective effects (data not shown). Thus, Trolox™ did not appear to be effective in counteracting the pro-oxidant effect of hydrogen peroxide. This was surprising given that diet trials with tank-reared fish have shown vitamin E to be a very effective antioxidant (e.g. Chaiyapechara *et al*. 2003, Huang *et al*. 2003, Lee and Dabrowski 2003, Ruff *et al*. 2003) Cell culture media are aqueous solutions. Thus, antioxidants must be water-soluble to be compatible with cell culture systems. Vitamin E (α-tocopherol) is not appreciably water soluble but Trolox™ is. However, although Trolox™ is water-soluble, it is only sparingly so. According to the product information from the supplier (Sigma-Aldrich Pty. Ltd.), Trolox™ is soluble in water up to a concentration of 0.5 mg/ml. This corresponds to a concentration of 2 mM. Thus, the concentrations we used were within the solubility range. However, with compounds that are sparingly soluble in water, it is often a better strategy to first make a stock solution in another solvent in which the compound is more soluble and then make dilutions of the stock solution in the aqueous medium. Trolox™ is much more soluble in ethanol (up to 160 mg/ml) than it is in water. Thus, future experiments should begin with the preparation of a Trolox™ stock solution in ethanol which can then be diluted in the aqueous cell culture growth medium.

Figure 5.8 shows the effects of vitamin C (ascorbic acid) on cell viability and intracellular ROS abundance. Vitamin C is highly water-soluble and therefore readily compatible with cell culture systems. However, contrary to expectations, vitamin C had little or no effect on either cell viability or intracellular ROS abundance. A possible explanation for this could be the instability of vitamin C. The cells were exposed to the antioxidants for 24 hours which may have exceeded the 'life-time' of vitamin C. Future experiments should focus on determining the minimum period of exposure to vitamin C.

Figure 5.9 shows the effects of butylated hydroxytoluene (BHT) on cell viability and intracellular ROS abundance. BHT is a commonly used preservative of fish

oils for fish feeds. However, it was ineffective in the cell culture system. This may be because BHT is oil soluble rather than water soluble. It was observed that BHT did not mix well with the aqueous cell culture medium and this may have prevented it from penetrating the cells.

Figure 5.10 shows the effects of ethoxyquin on cell viability and intracellular ROS abundance. Ethoxyquin is also a commonly used preservative of the oils in fish feeds. However, its use has recently been banned in some countries due to evidence that it may be carcinogenic. In the cell culture system, ethoxyquin did appear to increase cell viability and decrease intracellular ROS abundance. However, the effects on cell viability were not statistically significant and the effects on ROS abundance were variable. The explanation may be the same as for BHT.

Figure 5.11 shows the effects of sodium selenite on cell viability and intracellular ROS abundance. Sodium selenite is a source of selenium. Selenium is essential for the synthesis of the antioxidant enzyme glutathione peroxidase (Halliwell and Gutteridge, 2003). Glutathione peroxidase catalyses the detoxification of hydrogen peroxide and lipid hydroperoxides to form non-toxic products (Halliwell and Gutteridge, 2003). Thus, the effect of selenium is indirect via its interaction with glutathione peroxidase. At all concentrations tested, sodium selenite appeared to be toxic to the SBTG-1 cells. This was best illustrated by the results obtained with the Neutral Red assay in panel A. This assay showed that the cytotoxic effects of hydrogen peroxide and sodium selenite were additive. Considering the Neutral Red and the DCFH-DA assay results together, it is clear that the decrease in fluorescence (DCFH-DA assay) in the presence of sodium selenite is due to decreased cell viability (Neutral Red assay) and not due to fluorescence quenching by the antioxidant. This illustrates the importance of performing these two different assays in tandem to avoid misinterpretation of the results. In other words, if the DCFH-DA assay had been performed alone, the decrease in fluorescence might have been interpreted as fluorescence quenching (i.e., an antioxidant effect) by selenium. In fact, in this case, the decrease in fluorescence was due to cell death. Direct evidence that selenium is required for the production of the glutathione peroxidase enzyme in fish comes from the work of Bell *et al*. (1984, 1987) with rainbow trout and Atlantic salmon. Bell et al. (1984) injected live rainbow trout with radiolabelled [^{75}Se]selenomethionine and found that the label was incorporated into GPX when liver proteins were separated by polyacrylamide gel electrophoresis. In a related study, Atlantic salmon fed a diet deficient in selenium had much reduced levels of GPX enzyme activity, suggesting that selenium was an essential requirement for the synthesis of the GPX protein in this species as well (Bell *et al*., 1987). It will be interesting to investigate whether selenium starvation in the cell culture system and consequent lack of GPX enzyme activity leads to increased susceptibility to lipid peroxidation.

Figure 5.12 shows the effects of Herbalox® on cell viability and intracellular ROS abundance. Herbalox® is a rosemary oleoresin commercially available from Kalsec® (Kalamazoo, MI, United States of America). At the lowest concentration, i.e., 0.001% (w/v), Herbalox® had no significant effect on cell viability but at higher concentrations, it was clearly cytotoxic. Considering the Neutral Red and the DCFH-DA assay results together, it is clear that the decrease in fluorescence (DCFH-DA assay) in the presence of Herbalox® was due to decreased cell viability (Neutral Red assay) and not due to fluorescence quenching by the antioxidant. Again, this illustrates the importance of performing these two different assays in tandem to avoid coming to false conclusions about the effects of the antioxidants.

5.1.4 Conclusion

The DCFH-DA and the Neutral Red assays have both been successfully adapted for use with the SBTG-1 cell line. NatuRose® and Vin*life*® were both shown to be very effective antioxidants in the SBTG-1 cell culture system. Surprisingly, Trolox™ and vitamin C were not effective. This may be attributable to solubility and/or stability issues. Of great interest was the observation that NatuRose®, a mixture of 'free' astaxanthin, astaxanthin monoesters and other carotenoids was an effective antioxidant whereas 'free' astaxanthin alone was not. Again this might be explained by solubility and/or stability issues. The oil-soluble antioxidants BHT, ethoxyquin and Herbalox® are probably not worth testing further in the cell culture system. However, this is not a problem because Trolox™ and/or vitamin C can be used as a reference antioxidant once the problems with solubility and/or stability have been overcome. It could be interesting to try to completely remove selenium from the cell culture medium in order to test the effects on GPX enzyme activity and to determine whether the loss of GPX enzyme activity makes the cells significantly more susceptible to lipid peroxidation. Finally, the results have shown the importance of performing the two assays (DCFH-DA assay and Neutral Red assay) in tandem to be sure that decreased fluorescence (DCFH-DA assay) is due to an antioxidant effect rather than a cytotoxicity effect (Neutral Red assay).

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5.2 Year 2005-2007 Identification of the species of origin of the SBTG-1, SBTK and EPC-Geelong cell lines

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5.2.1 Introduction

The SBTG-1 cell line was developed during 2005 by PhD student Alexandra Korte working in Dr Kathryn Schuller's laboratory at Flinders University (A.B. Korte and K.A. Schuller, unpublished). At that time, the only other fish cell line in the Flinders University laboratory was an EPC cell line supplied to us by CSIRO Australian Animal Health Laboratory, Geelong. We refer to this cell line as EPC-Geelong. The original EPC cell line was derived from carp (*Cyprinus carpio*) and it had a karyotype of 84-107 chromosomes with a modal chromosome number of 96 (Fijan *et al*., 1983). Our investigations during 2006/07 showed that SBTG-1 had a karyotype of 51-54 chromosomes with a modal chromosome number of 53 and that primary cell cultures derived from southern bluefin Tuna (SBT) liver tissue had a chromosome number of 48 which was the same as had been reported for *Thunnus albacares*, *T. alalunga*, and *T. thunnus* (Klinkhardt *et al*., 1995). From these pieces of evidence we concluded that the species of origin of the SBTG-1 cell line was indeed SBT and that contamination of the SBTG-1 cell line with the EPC-Geelong cell line had not occurred.

During the period December 2007-August 2008, Dr Josephine Nocillado (Postdoctoral Research Fellow working on FRDC Project no. 2007/221), discovered, using karyotype and PCR analysis, that the species of origin of EPC-Geelong was in fact fathead minnow (*Pimephales promelas*) and that when the karyotypes and the PCR profiles of EPC-Geelong and SBTG-1 were compared, they were identical. This led to the conclusion that the species of origin for both EPC-Geelong and SBTG-1 was fathead minnow and that neither of these cell lines were what they had been supposed to be.

In the mammalian scientific literature, there are numerous cases of misidentified cell lines (e.g. Nelson-Rees and Flandermeyer 1977, Nelson-Rees *et al*. 1981, Chatterjee 2007, Hughes *et al*. 2007, Nardone 2007). The most high-profile example is the case of 40 different mammalian cell lines found to be contaminated with the HeLa cell line. The HeLa cell line, originally derived from Henrietta Lack's cervical cancer, is widely used in medical research laboratories. Here we report the identification of the species of origin of the SBTG-1, EPC-Geelong and SBTK cell lines using PCR and karyotyping. (Note: SBTK stands for SBT kidney).

Four different DNA markers were used for the PCR analyses:

- the mitochondrial DNA control region (or D-loop) used by Carlsson *et al*. (2004) to analyse population structure of Atlantic bluefin tuna in the Mediterranean Sea
- the mitochondrial cytochrome oxidase subunit I gene (*cox1*) used by Ward *et al*. (2005) for "DNA barcoding of Australia's fish species"
- the ribosomal DNA first internal transcribed spacer (ITS1) used by Chow *et al*. (2006) to investigate the phylogeny of *Thunnus* species
- microsatellites markers which were designed by Professor Abigail Elizur (University of the Sunshine Coast) to investigate the parentage of SBT fry

5.2.2 Methods

Extraction of genomic DNA

Genomic DNA was extracted following either QIAgen's DNeasy protocol for cells (QIAgen, Valencia, CA) or the phenol-chloroform extraction method (Sambrook *et al*., 1989). Genomic DNA was extracted from SBTG-1 cells at passages 3, 7, 19 and 22, SBTK cells at passage 5 and EPC-Geelong cells at passage 77. Aliquots of extracted DNA were stored at -20°C. SBTG-1 cells at passages 19 and 22 were from a culture previously maintained by A.B. Korte. SBTK cells at passage 5 were revived from liquid nitrogen-storage. EPC-Geelong cells (passage 77) were obtained from CSIRO Australian Animal Health Laboratory, Geelong. Methods for the routine culture and harvesting of the cells were as described above.

PCR amplification

The PCR primers used for the amplification of the D-loop, *cox1*, ITS1 and microsatellite markers are shown in Table 5.1.

The D-loop primers (Table 5.1) were designed based on a northern bluefin tuna (NBT) D-loop DNA sequence in the GenBank database (accession no. AY302574; Broughton and Reneau, 2006). D-loop DNA was amplified from SBT liver tissue and the SBTG-1 and EPC-Geelong cell lines. Either first-round or nested PCR was used. Nested PCR is where the first-round PCR product is used as the template for the second-round PCR. Nested PCR facilitates the amplification of low abundance targets. First-round PCR was performed with 200 ng template DNA together with the Tuna D loop 204 FOR and 811 REV primers or with 3.8μg template DNA together with either the Tuna D loop 204 FOR and 646 REV primers or the Tuna D loop 247 FOR and 646 REV primers. Nested PCR was performed with 200 ng template DNA together with the Tuna D loop 204 FOR and 811 REV primers for the first round and the Tuna D loop 247 FOR and 646 REV primers for the second round.

Table 5.1. Primers used for the PCR amplification of D-loop, *cox1*, ITS-1 and microsatellite markers.

