Evaluating fish cell cultures as a platform for testing the effectiveness of antioxidants in preserving flesh quality in southern bluefin tuna and yellowtail kingfish

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Non-technical summary

2007/221 Evaluating fish cell cultures as a platform for testing the effectiveness of antioxidants in preserving flesh quality in southern bluefin tuna and yellowtail kingfish

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OBJECTIVES:

1. To adapt existing assays for antioxidant potency from mammalian cell culture systems for use with primary fish cell cultures and immortal fish cell lines.
2. To use the adapted assays with primary fish cell cultures and immortal fish cell lines to screen a broad range of concentrations and combinations of traditional and novel antioxidants for their ability to inhibit lipid oxidation.
3. To rank the antioxidants with respect to their antioxidant potency relative to vitamin E, the 'industry standard' antioxidant.
4. To quantify how the antioxidant concentrations that are effective in primary fish cell cultures and immortal fish cell lines relate to the antioxidant concentrations that need to be added to aquafeeds.
5. To undertake a cost-benefit analysis of the various antioxidants and antioxidant combinations with respect to their eventual incorporation into feeds.
6. To investigate the impact of dietary antioxidants on cellular antioxidants and antioxidant enzymes in primary fish cell cultures and immortal fish cell lines.
7. To recommend the best concentrations and combinations of antioxidants to be added to southern bluefin tuna and yellowtail kingfish feeds to maximize fish health and flesh quality.

OUTCOMES ACHIEVED TO DATE

This project resulted in the successful isolation and culture of cells from various body tissues of yellowtail kingfish (*Seriola lalandi*) and southern bluefin tuna (*Thunnus maccoyii*). Cell-based toxicity tests and antioxidant assays developed during the project provide a means for aquaculture feed manufacturers to undertake preliminary screening of potential feed additives. It is anticipated that such preliminary testing will significantly reduce the cost and timeframe of future feed trials for large marine finfish. Cell-based experiments conducted during the project resulted in the characterisation of representative antioxidant compounds according to their ability to prevent oxidative damage to lipids. This information will assist producers involved in the development of new manufactured feeds designed to maximise the stability of lipids sensitive to oxidation, such as the omega-3 long-chain polyunsaturated fatty acids.

Cultured fish cells are particularly useful for the detection of species-specific viruses. Certain cell types isolated during the course of the project display good potential for used in virus screening and

characterisation. Further work will involve the transformation of the cells to immortal cell lines suitable for routine virus screening.

In summary, the outcomes of the project to date include:

- Reproducible and rapid methods for the testing of antioxidant potency and any possible cytotoxicity
- A reproducible method for the production of primary cultures from the important South Australian aquaculture species yellowtail kingfish (*Seriola lalandi*)
- A method for the production of relatively long-lived cell cultures from yellowtail kingfish (*Seriola lalandi*) and preliminary success with the production of cell cultures from southern bluefin tuna (*Thunnus maccoyii*)
- Standardised methods (karyotyping and PCR) for identifying the species of origin of fish cell cultures
- The karyotype of southern bluefin tuna
- A basis for the development of immortal cell lines from yellowtail kingfish and southern bluefin tuna for the purposes of virus isolation and identification

This project was largely based around the characterisation of antioxidants using cultured fish cells. Methods were developed for the isolation and growth in culture of cells isolated from the body tissues of yellowtail kingfish (*Seriola lalandi*) and southern bluefin tuna (*Thunnus maccoyii*). While such cell cultures are useful for a range of research activities, the current project focused on the development and implementation of cell-based assays for determining antioxidant efficacy, particularly in the context of preventing the oxidative degradation of polyunsaturated fatty acids. Aquaculture conditions, particularly during harvest, can promote stress in fish, which can lead to the deterioration of flesh quality and reduced shelf life. This deterioration has been linked to oxidative damage to cellular and tissue components, particularly long-chain polyunsaturated fatty acids. Dietary antioxidants can help to prevent such damage.

In this study, a range of antioxidants were characterised for their ability to inhibit cellular oxidative stress, which can lead to lipid oxidation, as well as their ability to directly inhibit lipid peroxidation in cultured fish cells. Our approach, based on the use of cultured cells, allowed the simultaneous testing of a large number of different concentrations of each antioxidant, enabling the determination of antioxidant potency and the comparison of different antioxidant compounds. We found that ascorbate, a representative vitamin C compound, strongly inhibited the formation of water-soluble reactive oxygen species (ROS) in cultured fish cells, but appeared to have a detrimental effect on lipid stability in high doses. Conversely, vitamin E (in particular alpha-tocopherol, a representative of the vitamin E family of antioxidants), strongly inhibited lipid oxidation, but had little effect on cellular ROS. High concentrations of vitamins C or E were well tolerated by cultured cells, with little to no toxicity evident, however another commonly used antioxidant, *tert*-butyl hydroquinone, exhibited cytotoxicity at moderate concentrations. The results from our cell-based assays indicate that to promote the stability of lipids such as omega-3 long chain polyunsaturated fatty acids, levels of vitamin E in aquaculture feeds should be maximised, while

vitamin C should be included at the minimal levels required for fish health and high concentrations avoided.