Primer (direction)	Target	5′ to 3′ sequence	Annealing temperature	Reference, or GeneBank Accession number, or source
Tuna D loop 204 FOR (forward)	D loop	GGCGATTAAACGAGATTTAAGACC	58˚C	Broughton and Reneau (2006)
Tuna D loop 247 FOR (forward)	D loop	CTAAGCCATACCAAGTCTCCTCAT	58˚C	Broughton and Reneau (2006)
Tuna D loop 646 REV (reverse)	D loop	GGTAATAGGAATTAAGCCATGCAG	58˚C	Broughton and Reneau (2006)
Tuna D loop 811 REV (reverse)	D loop	CACTCGAGATTTTCCTGTTTACG	58˚C	Broughton and Reneau (2006)
Fish F1 (forward)	Cox-1	TCAACCAACCACAAAGACATTGGCAC	54˚C	Ward *et al*. (2005)
Fish R1 (reverse)	Cox-1	TAGACTTCTGGGTGGCCAAAGAATCA	54˚C	Ward *et al*. (2005)
Fish R2 (reverse)	Cox-1	ACTTCAGGGGTGACCGAAGAATCAGAA	54˚C	Ward *et al*. (2005)
Tuna Cox-1 FOR1 (forward)	Cox-1	GCCTTAAGCTTGCTCATCCG	58˚C	DQ107637-41
Tuna Cox-1 nested FOR2 (forward)	Cox-1	GGACGACCAGATCTACAATGTA	54˚C	DQ107637-41
Tuna COX-1 REV1 (reverse)	Cox-1	TCCAGTCCTTGCCGCT	58˚C	DQ107637-41
Tuna COX-1 nested REV2 (reverse)	Cox-1	CTTCATCACAACAATTATCAATATG	54˚C	DQ107637-41
ITS1 For Chow (forward)	ITS-1	TCCGTAGGTGAACCTGCGG	58˚C	Chow *et al*. (2006)
ITS1 Rev Chow (reverse)	ITS-1	GCTACTTCTTGCGTCGC	58˚C	Chow *et al*. (2006)
SBT-ITS35-52For (forward)	ITS-1	ACAAGACGCCTCCGGACA	60˚C	AB212013-15 and AB27399
SBT-ITS209-230For (forward)	ITS-1	GCAAAACACCTCTCCTGGTCAG	60˚C	AB212013-15 and AB27399
SBT-ITS593-474Rev (reverse)	ITS-1	GAGACAAGTCGGGTTTGGAA	60˚C	AB212013-15 and AB27399
SBT-ITS626-605Rev (reverse)	ITS-1	GTTTTTACACCGCACAGAGGTT	60˚C	AB212013-15 and AB27399
Tth004 (forward and reverse)	Microsatellite	$(GT)_{16}$	60˚C	Prof. Abigail Elizur, University of the Sunshine Coast
Tth185 (forward and reverse)	Microsatellite	$(TG)_{11}$	60˚C	Prof. Abigail Elizur, University of the Sunshine Coast
Tth254 (forward and reverse)	Microsatellite	$(AG)_{21}$	60˚C	Prof. Abigail Elizur, University of the Sunshine Coast

Two different sets of primers were used for the *cox1* marker. One set was various combinations of the Fish F1, Fish R1 and Fish R2 "barcoding" primers designed by Ward *et al*. (2005) (Table 5.1). The other set was the 'SBT-specific' primers designed by ourselves based on SBT *cox1* DNA sequences in the GenBank database (accession nos. DQ107637-41) (Table 5.1). Template DNA was extracted from SBT liver tissue and the SBTG-1, SBTK and EPC-Geelong cell lines. When the "barcoding" primers were used, a first-round PCR with 200 ng of template DNA was performed. When the 'SBT-specific' primers were used, either a first-round PCR with 3.8μg of template DNA or a nested PCR with 200 ng of template DNA was performed. For the first-round PCR with 3.8μg of template DNA, the 'SBT-specific' primers used were various combinations of two forward (Tuna Cox-1 FOR1 and Tuna Cox-1 nested FOR2) and two reverse (Tuna COX-1 REV1 and Tuna COX-1 nested REV2). For the nested PCR with 200ng of template DNA, the 'SBT-specific' primers used were Tuna Cox-1 FOR1 and Tuna COX-1 REV1 for the first-round and Tuna Cox-1 nested FOR2 and Tuna COX-1 nested REV2 for the second round.

ITS1 DNA was amplified from the SBTG-1 and SBTK cell lines. Two hundred ng of template DNA was used together with the ITS1 For Chow and ITS1 Rev Chow primers (Chow *et al*., 2006; Table 5.1). 'SBT-specific' primers were also designed based on SBT ITS1 sequences in the GenBank database (Accession nos. AB212013-15 and AB27399; Table 5.1).

Microsatellite DNA markers were amplified using the Tth004, Tth185 and Tth254 primers (Table 5.1) together with 3.8μg of template DNA from the SBTG-1 and EPC-Geelong cell lines. These primers were kindly provided by Prof. Abigail Elizur (University of the Sunshine Coast, Queensland).

All PCR reactions consisted of 2.5μl 10X Platinum high fidelity PCR buffer, 1μl $MgSO_4$ (50mM), 1μl dNTP mixture (10 mM of each dNTP), 0.5μl forward and reverse primers (10μM of each primer), 0.625 units Platinum high fidelity DNA polymerase (Invitrogen, Carlsbad) and sterile water to 25μl. No-template controls were always performed. Thermal cycling included an initial denaturation step at 94°C for 2 min, followed by 35 cycles of 30 sec denaturation at 94°C, 30 sec annealing at the appropriate temperature and 45 sec extension at 68°C and a final extension at 68°C for 5 min. The number of cycles was reduced to 30 for the nested reactions. PCR products of the expected sizes were purified using Promega's Wizard SV Gel Clean-Up system following the manufacturer's protocol (Promega, Madison). The purified PCR products were ligated into the pGEMT-Easy vector (Promega). Transformed *E. coli* XL-1 blue (Stratagene, La Jolla) or DH5α (Invitrogen) competent cells were screened by PCR and restriction enzyme digestion. Plasmid DNA from positive colonies was purified using Promega's Wizard Plus Miniprep DNA Purification system. At least 6 clones were sequenced in both directions (carried out by the Australian Genome Research Facility; www.agrf.org.au). Sequence analyses were performed using Sequencher version 4.1.4 sofware (GeneCodes, Ann Arbor) and NCBI's BLAST

(blastn, megablast and discontiguous megablast) algorithm (www.ncbi.nlm.nih.gov). Sequence alignments and homology comparisons were performed using the ClustalW2 alignment program (www.ebi.ac.uk/Tools/clustalw2).

Karyotype analysis

Preparation and fixation of the cells for chromosome analysis was performed according to standard methods (Freshney, 2000) with slight modifications. Cells were incubated in colchicine (0.01mg.ml^{-1} final concentration) for 1-2 hours. Trypsinized cells were exposed for 5 min to a hypotonic solution consisting of a 1:1 mixture of 0.56% (w/v) KCl and 0.8% (w/v) trisodium citrate. Slides were stained with Leishman's stain for 3 min. Chromosome numbers from 30-50 metaphase spreads were counted using a light microscope at 1000X magnification. Photographs and karyotypes were prepared using a 'Cytovision' system (Applied Imaging International Ltd., Newcastle upon Tyne, U.K.). Karyotype analyses were performed on SBTG-1 cells at passages 31 and 40, SBTK cells at passage 5, EPC-Geelong cells at passages 79 and 86 and EPC-Flinders cells (passage number unknown). (Note: EPC-Flinders is a different stock of EPC-Geelong. EPC-Geelong arrived in the Flinders University laboratory in 2008 whereas EPC-Flinders arrived in the Flinders University laboratory in 2004).

5.2.3 Results

5.2.3.1 PCR analysis

Mitochondrial D-loop

Mitochondrial D-loop DNA was amplified from SBT liver tissue or the SBTG-1 cell line using either first-round or nested PCR, various amounts of template DNA and various primer combinations (Table 5.2).

Table 5.2. Mitochondrial D-loop DNA amplification sequence.

Source of template DNA	Amount of template DNA	First-round or nested PCR?	Primers	Expected size of PCR product (bp)	Obtained size of PCR product (bp)
SBT liver tissue	200 ng	first-round	Tuna D loop 204 FOR & 811 REV	608	608
SBTG-1 cell line	200 ng	first-round	Tuna D loop 204 FOR & 811 REV	608	no distinct product
SBTG-1 cell line	200 ng	nested	Tuna D loop 247 FOR & 646 REV	400	400
SBTG-1 cell line	3.8 μg	first-round	Tuna D loop 204 FOR & 646 REV	420	420
SBTG-1 cell line	3.8 μg	first-round	Tuna D loop 247 FOR & 646 REV	400	400

The results summarized in Table 5.1 show that PCR products were obtained from the SBTG-1 cell line only when the amount of template DNA was increased to 3.8 μg or when a nested PCR reaction was performed with the usual 200 ng of template DNA. This suggested that SBT D-loop DNA was in low abundance in the SBTG-1 cell line. Subsequent sequencing of the cloned PCR products and BLAST analysis of the sequences revealed that they all originated from *Thunnus* sp. As shown in Figure 5.13, the SBT liver tissue and SBTG-1 cell line D-loop sequences were 95% identical to a NBT D-loop sequence in the GenBank database. The SBT liver tissue and SBTG-1 cell line D-loop sequences were also 99-100% identical to one another.

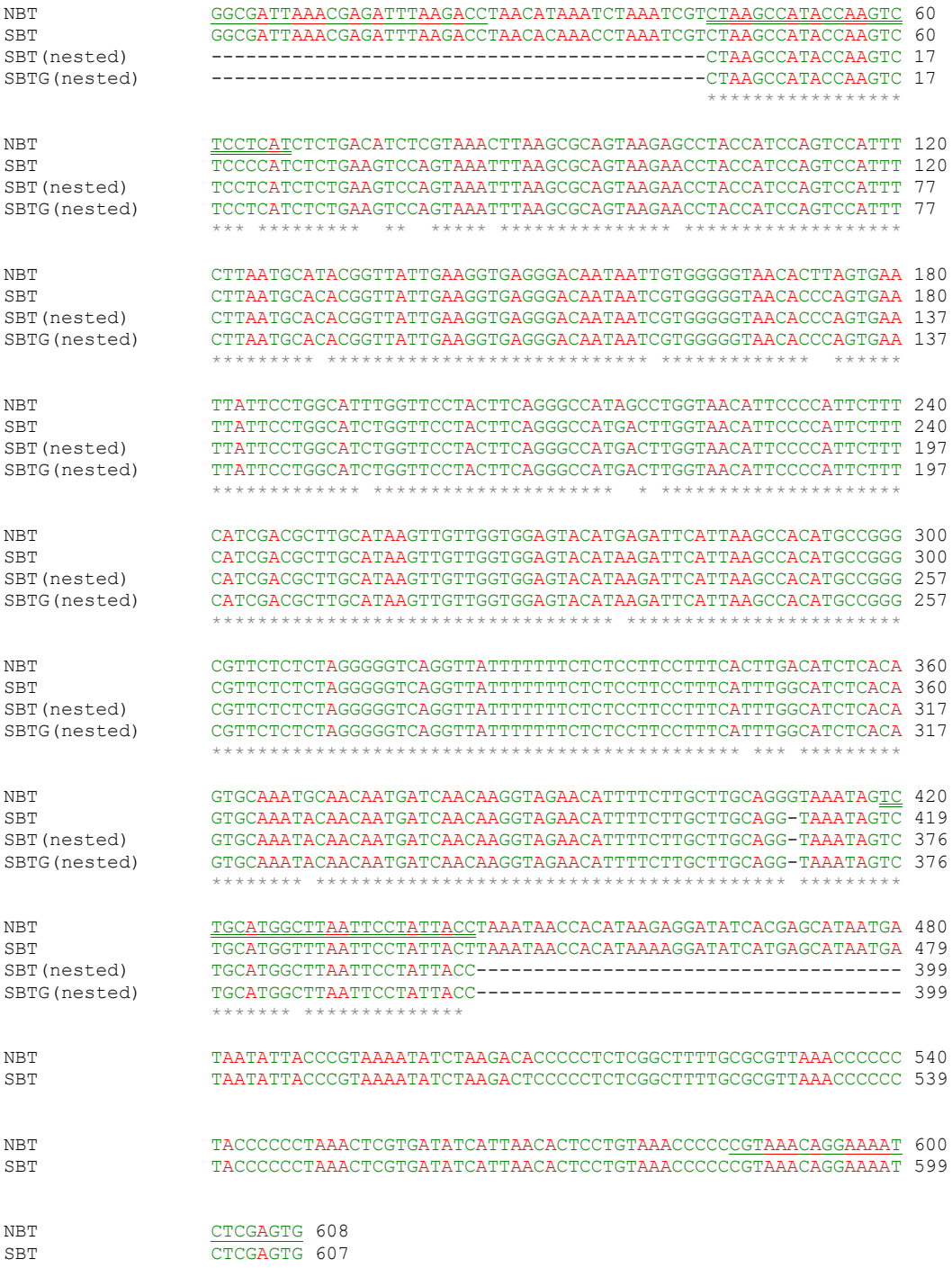

Figure 5.13. Comparison of SBT and putative SBT gonad cell line (SBTG) mtDNA control (D-loop) region sequences with that of NBT; GenBank Accesion no. AY302574). SBT and SBTG sequences are identical by 95% to the NBT sequence. SBT and SBTG sequences are identical by 99-100%. Alignment was performed using ClustalW (www.ebi.ac.uk/Tools/clustalw2). Asterisks denote conserved nucleotides. Gaps were introduced to maximise alignment. First round forward (Tuna D loop 204 FOR) and reverse (Tuna D loop 811 REV) PCR primers are underlined. Nested forward (Tuna D loop 247 FOR) and reverse (Tuna D loop 646 REV) PCR primers are double-underlined.

The SBT D-loop primers were also used to test the EPC-Geelong cell line. To our surprise, the PCR products obtained for the EPC-Geelong cell line were the same size as those obtained for SBT liver tissue and the SBTG-1 cell line. Subsequent sequencing and BLAST analysis revealed that the EPC-Geelong cell line, SBT liver tissue and SBTG-1 cell line D-loop sequences were all identical to one another. This suggested either that the EPC-Geelong cell line had become contaminated with the SBTG-1 cell line (or *vice versa*) or that the EPC-Geelong and SBTG-1 PCR reactions had become contaminated with the PCR product from the SBT liver tissue PCR reaction.

Mitochondrial cox-1

Mitochondrial *cox-1* DNA was amplified from the SBTG-1, SBTK and EPC-Geelong cell lines and SBT liver tissue. Two hundred ng of template DNA was used together with either the Fish F1 and Fish R1 or Fish F1 and Fish R2 *cox1* "barcoding" primers of Ward *et al*. (2005). In all cases, a 700 bp PCR product was obtained. Subsequent cloning, sequencing and BLAST analysis revealed that the SBT liver tissue sequence was 99-100% identical to *Thunnus maccoyii cox1* sequences in the GenBank database (accession nos. DQ107637-41). Surprisingly, however, the SBTG-1, SBTK and EPC-Geelong cell line sequences were 98-100% identical to a fathead minnow (*Pimephales promelas*) sequence (GenBank accession no. EU525095) and only 79% identical to the SBT liver tissue sequence (Figure 5.14). This suggested that all three cell lines originated from fathead minnow and not from either SBT (*Thunnus maccoyii*) or carp (*Cyprinus carpio*) as had been supposed.

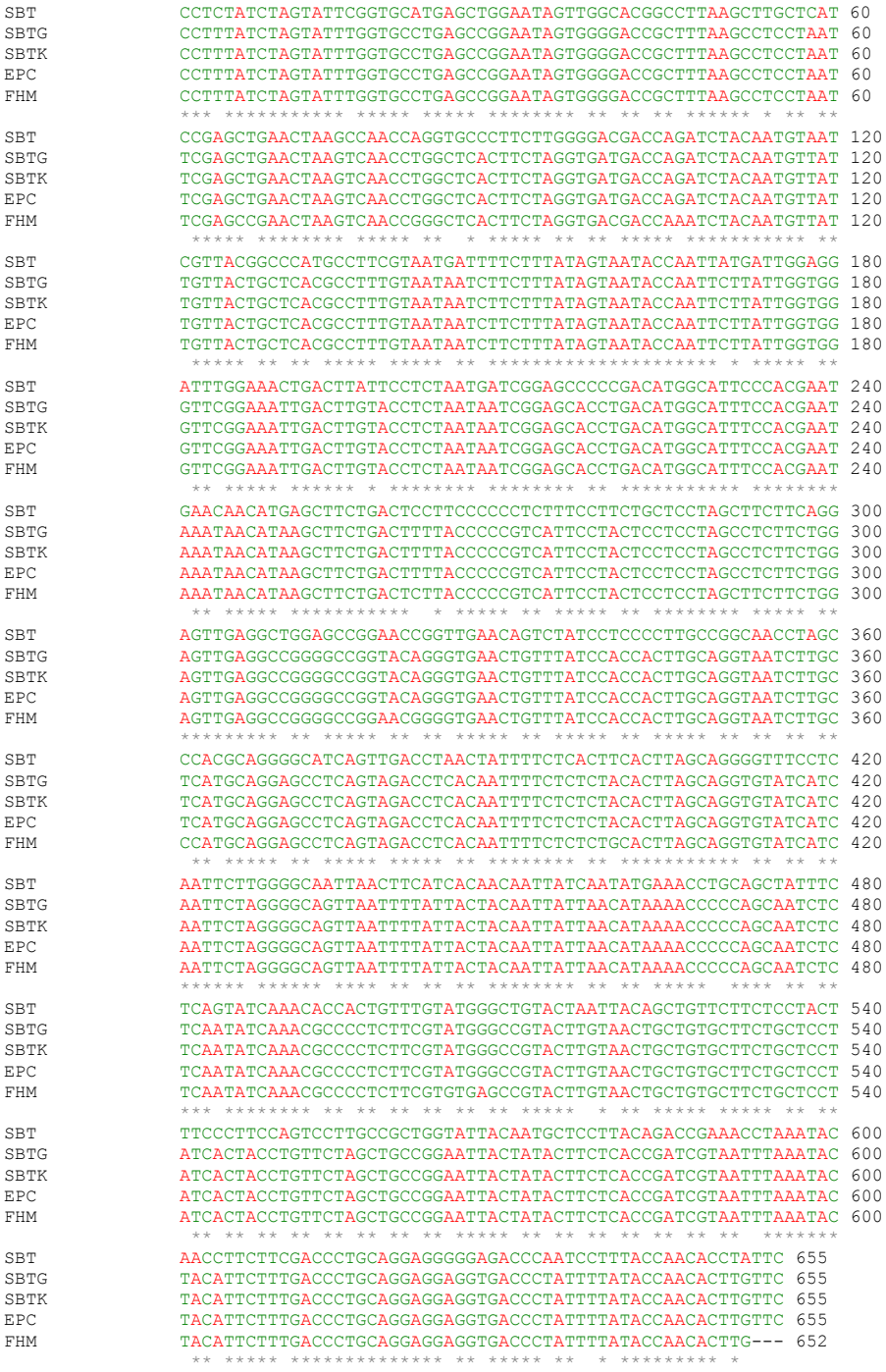

Figure 5.14. Alignment of COX-1 sequences obtained from SBT, putative SBT gonad (SBTG) and kidney (SBTK) cell lines and Epithelioma Papulosum Cyprini (EPC) cell line. Also included in the figure is the fathead minnow (FHM) COX-1 sequence (GenBank Accession no. EU525095). Alignment was performed using ClustalW (www.ebi.ac.uk/Tools/clustalw2). The universal COX-1 primers (Ward *et al*. 2005) were removed prior to alignment. Asterisks denote conserved nucleotides. The SBTG, SBTK, EPC and FHM sequences are identical by 79% to the SBT sequence. The SBTG, SBTK, EPC and FHM sequences are identical by 98-100%. The SBT sequence obtained is 99-100% identical to the SBT COX-1 sequences on the GenBank (Accession nos. DQ107637-41).

Mitochondrial *cox1* DNA was also amplified using primers based on the SBT liver tissue *cox1* sequence. These are referred to as 'SBT-specific' primers. Products of the expected sizes were obtained for the SBTG-1 cell line and also for SBT liver tissue. However, as described above for the mitochondrial D-loop, successful amplification depended on the amount of template DNA. With 200 ng of template DNA, a nested PCR reaction was required to obtain the expected 364 bp product from the SBTG-1 cell line. In contrast, with the same amount of template DNA, only first-round PCR was required to obtain the expected 520 bp product from SBT liver tissue. When the amount of template DNA was increased to 3.8 μg and the Tuna Cox-1 nested FOR2 and Tuna COX-1 REV1 primers were used only first-round PCR was required to yield the expected 469 bp PCR product from the SBG-1 cell line. Another pair of primers, Tuna Cox-1 nested FOR2 and Tuna COX-1 nested REV2, also yielded the expected 366 bp product from the larger amount (3.8 μg) of template DNA. All of the sequences were 99-100% identical to SBT *cox1* sequences in the GenBank database (accession nos. DQ107637-41; Figure 5.15).

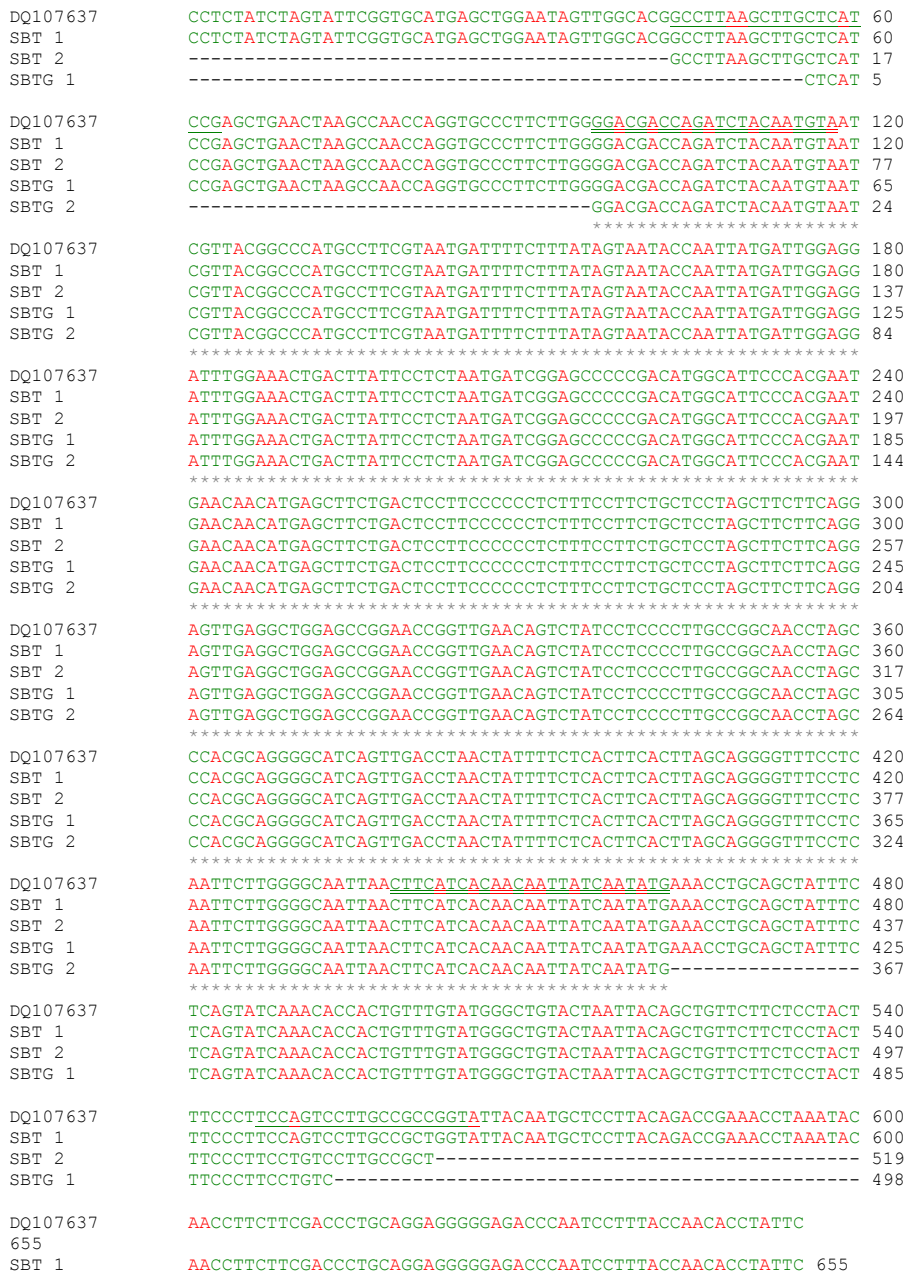

Figure 5.15. Comparison of COX-1 sequences obtained from southern bluefin tuna (SBT) and putative SBT gonad (SBTG) using SBT-specific primers (SBT 2, SBTG 1, SBTG 2) with SBT COX-1 sequence from the GenBank (DQ107637) and with SBT 1 sequence, which was obtained using Ward *et al*.'s (2005) universal primers. Alignment was performed using ClustalW (www.ebi.ac.uk/Tools/clustalw2). The first round PCR primers are underlined. The nested PCR primers are double-underlined. SBTG 1 sequence was obtained from primer pair combinations of the four PCR primers. SBTG 2 sequence was obtained from the fully nested primer pairs. The compared sequences are identical by 99-100%.

PCR reactions using EPC-Geelong cell line DNA as the template and various combinations of the 'SBT-specific' *cox1* primers yielded products of the correct sizes from two different primers pairs, i.e., Tuna Cox-1 nested FOR2 together with Tuna COX-1 REV1 (469 bp) or Tuna Cox-1 nested FOR2 together with Tuna COX-1 nested REV2 (366 bp). BLAST analysis of the sequences obtained for the PCR products revealed that they were all *Thunnus maccoyii cox1* sequences.

Microsatellites

Three microsatellite primers (Tth004, Tth185 and Tth254), previously shown to amplify the expected targets from *T. maccoyii* (A. Elizur and W. Knibb, unpublished data) were tested on 3.8 µg of the SBTG-1 cell line template DNA. No PCR products were obtained from any of the primer pairs tested. EPC-Geelong cell line DNA was used as a negative control and again no products were obtained.