In human and mammalian animal health research, cell cultures are used in a wide range of applications, from virus screening to toxicology, to detailed investigations of gene regulation and protein function. Compared to the variety of mammalian cell lines available to researchers, there are relatively few fish cell lines in culture collections, with marine species particularly under-represented. Certain types of cells isolated during the course of this project display characteristics suited to the isolation and characterisation of viruses of commercially important marine fish species. Planned work involves transforming these cells into immortal cell lines suitable for routine virus screening.

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Background

Fish lipids are rich in long-chain polyunsaturated fatty acids (LC-PUFA), especially the omega-3 fatty acids EPA (eicosapentaenoic acid) and DHA (docosahexanoic acid) which are associated with the human health benefits of a seafood-rich diet e.g. reduced risk of cardiovascular and inflammatory diseases. Unfortunately, LC-PUFA are highly susceptible to oxidation as a result of oxidative stress. Oxidative stress occurs when an animal's antioxidant defence system is overwhelmed by high rates of oxygen radical production or insufficient intake of dietary antioxidants (e.g. vitamin C and vitamin E). Cellular lipids rich in PUFA are especially susceptible to attack by oxygen radicals. Oxygen radical attack on cellular lipids produces lipid hydroperoxyl radicals which can set off a chain reaction of lipid peroxidation. The short term outcome of this is the destruction of fatty acids including the healthy omega-3 fatty acids. The longer term outcome is the production of aldehydes which give rancid fish their characteristic 'off' odour and flavour.

Oxygen radical production is a normal consequence of aerobic respiration. Thus, it increases when respiration increases such as during the harvesting of farmed fish. Lipid hydroperoxyl radicals formed during harvest continue to cause damage post-mortem. This is at least part of the explanation for the poorer flesh quality of stressed fish. Inadvertent feeding with partially rancid feeds also causes oxidative stress. Lipid hydroperoxyl radicals present in the feeds can carry over into the flesh of the farmed fish, setting off the chain reaction of lipid oxidation described above. The other factors detrimentally affecting flesh quality and shelf life post-mortem are (1) the cessation of dietary intake of antioxidants and (2) the degradation of the cellular antioxidants (e.g. glutathione) and antioxidant enzymes (e.g. glutathione peroxidase) that together with the dietary antioxidants constitute the antioxidant defence system.

Thus, deposition of larger quantities of antioxidants in the flesh of farmed fish prior to slaughter may better preserve the healthy omega-3 fatty acids and also improve the quality and extend the shelf life of the seafood product. In FRDC Project No. 2004/209, Dr Philip Thomas and co-workers showed that there was a strong positive relationship between dietary vitamin E concentration and vitamin E deposition in the flesh of farmed flesh. There was also a positive effect on colour shelf life and lipid stability during simulated retail display. However, this project did not investigate the stability of the individual fatty acids that make up the lipids which is an important factor to consider when marketing seafood for its human health value.

This project addresses the need for a preliminary screening system for dietary antioxidant supplements proposed as feed additives for marine finfish aquaculture. On-farm diet trials with southern bluefin tuna (SBT) such as those carried out in FRDC Project No. 2004/209 are time-consuming and expensive. For example, it can take 2-3 years to test just 3 or 4 different dietary antioxidant supplements in on-farm diet trials. In addition, the statistical power of these experiments is limited by the high economic value of SBT. For example, instead of taking one sea cage as the unit of replication, the researcher is forced to take individual fish within the same cage or analyse trends over several growing seasons. The use of fish cell lines and primary cell cultures has the potential to overcome these problems. In one experiment which typically lasts one week, it is possible to test a large number of different concentrations and/or combinations of antioxidants.

This project evaluated the use of fish cell lines and primary cell cultures as a platform for pre-screening dietary antioxidants prior to testing the most promising candidates in diet trials with live fish. *In vitro* testing employing cultured cells will reduce the number of experiments that need to be carried out with live fish. This will save time and the cost of live fish trials.

Need

The vast majority of the Southern Bluefin Tuna (SBT) farmed in South Australia is sold fresh for sashimi production in Japan. Premium prices are obtained for sashimi grade tuna with a high fat content. Unfortunately, fatty fish tissues with their high long-chain polyunsaturated fatty acid (LC-PUFA) content are prone to oxidation. This results in poor flesh quality, reduced shelf life and significant trimming of the tuna carcass at the market.

FRDC Project No. 2004/209 investigated the impact of dietary supplements on the flesh quality of farmed SBT. Specifically, it investigated the impact of the dietary antioxidants vitamins C and E and the mineral selenium. Project No. 2004/209 showed that these supplements can extend the shelf life of muscle samples taken from farmed SBT fed diets containing higher than normal concentrations of the antioxidants.

Although Project No. 2004/209 has been very successful, performing diet trials with live SBT is expensive and logistically difficult. It is also time-consuming and the statistical power of the experiments is limited by the fact that it is economically unfeasible to have multiple replicate sea-cages for each dietary treatment. As a result of these constraints, experiments must be repeated over several years to obtain statistically valid results.