ITS1

ITS1 DNA was amplified from SBTG-1 and SBTK cell line DNA using two primer sets, one designed by Chow *et al*. (2006) and the other designed by ourselves based on *T. maccoyii* ITS1 sequences in the GenBank database (accession nos. AB212013-15 and AB27399). Using the primers designed by Chow *et al*. (2006), a PCR product of less than 400 bp was obtained from 200 ng of SBTG-1 or SBTK template DNA. Following cloning and sequencing, BLAST analysis revealed that the sequence of this 400 bp product was 81-87% identical to cyprinid ITS1 sequences in the GenBank database (accession nos. AM183345, AM183339, AY524426, AY524428, AY524427, AM183346, AM183341, AM183342). In addition, the SBTG-1 and SBTK ITS1 sequences were 98% identical to one another (Figure 5.16).

```
SBTG       CGCTGCGTTCTTCATCGACTCACGAGCCGAGTGATCCACCGCTAAGAGTTGTACTCTTGG 60
SBTK       CGCTGCGTTCTTCATCGACTCACGAGCCGAGTGATCCACCGMTAAGAGTTGTACTMTTGG 60
           ************************************** ************* ****

SBTG       TTTTTTTGGTTTTTCGAGGCCGACCGACACAAACCGGGGGAATTGGGGAACAAAAAAAATR 120
SBTK       TTTTTTTGGTTTTTCGAGGCCGACCGACACAAACCGGGGGAATTGGGGAACAAAAAAAATA 120
           ************************************************************

SBTG       AGTGGTGGCGAGACCCGGACGGGCGCCCCCTCCCCCCCGGTCGAGGGATGGGGAGGAGGT 180
SBTK       AGTGGTGGCGAGACCCGGACGGGCGCCCCCTCCCCCCCGGTCGAGGGATGGGGAGGAGGT 180
           ************************************************************

SBTG       CTTTAAACCCACGGCCCCCCCACYGGGGGGGAGACCTAACGGGTACCCGTCGGGGTTGCGA 240
SBTK       CTTTAAACCCACGGCCCCCCCACTGGGGGGGAGACCTAACGGGTACCCGTCGGGGTTGCGA 240
           ********************** ***********************************

SBTG       CTCTCGCCACTCGTTTTTCACCGAAGGGGGTTCCGGGCCCGCCGGGGCGGACCCGAGGCG 300
SBTK       CTCTCGCCACTCGTTTTTCACCGAAGGGGGTTCCGGGCCCGCCGGGGCGGACCCGAGGCG 300
           ************************************************************

SBTG       TCTGGTTTGTTTCGTT 316
SBTK       TCTGGTTTGTTTCGTT 316
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Figure 5.16. Alignment of ITS-1 sequences obtained from the putative SBT gonad (SBTG) and kidney (SBTK) cell lines. The universal ITS-1 primers designed by Chow *et al*. (2006) were used for PCR amplification. The SBTG and SBTK sequences are identical by 98%. Alignment was performed using ClustalW (www.ebi.ac.uk/Tools/clustalw2).

ITS1 amplification using 'SBT-specific' ITS1 primers with SBTG-1 cell line template DNA yielded the expected products from three primer combinations, namely: SBT-ITS35-52For and SBT-ITS593-474Rev (552 bp); SBT-ITS35-52For and SBT-ITS626-605Rev (592 bp); SBT-ITS209-230For and SBT-ITS593-474Rev (385 bp). For SBTK, products of the correct sizes were obtained from two primer combinations: SBT-ITS35-52For and SBT-ITS626-605Rev (592 bp); SBT-ITS209-230For and SBT-ITS593-474Rev (385 bp). BLAST analysis of the sequences of the clones of these products did not result in any significant matches to any sequences in the GenBank database. However, the SBTG-1 and SBTK cell line DNA sequences were 99% identical to one other (Figure 5.17).

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SBTG            GTTTTTACACCGCACAGAGGTTAGATATTGCGTGCATATGCATAACGAGGAACAAGTTAG 60
SBTK            ------------CACAGAGGTTAGATATTGCGTGCATATGCATAACGAGGAACAAGTTAG 48
                            *********************************************

SBTG            ATATATCATGCAGTGCGATGAAACACATCTTAGGTGTTTTCTTTTCATTGAAGAGAGGTT 120
SBTK            ATATATCATGCAGTGCGATGAAACACATCTTAGGTGTTTTCTTTTCATTGAAGAGAGGTT 108
                ************************************************************

SBTG            AGTTATTGCGTGCATATGCATAACGAGAAACAAGTTATATCTATCATTCAATGCAATAAA 180
SBTK            AGTTATTGCGTGCATATGCATAACGAGAAACAAGTTATATCTAYCATTCAATGCAATAAA 168
                ******************************************* ****************

SBTG            ACACATGTTAGGTTTTTTCTTTTCATTGCACAGAGGTTAGATATTGAGTGAATATGCATG 240
SBTK            ACACATGTTAGGTTTTTTCTTTTCATTGCACAGAGGTTAGATATTGAGTGAATATGCATG 228
                ************************************************************

SBTG            ACGAGGAACAAGTTAGATATGTCATGCAATGCACTGAAACACATGTTAGGTGTTTTCTTT 300
SBTK            ACGAGGAACAAGTTAGATATGTCATGCAATGCACTGAAACACATGTTAGGTGTTTTCTTT 288
                ************************************************************

SBTG            TCACTGCAGAGAGGTTAGTTATTGCGTGCATATACATAACGAGGAACAAGTTAGATAGGT 360
SBTK            TCACTGCAGAGAGGTTAGTTATTGCGTGCATATACATAACGAGGAACAAGTTAGATAGGT 348
                ************************************************************

SBTG            CATACAATGCAATGAAACACATCTTAGGTACTTTCTTTTCATTGCACAGAAGTTAGTTAT 420
SBTK            CATACAATGCAATGAAACACATCTTAGGTATTTTCTTTTCATTGCACAGAAGTTAGTTAT 408
                ****************************** ******************************

SBTG            TGCGTGTGTGACATTATTAGTTAAGTGATTGACAGTTGCGGCATCCTATCAGGGGATAGT 480
SBTK            TGCGTGTGTGACATTATTAGTTAAGTGATTGACAGTTGCGGCATCCTATCAGGGGATAGT 468
                ************************************************************

SBTG            ATCATCTACTTAATGCGGGTTGGCTGTGTCCGGAGGCGTCTTGT 524
SBTK            ATCATCTATTTAATGCGGGTTGGCTGTGTCCGGAGGCGTCTTGT 512
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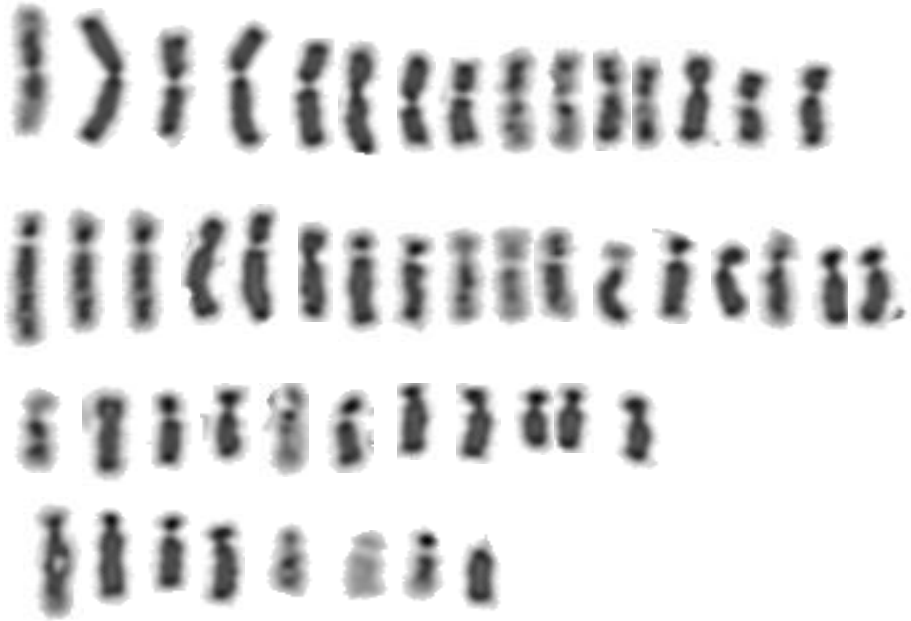
Figure 5.17. Alignment of SBTG and SBTK ITS-1 sequences obtained using primers designed from *Thunnus maccoyii* ITS-1 sequences (Accession numbers AB212013-15 and AB27399). Alignment was performed using ClustalW (www.ebi.ac.uk/Tools/clustalw2). The sequences are identical by 99%.

5.2.3.2 Karyotype analysis

The chromosome numbers for the SBTG-1, SBTK, EPC-Geelong and EPC-Flinders cell lines were within similar ranges. The SBTG-1 cell line had a chromosome number ranging from 51-54 (Figure 5.18a and 5.18b), the SBTK cell line had a chromosome number ranging from 31-58 (Figure 5.19a and 5.19b), the EPC-Geelong cell line had a chromosome number ranging from 45-55 (Figure 5.20a and 5.20b) and the EPC-Flinders cell line had a chromosome number ranging from 47-57 (Figure 5.21a and 5.21b). The chromosomes were also similar morphologically. This was evident when the chromosomes were classified as either metacentric, sub-metacentric or telocentric.

A



B

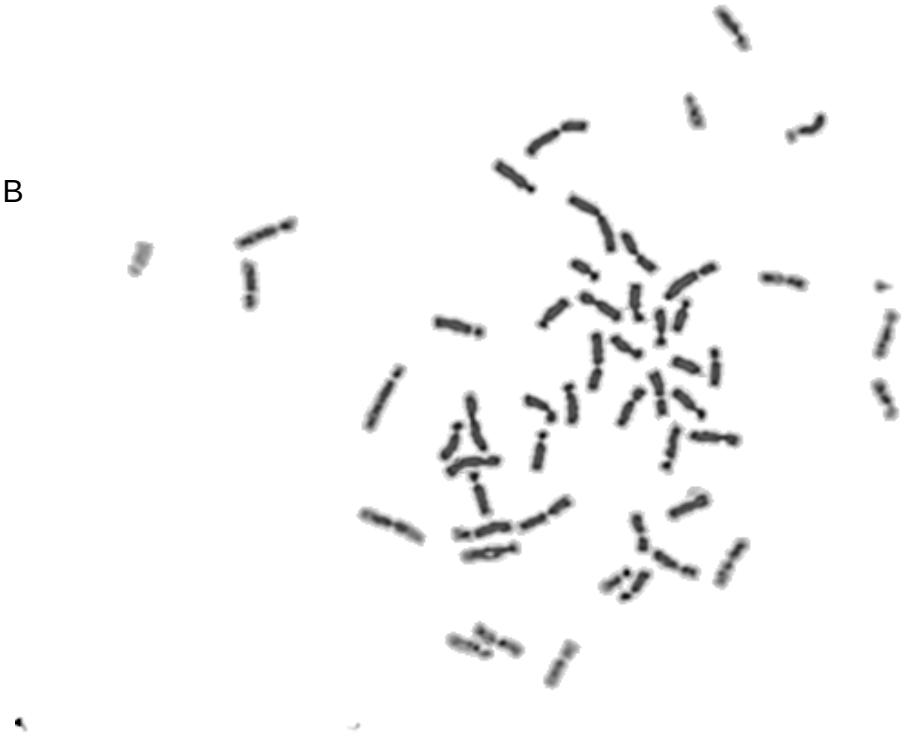


Figure 5.18. Representative karyotype of the putative SBT gonad cell line (passage 40). A – Arranged chromosomes based on morphology; B –metaphase spread.

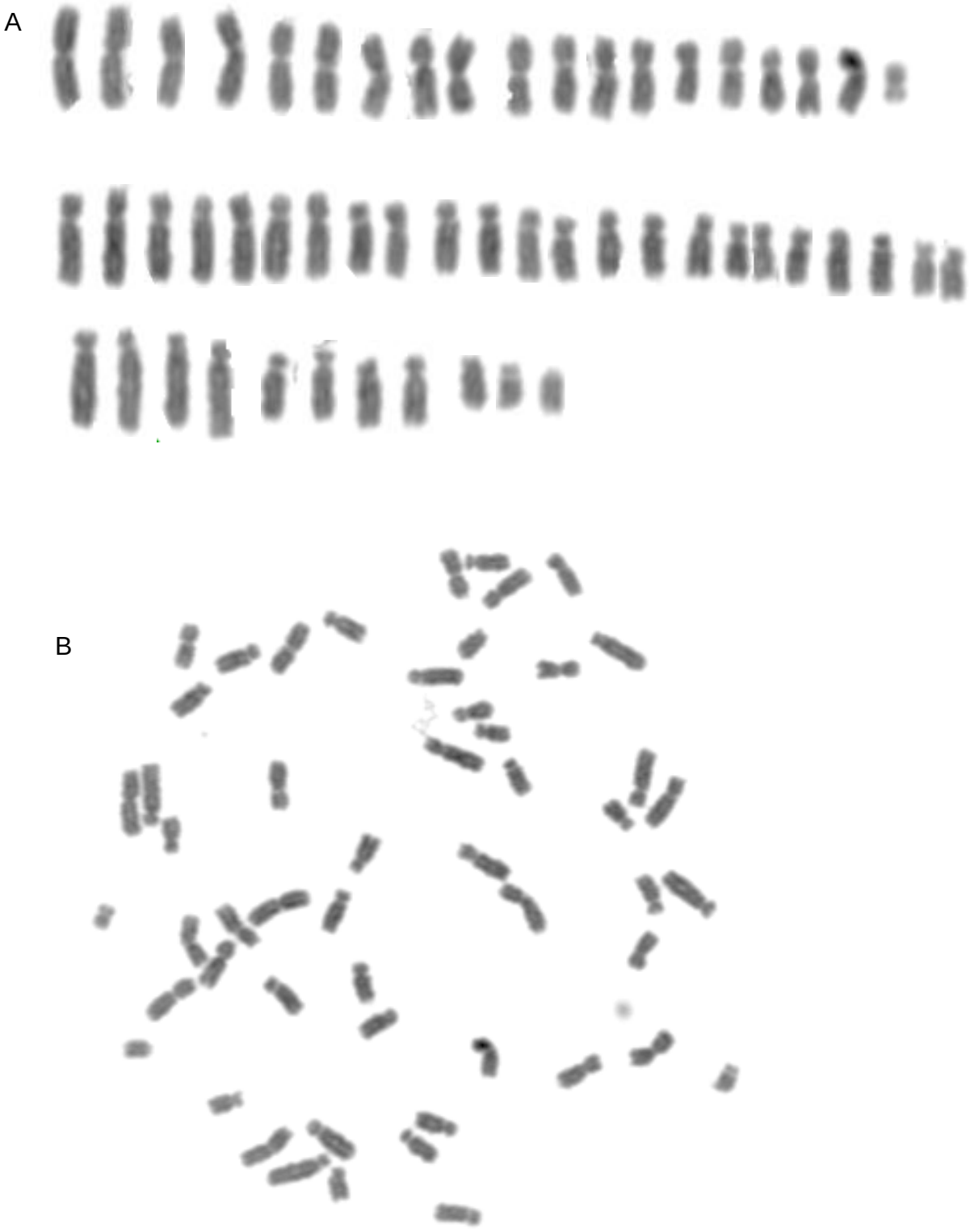


Figure 5.19. Representative karyotype of the putative SBT kidney cell line (passage 5). A – Arranged chromosomes based on morphology; B –metaphase spread.

A

B

Figure 5.20. Representative karyotype of the EPC-Geelong cell line (passage 86). This cell line was obtained from CSIRO Geelong at passage 77. A – Arranged chromosomes based on morphology; B –metaphase spread.

A

B

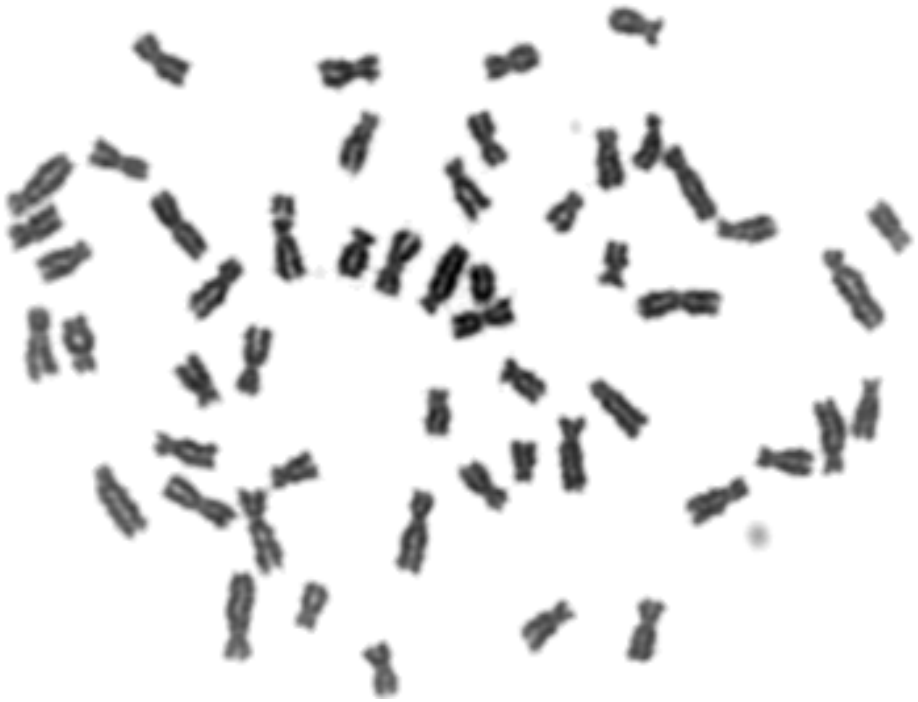


Figure 5.21. Representative karyotype of the EPC-Flinders cell line (passage number unknown). This cell line was originally obtained from CSIRO Geelong but has been maintained at Flinders University for a period of time. A – Arranged chromosomes based on morphology; B –metaphase spread.

5.2.4 Discussion

PCR and karyotype analysis has revealed that the SBTG-1 and SBTK cell lines have been contaminated with a fathead minnow cell line and that the source of the fathead minnow cells was a misidentified EPC cell line obtained from CSIRO Australian Animal Health Laboratory, Geelong (referred to here as EPC-Geelong). The original EPC cell line was derived from carp (*Cyprinus carpio*) and it had a chromosome number ranging from 84 to 107 (Fijan *et al*., 1983). (Note: Variation in chromosome number is common in immortal cell lines). In contrast, the EPC-Geelong, SBTG-1 and SBTK cell lines had chromosome numbers ranging from 31 to 58 (our work). This compares with the 'normal' chromosome numbers for these species which are 100 for carp (Fijan *et al*., 1983), 48 for SBT (A.B. Korte and K.A. Schuller, unpublished) and 50 for fathead minnow (Levan *et al*., 1966). Thus, the EPC-Geelong cell line had only half as many chromosomes as the original EPC cell line and only half as many chromosomes as the 'normal' chromosome number for carp. Therefore, the EPC-Geelong cell line was not the same as the original EPC cell line and it was not derived from carp.

The true species of origin of the EPC-Geelong, SBTG-1 and SBTK cell lines was determined using PCR analysis. The most convincing results were obtained using the *cox1* "barcoding" primers of Ward *et al*. (2005). Using these primers, the *cox1* DNA sequences for the EPC-Geelong, SBTG-1 and SBTK cell lines were found to be 98-100% identical to fathead minnow *cox1* DNA sequences in the GenBank database. Several different passages (both early and late) of the SBTG-1 and SBTK cell lines were tested and the results, in all cases, were the same. Thus, it is unlikely that any uncontaminated stocks of the SBTG-1 or SBTK cell lines remain. In addition, these results reveal that the EPC-Geelong cell line is derived fathead minnow and not from carp as was originally thought.

Further evidence for the true species of origin of the SBTG-1 and SBTK cell lines came from PCR analyses using the ITS1 marker. The ITS1 PCR product obtained for the SBTG-1 and SBTK cell lines was less than 400 bp in length. This compared with the ITS1 PCR products for cyprinids which were 330-350 bp in length and for tuna species which were 643-656 bp in length (Chow *et al*., 2006). No ITS1 sequences are available for Fathead minnow but Fathead minnow, like carp, is a cyprinid.

PCR analysis was also done using primers we designed ourselves based on SBT *cox1* sequences in the GenBank database. We refer to these primers as 'SBT-specific' *cox1* primers. The results obtained with the 'SBT-specific' *cox1* primers were less clear-cut than those obtained with the *cox1* "barcoding" primers. With the 'SBT-specific' *cox1* primers, 200 ng of template DNA was sufficient to amplify a PCR product from SBT liver tissue but not enough to amplify a PCR product from either the SBTG-1 or SBTK cell line. To obtain a product from either the SBTG-1 or SBTK cell line, either the amount of template DNA had to be increased more than 10-fold to 3.8 μg or a nested PCR reaction had to be performed. In nested PCR, the PCR product from the first-round PCR

acts as the template for the second-round PCR. All of the *cox1* PCR products obtained under all of these conditions were 99-100% identical to the SBT *cox1* sequences in the GenBank database. Taken together, these results suggested that (a) SBT cells were in low abundance in the SBTG-1 and SBTK cell cultures or (b) that the PCR reactions for the cell lines had become contaminated with the SBT liver tissue PCR product. Spurious PCR products amplified from trace amounts of contaminating DNA are a well known problem in forensic DNA analysis and molecular biology research laboratories. Thus, we favour explanation (b) that the PCR reactions for the cell lines had become contaminated with the SBT liver tissue PCR product.

Further evidence for the PCR contamination hypothesis comes from our observation that PCR analyses of the EPC-Geelong cell line using the SBT-specific *cox1* primers also yielded DNA sequences that were virtually identical to SBT *cox1* sequences in the GenBank database.

The results obtained for the D-loop PCR analyses using 'SBT-specific' primers were essentially the same as the results obtained for the *cox1* PCR analyses using 'SBT-specific primers. In other words, when 'SBT-specific' D-loop primers were used, only 200 ng of template DNA was required to obtain a PCR product from SBT liver tissue whereas 3.8 μg of template DNA (more than ten times as much) was required to obtain a PCR product from the SBTG-1 cell line or a nested PCR reaction had to be run with the SBTG-1 cell line to obtain a product. The D-loop sequences obtained for the SBTG-1 cell line were 99-100% identical to those obtained for SBT liver tissue. Again, there are the same two likely explanations from these results: (a) SBT cells are in low abundance in the SBTG-1 cell line or (b) the SBTG-1 cell line PCR reaction became contaminated with the PCR product from the SBT liver tissue PCR reaction. Again, we favour explanation (b).

During the course of the work described here, it came to our attention that the EPC cell line (CRL-2872™) in the ATCC (American Type Culture Collection) had also been misidentified and that it too had been shown to be derived from fathead minnow. The ATCC is an internationally-recognized supplier of cell cultures for research and diagnostics. Thus it is highly likely that a large number of laboratories around the world are using fathead minnow cells in the belief that they are using EPC cells. This highlights the importance of authenticating the identity of all cell lines whether newly developed or supplied by a reputable source. The tools we describe here, especially PCR analysis using the *cox1* "barcoding" primers and karyotype analysis, should prove particularly useful for future fish cell line research.

The cell line cross-contamination described in this report is by no means an isolated case. Recent data has shown that between 18% and 36% of cell lines submitted to major cell repositories are either contaminated or misidentified (Hughes *et al*., 2007). The incidence of research papers containing flawed data

due to the use of cross-contaminated and misidentified cell lines is estimated to be between 15% and 20% (Nardone, 2007). In addition, an estimated 35% to 40% of published cell biology papers should be retracted because the researchers were using misidentified cells lines (Chatterjee, 2007).

In the medical research community, there is a growing awareness of the waste of time and money as a result of the use of misidentified cell lines. Some scientific journals, e.g. BioTechniques and Journal of the National Cancer Institute, will soon be asking authors to state their methods of cell line authentication before they are allowed to publish (McCormick, 2008; Chatterjee, 2007). It has also been proposed that publication of manuscripts dealing with the development of new cell lines be made contingent upon documented authentication using DNA profiling or karyotyping (MacLeod *et al*., 1999).

The techniques described in this report are widely accepted procedures employed to authenticate cell lines. Other DNA profiling techniques, such as short tandem repeat polymorphisms, mini- and microsatellites, are being utilised to detect intraspecies contamination, particularly of human-derived cell lines (Yoshino *et al*., 2006; MacLeod *et al*., 1999). At the other end of the spectrum, there are simple enough techniques that can prevent the occurrence of contamination or cell line misidentification. Handling of only one cell line at a time and the use of separate media and reagents for each cell line are effective methods (Liscovitch and Ravid, 2007). Grounds for suspicion regarding newly established cell lines, such as unexpected success in establishing cultures from refractory primary material, should not be ignored (MacLeod *et al*., 1999). Collection and storage of additional tissue samples from the donor individual is necessary to facilitate authentication when a cell line is finally established (review by Chatterjee, 2007). Regular authentication of stocks can prevent contamination being carried over to other cell lines or perpetuated from one laboratory to another. Although demanding and tedious, strict adherences to methods that prevent cell line contamination or misidentification are absolutely necessary.

5.2.5 Acknowledgements

The karyotype analyses described here were conducted in collaboration with Dr Rhonda Hutchinson from the Women's and Children's Hospital of Adelaide.

5.2.6 References

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Appendix 1

a. Sensory evaluation: Comments made by consumers on tuna fed different diets

Sample	Comment
Tuna fed Mixed Energy Diet A (470)	• This piece shared characteristics similar to that of the reference • 470 was a lot creamier on the palate and to my taste was a lot nicer than 768 • Not a strong flavour • had a better texture but did not have the flavour of the reference • Taste of 470 is better than any samples before. Colour is more natural. It looks fresh • The odour of 470 was milder than the reference. The texture was a lot firmer, but the colour to me wasn't as eye appealing as the reference • sweet flavour like…… lemon squeezed • sweeter flavour • Fairly close to reference. Colour a bit more red • much nicer colour & flavour (a lot stronger) • nice • chewy • texture in sample 470 not as smooth • strong smell and flavour stand out for me • this sample had a lot of similarities to the reference • seemed close to the sample in all aspects • B • C • Very dark in colour. Pleasant flavour & texture
Tuna fed Mixed Energy Diet B (180)	• tasted like local pilchards • While I didn't like the pale colour much, texture and flavour were good. My sense of smell is poor today • this was a very tasty sample quite similar to the reference in all aspects except flavour. The flavour was nice and smooth and unassuming • not the same sea flavour but enjoyable • much more like the reference in all ways • The colour looks pale (not natural) but the odour is better than the reference • Colour was not eye appealing. It has a nice white feathering in the flesh but the colour overall was too light. It was very light on in the odour, but due to the amount of time the reference was sitting at room temperature its odour increased. Its texture was firm but I prefer that

Tuna fed Mixed Energy Diet B (180)

- good texture and taste
- the reference tastes a bit better than the sample, flavour wise, but the colour and flavour of the sample is good
- again more red colour in flesh but firmer texture
- weird flavour
- flesh fairly soft, flavour fairly similar to reference, slightly different aftertaste
- straight down the middle - nothing special at all
- flavour very tart
- sample 180 seemed to have more moisture coming out of it
- good blend of colour and fat content
- this sample tasted sweeter than the reference
- slight
- nice I like it
- B-
- A
- stronger flavour, colour darker and texture slightly stringy

Tuna fed Mixed Energy Diet C (600)

- appearance not as appealing as previous sample
- Flavour not quite as nice as reference, but I still like it overall
- the flavour of sample 600 was stronger and left a stronger aftertaste and the meat a little more "blood" red
- exhibited very small blood clots and a blood vein -not appealing to look at by comparison
- sample contained more oil with smoother texture
- sample does not smell fishy. Texture is nice and soft taste has improved when compared to the reference
- I felt that sample 600 had a stronger odour than the reference. The colour looked similar but the white pattern in the flesh was more intense (looked good) the texture was similar to that of the reference
- very nice texture
- better flavour
- little differentiation in colour across sample
- fat content much higher than other samples. Texture not appealing
- I prefer more red meat (akami) & less toro in my slices. This one appeared to be mainly toro (lighter meat)
- less flavour, more texture (firmer) than reference
- better than reference
- sample 600 was almost colourless
- flavour & texture were similar but there was very little smell and it was more brown in appearance where as the reference was pinker
- Very similar to reference. Very nice

Tuna fed Mixed Energy Diet C (600)

- a bit ordinary in comparison to other samples
- this sample seemed more solid to bite through
- liked this sample
- the colour of 600 is darker than the other but the flavour is the same
- C-
- B
- texture not as good or smooth as the reference or samples 768 or 545

Tuna fed Local Baitfish (545)

- similar to reference in many characteristics
- Bright colour, nice texture but not as tasty
- the reference samples smell was stronger than this sample, the samples colour was very similar to the reference, the taste was stronger
- sample was fattier than reference
- I think this was the best of a very good lot of samples
- flesh on sample had a brown tinge rather than the reference which has a pink tinge
- looks pale (not natural) compared to the reference
- 545 were lighter in colour than the reference. It seems to have a similar odour. The texture by touch to me seemed a better texture than the reference. It was a better texture than the first sample
- very good taste and look
- better texture and overall better flavour
- Not as much colour distinction between darker red and outside flesh c.f. reference. Reference has lighter coloured outside flesh
- almost a sour flavour (not good)
- More flavour, slightly heavier texture. More colour than reference
- sample 545 was relatively odourless
- tougher than the reference but similar in every other way
- the flavour is stronger in this sample
- Minimal fishy smell. Comfortable eating texture
- strange flavour, a little more acidic
- sample different - pleasant though
- no
- C+
- B
- a bit stronger in flavour than the reference & sample 768 - texture good

b. Questionnaires

Name…… Consumer 1A

26-AUG-2005 TIME……

Do not eat the sample yet!

You will be given a tuna sashimi sample labelled **Reference** and a series of samples labelled with 3-digit codes.

1. Evaluate the **Reference** sample first and then the coded samples. You do not score the reference sample only the **coded** samples.
2. Indicate the size of the difference of the **coded** samples relative to the **Reference** sample using the scales below.
3. Also please indicate how much you like or dislike the coded sample for the attribute specified.

Remember a duplicate reference sample may be included in the coded samples given to you.

Please smell sample **768** and mark using a vertical stroke I on the line scale below what you think is the size of the difference relative to the reference in **odour**.

1. Odour of sample **768** relative to the reference?

No difference Extremely different

2. How much do you like or dislike the **odour** of sample **768?**

Dislike extremely Neither like nor dislike Like extremely

Look at sample **768** and mark using a vertical stroke I on the line scale below what you think is the size of the difference relative to the reference in terms of **colour**.

3. Colour of sample **768** relative to the reference?

No difference Extremely different

4. How much do you like or dislike the **colour** of sample **768?**

Dislike extremely Neither like nor dislike Like extremely

Now eat some of the sample and answer the following questions.

Mark using a vertical stroke I on the line scale below what you think is the size of the difference relative to the reference in terms of **texture**.

5. Texture of sample **768** relative to the reference?

No difference Extremely different

6. How much do you like or dislike the **texture** of sample **768**?

Dislike extremely Neither like nor dislike Like extremely

Mark using a vertical stroke I on the line scale below what you think is the size of the difference relative to the reference in terms of **flavour**.

7. Flavour of sample **768** relative to the reference?

No difference Extremely different

8. How much do you like or dislike the **flavour** of sample **768?**

Dislike extremely Neither like nor dislike Like extremely

9 Overall, how much do you like or dislike sample **768?**

Dislike extremely Neither like nor dislike Like extremely

Do you have any comments about this sample?

Comments:……
……
……

Name:…….. **Consumer 1A**

26-AUG-2005 TIME:……

Please answer the questions by placing a cross in the box to indicate your choice ☒

Please take the time to answer these general questions!

1. What is your gender? ☐ male ☐ female

2. Which age group do you belong to?

☐18 – 30 ☐31 – 40 ☐41 – 50 ☐51 – 60 ☐61 – 75

3. What is your country of birth?

☐ Australia ☐ Japan ☐ Other please specify……..

4. How long have you lived in Australia?

- ☐ 1 year or less
- ☐ 1 to 2 years
- ☐ 2 to 3 years
- ☐ 3 to 5 years
- ☐ more than 5 years

5. How often do you eat **tuna sashimi**?

- ☐ About one or more time(s) a week
- ☐ About once per fortnight
- ☐ About once per month
- ☐ About once every 6 months
- ☐ About once a year

6. When eating eating **tuna sashimi** do you eat it

- ☐ By itself with no sauces
- ☐ Other
- ☐ please specify……...

7. Do you work for

- ☐ A tuna farming company
- ☐ Not for a tuna farming company but within the fishing or aquaculture industry
- ☐ Neither of the above

c. Consumer Demographics

Of the 35 consumers who evaluated the tuna sashimi, 74% were male and 26% were female (Figure A1.1).

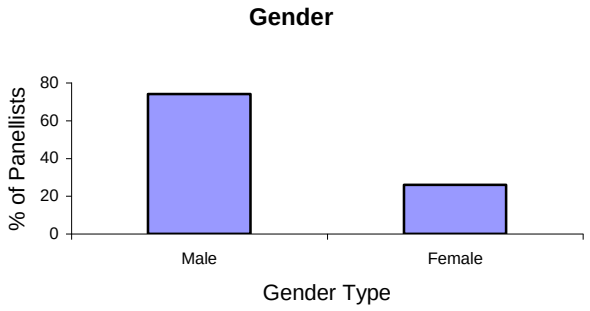

Figure A1.1. The gender of the consumers who evaluated the tuna sashimi (n=35).

The distribution across the different age groups of the consumers is illustrated in Figure A1.2, demonstrating that the greater percentage of consumers were in the 18-30 year age group.

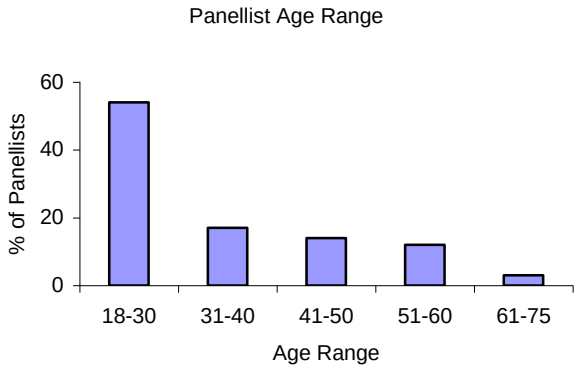

Figure A1.2. The age groups of the consumers who evaluated the tuna sashimi (n=35).

With reference to the consumers' country of birth, Figure A1.3 indicates that 88% of the consumers were born in Australia, 6% were born in Japan and 6% were born in other countries not specifically named.

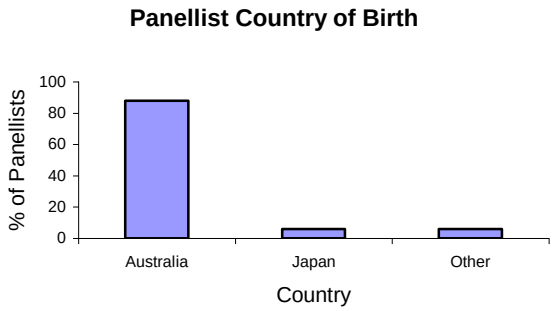

Figure A1.3. Country of birth of the consumers who evaluated the tuna sashimi (n=35).

Of the consumers who participated in the tasting, 88% had lived in Australia for more than five years with 12% living in Australia less than 5 years (Figure A1.4).

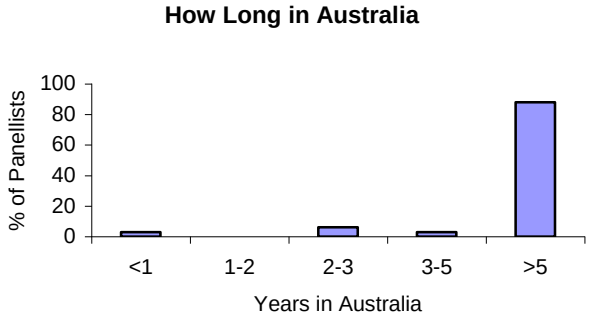

Figure A1.4. How many years the consumers who evaluated the tuna sashimi had lived in Australia for (n=35).

Only 97% of the consumers participating in this tuna trial responded to the question relating to regularity of consumption of sashimi. Of this, 18% ate sashimi weekly or fortnightly, 38% monthly, 26% six monthly and 18% yearly (Figure A1.5). So while the majority of the consumers ate sashimi once a month or more frequently there were still 44% who only ate sashimi on a six monthly or yearly basis. The frequency of consumption is described in the graph below.

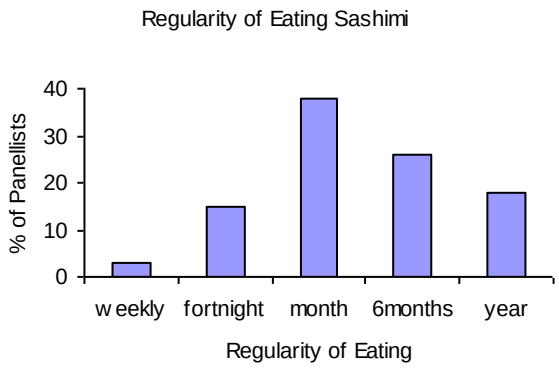

Figure A1.5. Regularity of consumption of sashimi of the consumers who evaluated the tuna sashimi (n=34)

With reference as to whether or not consumers ate sashimi with or without the addition of a sauce or condiment, 53% preferred consumption with sauce or other condiments, whereas one consumer had no preference either way. 32 % of the consumers ate the sashimi with or without additional condiments. Only 15% ate the sashimi without sauce as displayed in Figure A1.6. One consumer had no preference either way (i.e. did not eat sashimi).

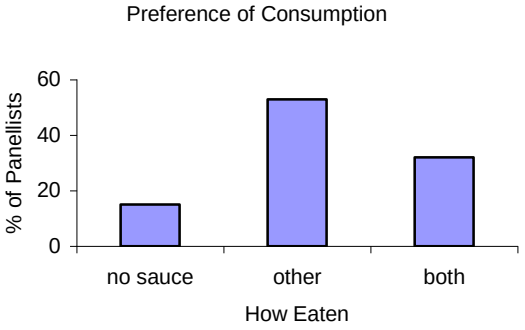

Figure A1.6. Preference of consumption of sashimi by the consumers who evaluated the tuna sashimi (n=34).

To gain some understanding of the background for consumption of sashimi, consumers were asked to indicate their main work area. The majority, (69%) did not work in the fishing industry (Figure A1.7). Only 11% of those surveyed were involved specifically in tuna farming. The remaining 20% were involved in another area of the fishing industry.

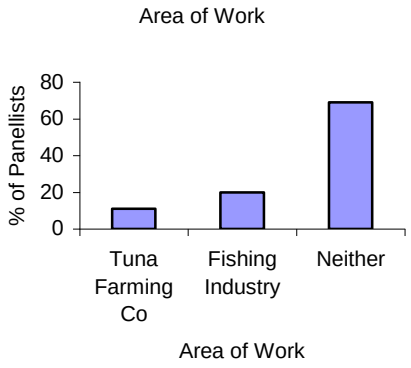

Figure A1.7. Area of work of the consumers who evaluated the tuna sashimi (n=35).

Appendix 2: 2007 Tuna surrogate experiment - supporting statistical tables and graphs

Table A2.1. Average temperature (°C) and dissolved oxygen levels (% saturation) of the YTK experimental tanks.

Date	Average temperature (°C)	Average dissolved oxygen (% saturation)	n
04/04/2007	21.5	92.1	8
11/04/2007	21.3	92.6	12
13/04/2007	21.7	92.3	11
16/04/2007	20.0	93.5	12
20/04/2007	20.1	92.1	24
24/04/2007	19.8	92.4	24
27/04/2007	19.8	94.2	24
30/04/2007	19.2	95.4	24
02/05/2007	19.5	94.1	24
04/05/2007	18.9	95.8	24

Table A2.2. Tests of between-subjects effects for proximate analysis of moisture (%) for YTK.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	42.843(b)	7	6.120	5.370	.003	.701	37.587	.972
Intercept	126910.944	1	126910.944	111340.447	.000	1.000	111340.447	1.000
Vitamin E	9.325	1	9.325	8.181	.011	.338	8.181	.766
Vitamin C	23.960	1	23.960	21.020	.000	.568	21.020	.990
Selenium	2.184	1	2.184	1.916	.185	.107	1.916	.256
VitaminE * VitaminC	5.171	1	5.171	4.536	.049	.221	4.536	.517
VitaminE * Selenium	1.622	1	1.622	1.423	.250	.082	1.423	.202
VitaminC * Selenium	.138	1	.138	.121	.732	.008	.121	.062
VitaminE * VitaminC * Selenium	.443	1	.443	.388	.542	.024	.388	.090
Error	18.238	16	1.140					
Total	126972.025	24						
Corrected Total	61.081	23						

a Computed using alpha = .05
b R Squared = .701 (Adjusted R Squared = .571)

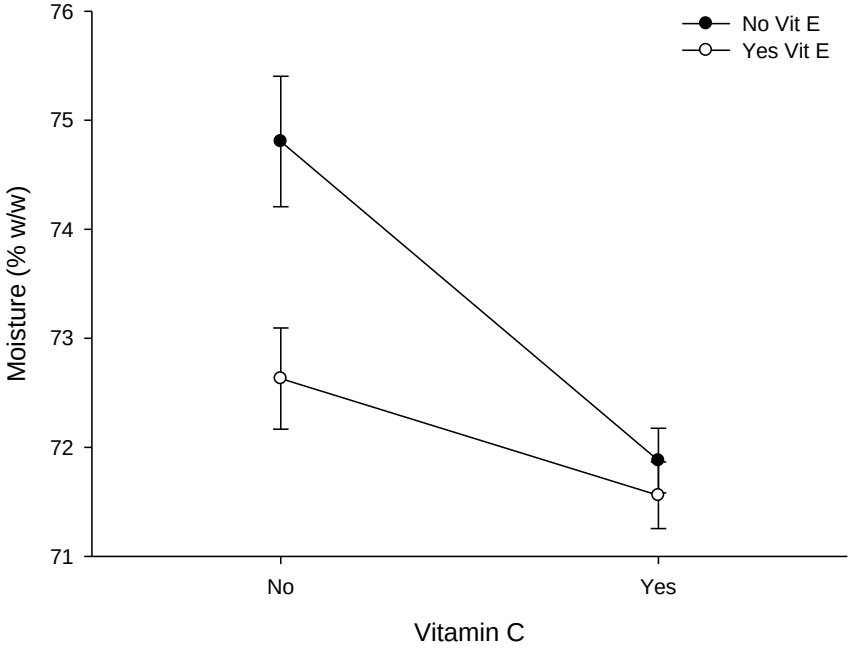

Figure A2.1. Interaction effects of added vitamin E and C in the diet on proximate analysis of mean moisture (%) in YTK.

Table A2.3. Tests of between-subjects effects for proximate analysis of fat (%) for YTK.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	6.918(b)	7	.988	.774	.618	.253	5.419	.233
Intercept	645.430	1	645.430	505.575	.000	.969	505.575	1.000
Vitamin E	.352	1	.352	.276	.607	.017	.276	.078
Vitamin C	2.244	1	2.244	1.758	.204	.099	1.758	.239
Selenium	.288	1	.288	.226	.641	.014	.226	.073
Vitamin E * Vitamin C	.027	1	.027	.021	.886	.001	.021	.052
Vitamin E * Selenium	.048	1	.048	.038	.848	.002	.038	.054
Vitamin C * Selenium	1.134	1	1.134	.888	.360	.053	.888	.144
Vitamin E * Vitamin C * Selenium	2.824	1	2.824	2.212	.156	.121	2.212	.288
Error	20.426	16	1.277					
Total	672.773	24						
Corrected Total	27.344	23						

a Computed using alpha = .05 b R Squared = .253 (Adjusted R Squared = -.074)

Table A2.4. Tests of between-subjects effects for proximate analysis of crude protein (%) for YTK.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	13.944(b)	7	1.992	2.516	.060	.524	17.615	.700
Intercept	7747.945	1	7747.945	9787.909	.000	.998	9787.909	1.000
VitaminE	4.524	1	4.524	5.715	.029	.263	5.715	.612
VitaminC	7.459	1	7.459	9.423	.007	.371	9.423	.822
Selenium	1.421	1	1.421	1.795	.199	.101	1.795	.243
VitaminE * VitaminC	.040	1	.040	.051	.825	.003	.051	.055
VitaminE * Selenium	.101	1	.101	.128	.725	.008	.128	.063
VitaminC * Selenium	.052	1	.052	.066	.800	.004	.066	.057
VitaminE * VitaminC * Selenium	.346	1	.346	.437	.518	.027	.437	.095
Error	12.665	16	.792					
Total	7774.554	24						
Corrected Total	26.609	23						

a Computed using alpha = .05 b R Squared = .524 (Adjusted R Squared = .316)

Figure A2.2. Interaction effects of added vitamin C in the diet on proximate analysis of crude protein (%) in YTK.

Figure A2.3. Main effect of added vitamin E in the diet on proximate analysis of mean crude protein (%) in YTK.

Table A2.5. Tests of between-subjects effects for specific growth rate of YTK.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	2.277(b)	7	.325	4.789	.005	.677	33.523	.952
Intercept	42.691	1	42.691	628.488	.000	.975	628.488	1.000
Selenium	.123	1	.123	1.816	.197	.102	1.816	.245
Vitamin C	.373	1	.373	5.497	.032	.256	5.497	.596
Vitamin E	.300	1	.300	4.419	.052	.216	4.419	.506
Selenium * Vitamin C	.254	1	.254	3.737	.071	.189	3.737	.443
Selenium * Vitamin E	.135	1	.135	1.994	.177	.111	1.994	.264
Vitamin C * Vitamin E	.907	1	.907	13.355	.002	.455	13.355	.929
Selenium * Vitamin C * Vitamin E	.184	1	.184	2.706	.120	.145	2.706	.340
Error	1.087	16	.068					
Total	46.055	24						
Corrected Total	3.364	23						

a Computed using alpha = .05 b R Squared = .677 (Adjusted R Squared = .536)

Table A2.6. Tests of between-subjects effects for hepatomsomatic index of YTK.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	.293(b)	7	.042	2.408	.025	.132	16.859	.845
Intercept	95.067	1	95.067	5461.518	.000	.980	5461.518	1.000
Vitamin C	.002	1	.002	.116	.734	.001	.116	.063
Vitamin E	.086	1	.086	4.932	.028	.043	4.932	.595
Selenium	.000	1	.000	.012	.914	.000	.012	.051
Vitamin C * Vitamin E	.138	1	.138	7.913	.006	.067	7.913	.796
Vitamin C * Selenium	.054	1	.054	3.095	.081	.027	3.095	.415
Vitamin E * Selenium	.010	1	.010	.598	.441	.005	.598	.120
Vitamin C * Vitamin E * Selenium	.002	1	.002	.124	.725	.001	.124	.064
Error	1.932	111	.017					
Total	97.409	119						
Corrected Total	2.226	118						

a Computed using alpha = .05…b R Squared = .132 (Adjusted R Squared = .077)

Table A2.7. Tests of between-subjects effects for vitamin C concentration ($mg.kg^{-1}$) in the muscle of YTK.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	897.401(b)	7	128.200	93.360	.000	.976	653.523	1.000
Intercept	1587.742	1	1587.742	1156.258	.000	.986	1156.258	1.000
Selenium	5.300	1	5.300	3.860	.067	.194	3.860	.455
VitaminC	877.218	1	877.218	638.825	.000	.976	638.825	1.000
VitaminE	4.679	1	4.679	3.407	.084	.176	3.407	.411
Selenium * VitaminC	4.676	1	4.676	3.405	.084	.175	3.405	.411
Selenium * VitaminE	2.265	1	2.265	1.649	.217	.093	1.649	.227
VitaminC * VitaminE	2.559	1	2.559	1.863	.191	.104	1.863	.250
Selenium * VitaminC * VitaminE	.706	1	.706	.514	.484	.031	.514	.104
Error	21.971	16	1.373					
Total	2507.114	24						
Corrected Total	919.371	23						

a Computed using alpha = .05 b R Squared = .976 (Adjusted R Squared = .966)

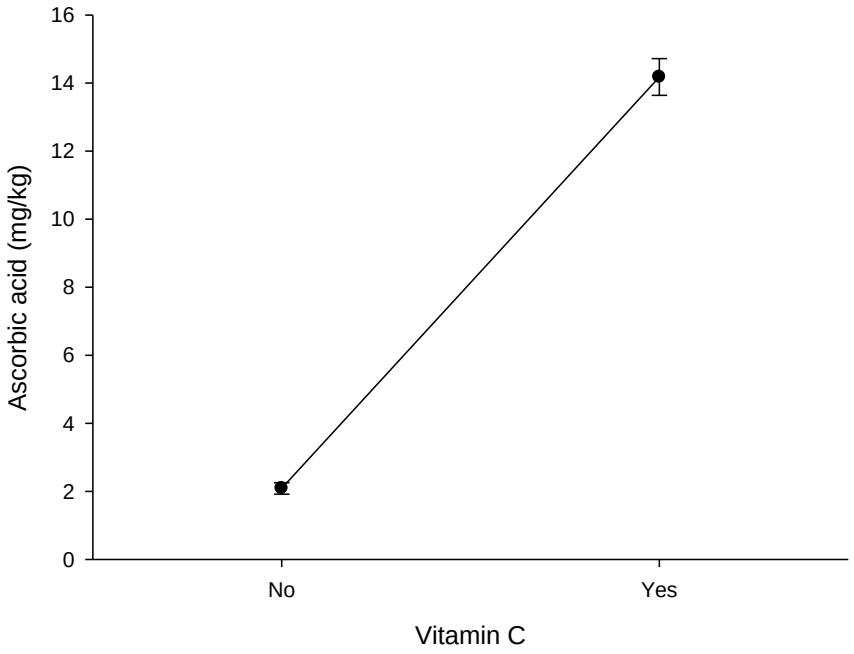

Figure A2.4. Main effect of added vitamin C in the diet on mean vitamin C (ascorbic acid) concentration ($mg.kg^{-1}$) in the muscle of YTK.

Table A2.8. Tests of between-subjects effects for vitamin E concentration ($mg.kg^{-1}$) in the muscle of YTK.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	17.590(b)	7	2.513	15.707	.000	.873	109.951	1.000
Intercept	92.073	1	92.073	575.530	.000	.973	575.530	1.000
Selenium	.253	1	.253	1.579	.227	.090	1.579	.219
Vitamin C	.469	1	.469	2.933	.106	.155	2.933	.363
Vitamin E	14.830	1	14.830	92.701	.000	.853	92.701	1.000
Selenium * Vitamin C	.025	1	.025	.154	.700	.010	.154	.066
Selenium * Vitamin E	1.419	1	1.419	8.871	.009	.357	8.871	.798
Vitamin C Vitamin E	.333	1	.333	2.080	.169	.115	2.080	.274
Selenium * Vitamin C Vitamin E	.261	1	.261	1.633	.220	.093	1.633	.225
Error	2.560	16	.160					
Total	112.223	24						
Corrected Total	20.150	23						

a Computed using alpha = .05 b R Squared = .873 (Adjusted R Squared = .817)

Table A2.9. Tests of between-subjects effects for selenium concentration ($mg.kg^{-1}$) in the muscle of YTK.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	.104(b)	7	.015	70.474	.000	.969	493.321	1.000
Intercept	3.747	1	3.747	17807.420	.000	.999	17807.420	1.000
Selenium	.097	1	.097	459.864	.000	.966	459.864	1.000
Vitamin C	.002	1	.002	9.000	.008	.360	9.000	.804
Vitamin E	.000	1	.000	1.494	.239	.085	1.494	.210
Selenium * Vitamin C	.001	1	.001	6.531	.021	.290	6.531	.670
Selenium * Vitamin E	.002	1	.002	11.864	.003	.426	11.864	.898
Vitamin C Vitamin E	.001	1	.001	4.457	.051	.218	4.457	.510
Selenium * Vitamin E	2.34E-005	1	2.34E-005	.111	.743	.007	.111	.061
Error	.003	16	.000					
Total	3.854	24						
Corrected Total	.107	23						

a Computed using alpha = .05 b R Squared = .969 (Adjusted R Squared = .955)

Table A2.10. Tests of between-subjects effects for vitamin C concentration ($mg.kg^{-1}$) in the liver of YTK.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	14925.669(b)	7	2132.238	90.199	.000	.975	631.396	1.000
Intercept	25097.652	1	25097.652	1061.698	.000	.985	1061.698	1.000
Selenium	4.364	1	4.364	.185	.673	.011	.185	.069
VitaminC	14758.560	1	14758.560	624.326	.000	.975	624.326	1.000
VitaminE	21.838	1	21.838	.924	.351	.055	.924	.148
Selenium * VitaminC	.079	1	.079	.003	.955	.000	.003	.050
Selenium * VitaminE	39.470	1	39.470	1.670	.215	.094	1.670	.229
VitaminC * VitaminE	57.351	1	57.351	2.426	.139	.132	2.426	.310
Selenium * VitaminC * VitaminE	44.008	1	44.008	1.862	.191	.104	1.862	.250
Error	378.227	16	23.639					
Total	40401.547	24						
Corrected Total	15303.895	23						

a Computed using alpha = .05 b R Squared = .975 (Adjusted R Squared = .964)

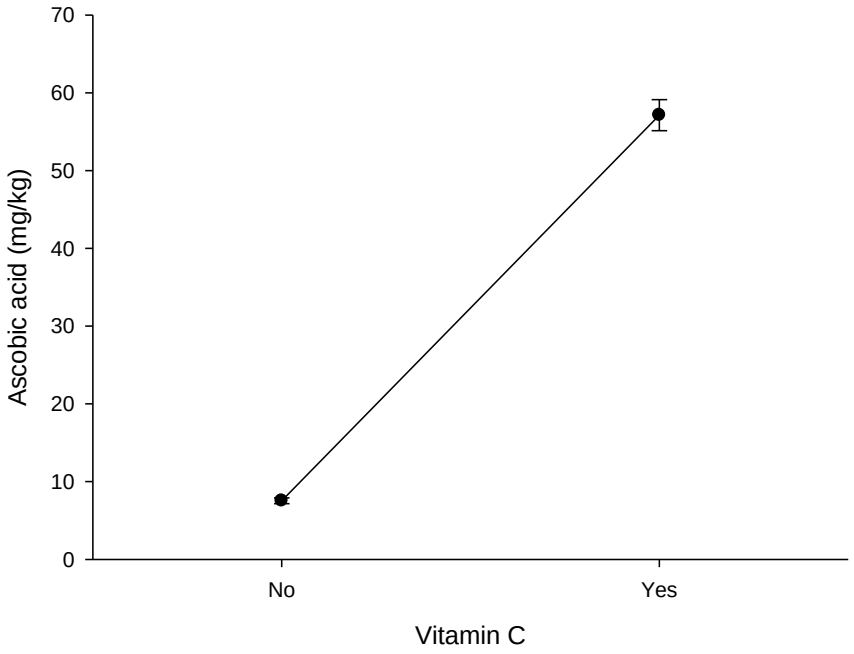

Figure A2.5. Main effect of added vitamin C in the diet on mean vitamin C (ascorbic acid) concentration ($mg.kg^{-1}$) in the liver of YTK.

Table A2.11. Tests of between-subjects effects for vitamin E concentration ($mg.kg^{-1}$) in the liver of YTK.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	1571.333(b)	7	224.476	15.222	.000	.869	106.555	1.000
Intercept	4004.425	1	4004.425	271.547	.000	.944	271.547	1.000
Selenium	3.951	1	3.951	.268	.612	.016	.268	.078
VitaminC	92.921	1	92.921	6.301	.023	.283	6.301	.655
VitaminE	1393.850	1	1393.850	94.520	.000	.855	94.520	1.000
Selenium * VitaminC	3.259	1	3.259	.221	.645	.014	.221	.073
Selenium * VitaminE	59.396	1	59.396	4.028	.062	.201	4.028	.471
VitaminC * VitaminE	10.049	1	10.049	.681	.421	.041	.681	.121
Selenium * VitaminC * VitaminE	7.905	1	7.905	.536	.475	.032	.536	.106
Error	235.947	16	14.747					
Total	5811.705	24						
Corrected Total	1807.280	23						

a Computed using alpha = .05 b R Squared = .869 (Adjusted R Squared = .812)

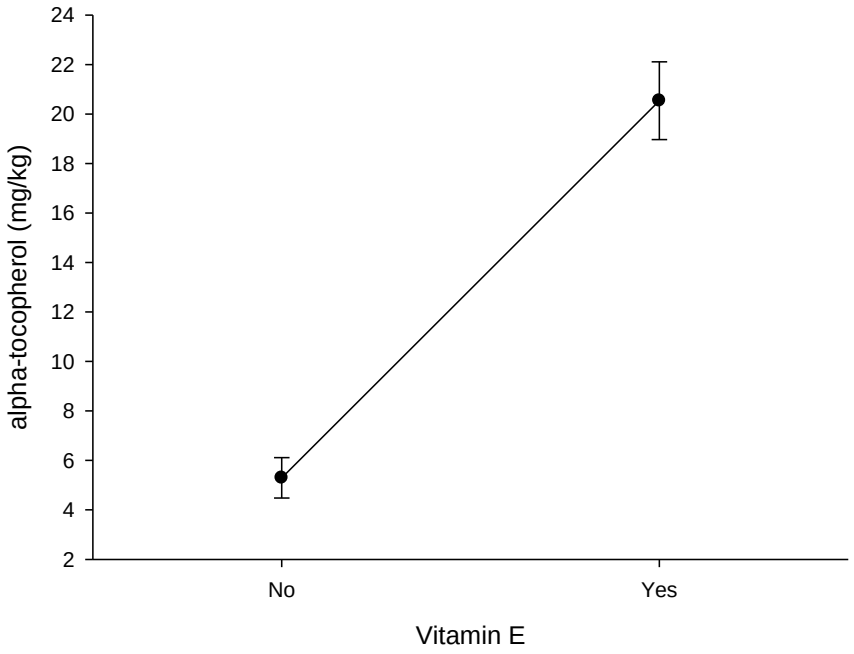

Figure A2.6. Interaction effects of added vitamin E in the diet on mean vitamin E (α tocopherol) concentration ($mg.kg^{-1}$) in the liver of YTK.

Figure A2.7. Main effect of added vitamin C in the diet on mean vitamin E (alpha-tocopherol) concentration ($mg.kg^{-1}$) in the liver of YTK.

Table A2.12. Tests of between-subjects effects for selenium concentration ($mg.kg^{-1}$) in the liver of YTK.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	2.505(b)	7	.358	12.386	.000	.844	86.700	1.000
Intercept	117.347	1	117.347	4061.120	.000	.996	4061.120	1.000
Selenium	.721	1	.721	24.969	.000	.609	24.969	.997
Vitamin C	1.512	1	1.512	52.339	.000	.766	52.339	1.000
Vitamin E	.030	1	.030	1.029	.325	.060	1.029	.159
Selenium Vitamin C	.033	1	.033	1.148	.300	.067	1.148	.172
Selenium Vitamin E	.093	1	.093	3.211	.092	.167	3.211	.392
Vitamin C * Vitamin E	.116	1	.116	4.003	.063	.200	4.003	.468
Selenium * Vitamin C * Vitamin E	2.60E-006	1	2.60E-006	.000	.993	.000	.000	.050
Error	.462	16	.029					
Total	120.314	24						
Corrected Total	2.968	23						

a Computed using alpha = .05 b R Squared = .844 (Adjusted R Squared = .776

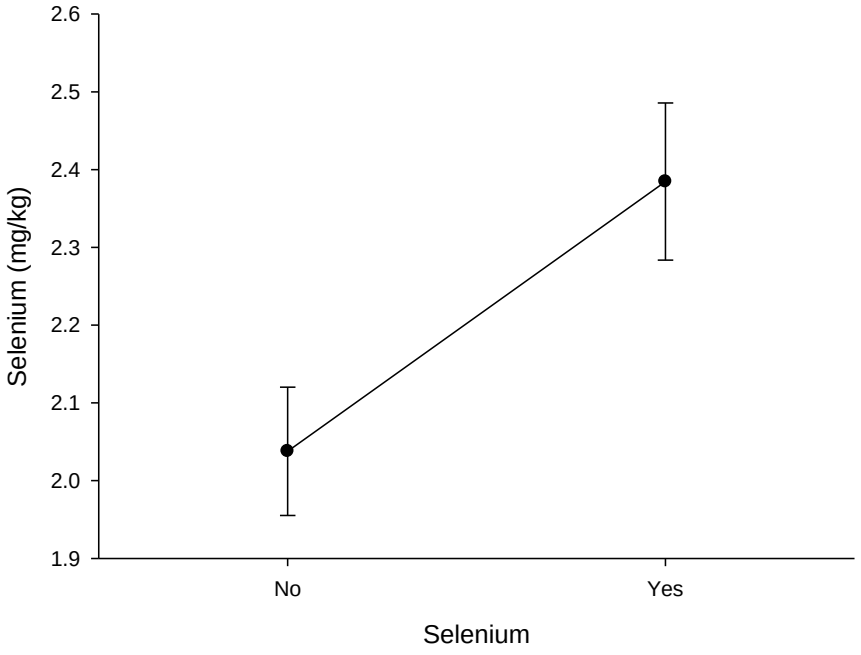

Figure A2.8a. Main effect of added selenium in the diet on mean selenium concentration ($mg.kg^{-1}$) in the liver of YTK.

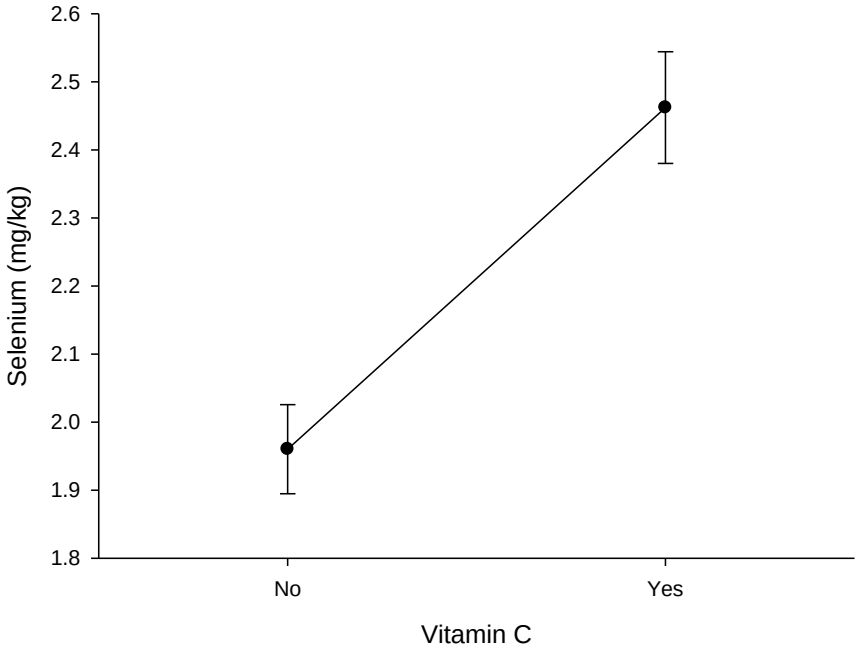

Figure A2.8b. Main effect of added vitamin C in the diet on mean selenium concentration (mg.kg^{-1}) in the liver of YTK.

Table A2.13. Tests of between-subjects effects for malonaldehyde concentration ($mg.kg^{-1}$) in the muscle of YTK at harvest.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	.031(b)	7	.004	1.821	.152	.443	12.744	.535
Intercept	2.439	1	2.439	1015.980	.000	.984	1015.980	1.000
Vitamin E	.000	1	.000	.089	.769	.006	.089	.059
Vitamin C	.004	1	.004	1.596	.225	.091	1.596	.221
Selenium	.011	1	.011	4.534	.049	.221	4.534	.516
Vitamin E * Vitamin C	.002	1	.002	.974	.338	.057	.974	.153
Vitamin E * Selenium	.005	1	.005	1.981	.178	.110	1.981	.263
Vitamin C * Selenium	.006	1	.006	2.633	.124	.141	2.633	.332
Vitamin E * Vitamin C * Selenium	.002	1	.002	.935	.348	.055	.935	.149
Error	.038	16	.002					
Total	2.508	24						
Corrected Total	.069	23						

a Computed using alpha = .05 b R Squared = .443 (Adjusted R Squared = .200)

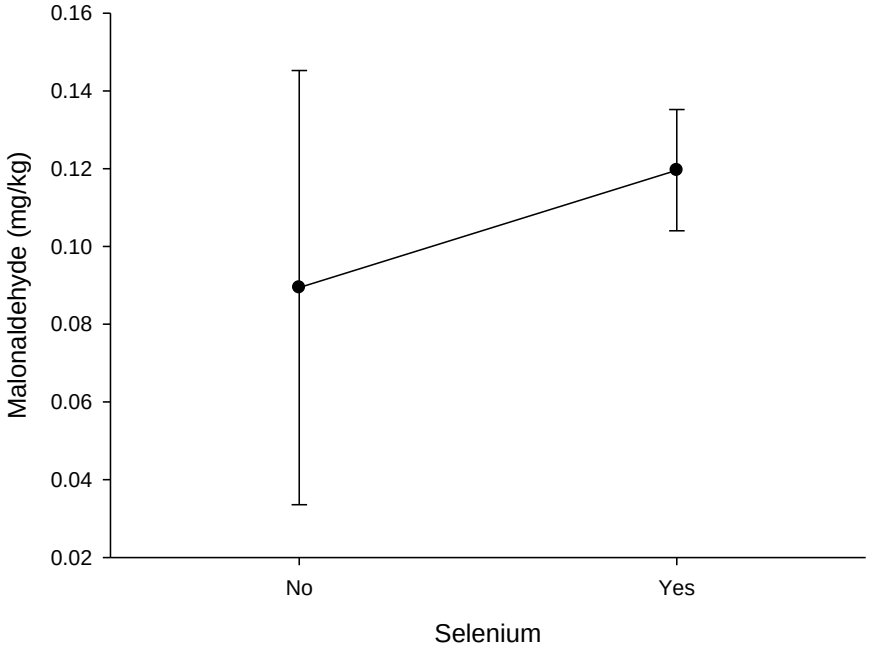

Figure A2.9. Main effect of added selenium in the diet on mean malonaldehyde concentration ($mg.kg^{-1}$) in the muscle of YTK at harvest.

Table A2.14. Tests of between-subjects effects for malonaldehyde concentration ($mg.kg^{-1}$) in the liver of YTK at harvest.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	.005(b)	7	.001	.403	.887	.150	2.822	.134
Intercept	.774	1	.774	467.302	.000	.967	467.302	1.000
Selenium	.000	1	.000	.115	.739	.007	.115	.062
Vitamin C	.001	1	.001	.563	.464	.034	.563	.109
Vitamin E	.001	1	.001	.369	.552	.023	.369	.088
Selenium * Vitamin C	.001	1	.001	.585	.456	.035	.585	.111
Selenium * Vitamin E	.002	1	.002	1.028	.326	.060	1.028	.159
Vitamin C * Vitamin E	.000	1	.000	.125	.729	.008	.125	.063
Selenium * Vitamin C * Vitamin E	6.27E-005	1	6.27E-005	.038	.848	.002	.038	.054
Error	.026	16	.002					
Total	.805	24						
Corrected Total	.031	23						

a Computed using alpha = .05 b R Squared = .150 (Adjusted R Squared = -.222)

Table A2.15. Tests of between-subjects effects for malonaldehyde concentration ($mg.kg^{-1}$) in the muscle of YTK 5 days post harvest.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power(a)
Corrected Model	.411(b)	7	.059	3.738	.014	.621	26.168	.881
Intercept	31.776	1	31.776	2023.387	.000	.992	2023.387	1.000
Selenium	.011	1	.011	.684	.420	.041	.684	.122
Vitamin C	.259	1	.259	16.516	.001	.508	16.516	.968
Vitamin E	.003	1	.003	.159	.695	.010	.159	.066
Selenium * Vitamin C	.005	1	.005	.318	.580	.020	.318	.083
Selenium * Vitamin E	.032	1	.032	2.014	.175	.112	2.014	.266
Vitamin C * Vitamin E	.043	1	.043	2.750	.117	.147	2.750	.345
Selenium * Vitamin C * Vitamin E	.059	1	.059	3.725	.072	.189	3.725	.442
Error	.251	16	.016					
Total	32.438	24						
Corrected Total	.662	23						

a Computed using alpha = .05 b R Squared = .621 (Adjusted R Squared = .455)

Figure A2.10. Main effect of added vitamin C in the diet on mean malonaldehyde concentration (mg.kg-1) in the muscle of YTK 5 days post harvest.

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Appendix 4: Intellectual property

The information within this volume is subject to the IP restrictions imposed by the Aquafin CRC within the IP Register. That said, in summary the information of this volume has no or undetermined IP value.

The research reported in section 6.1 was carried out by PhD candidate Alex Korte as part of her postgraduate studies.
The research reported in section 6.2 was carried out by Josephine Nocillado as part of FRDC Project. 2007/221.