This project evaluated the use of generic fish cell lines and primary fish cell cultures as a platform for testing dietary antioxidant supplements for large marine finfish such as southern bluefin tuna and yellowtail kingfish.

Objectives

1. To adapt existing assays for antioxidant potency from mammalian cell culture systems for use with primary fish cell cultures and immortal fish cell lines.
2. To use the adapted assays with primary fish cell cultures and immortal fish cell lines to screen a broad range of concentrations and combinations of traditional and novel antioxidants for their ability to inhibit lipid oxidation.
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Methods

N.B. **Appendix 3** contains a full list of chemicals, consumables and suppliers.

Routine cell culture

For the routine maintenance of cell lines used in the project, cells were grown in Liebovitz' L-15 medium supplemented with 10% (v/v) foetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 20 mM HEPES buffer, pH 7.4. For routine maintenance, the cells were grown in filter-capped 75 cm^2 flasks in a humidified incubator at 25°C under a standard ambient atmosphere. For routine subculturing, cells were washed three times with phosphate-buffered saline (PBS) and detached from the flask surface by incubating with trypsin-EDTA solution for 5 to 10 minutes at 25°C. After resuspending in fresh growth medium, cells were distributed to new flasks and medium added to a total of 15 mL per 75 cm^2 flask. Cells were subcultured every 3 to 7 days depending on the split ratio.

To prepare cells for long-term cryostorage in liquid nitrogen, monolayers were rinsed twice with PBS and cells were detached using trypsin-EDTA. After resuspending in normal growth medium (supplemented as described above), cells were transferred to sterile centrifuge tubes and pelleted at 1750 x *g* for 5 minutes. Supernatants were discarded and the cells resuspended in growth medium containing 10% (v/v) DMSO and transferred to cryotubes. To facilitate slow cooling, tubes were wrapped in a thick layer of cotton wool and placed in a -80°C freezer overnight. The tubes were then transferred to a liquid nitrogen storage dewar.

Isolation of hepatocytes and fibroblasts from YTK and SBT

Fish were killed by anaesthetic overdose (using a clove oil-based anaesthetic) or accessed immediately after harvest (in the case of ranched Southern Bluefin Tuna). Tissue sampling was carried out immediately after death or following a short incubation on ice of 30 minutes or less.

Tissue samples were prepared for cell isolation by rinsing briefly with 70% (v/v) ethanol, followed by rinsing thoroughly at least five times with sterile PBS. Prior to performing cell isolations, tissue samples were incubated for at least 30 minutes in Liebovitz' L-15 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B (also containing 20% (v/v) FBS and the pH stabilised by the addition of 20 mM HEPES buffer, pH 7.4) to minimise the chances of microbial contamination. In the case of southern bluefin tuna (SBT) liver samples, the medium was further supplemented with 5 µg/mL chloramphenicol and the tissue transported in this medium from the sampling site to the laboratory (approximately 4 to 6 hours travel time).

Prior to isolating cells, the tissue was rinsed thoroughly in sterile PBS to remove blood cells and growth medium. Tissue samples were minced into small pieces using two scalpels before incubating for 30 minutes to 1 hour with occasional agitation in 1 mg/mL collagenase solution (approximately 10 times the volume of the tissue sample) prepared in PBS containing 10 mM calcium chloride. This preparation was then passed repeatedly through a pipette to break up any remaining pieces of tissue before being filtered through a sterile 70 µm sieve. The resulting cell suspension was diluted

approximately ten-fold in L-15 medium supplemented with 20% (v/v) foetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B and 20 mM HEPES buffer, pH 7.4.

For assays involving the use of primary hepatocytes, viable cells were counted using trypan blue exclusion and plates were seeded at a density appropriate for the assay. Cells were incubated for 24 hours to allow attachment of hepatocytes before changing the growth medium and proceeding with the assay.

For the propagation of fibroblast-like cells, epithelial cells and other cells capable of attaching to the surface of tissue culture vessels, cells were seeded into 25 cm^2 or 75 cm^2 flasks and incubated for 24 hours before changing growth media. Standard culture conditions consisted of incubation at 25°C in a humidified incubator (capable of heating and refrigeration) under a standard atmosphere. Growth media were changed every 4 to 7 days. When confluence was achieved (for cells capable of proliferation in culture), cells were rinsed three times with PBS, detached from the flask surface with trypsin-EDTA and resuspended in growth medium before distributing to new flasks and adding fresh medium.

Identification of species of origin of cell cultures

Species identification by sequence analysis

For cell lines and proliferating cells grown from primary fish cell cultures, the species of origin was determined by determining the DNA sequence of a fragment of the mitochondrial gene encoding *cytochrome c oxidase subunit I* (*cox1*) and comparing the resulting sequence with a database of known *cox1* sequences. This gene is used extensively for determining the relatedness of different fish species and for species identification (Ward et al., 2005).

Genomic DNA was isolated from approximately 1 x 10^6 cells using a spin-column kit (Promega) according to the manufacturer's instructions. A fragment of the *cox1* gene was amplified by PCR according to published methods (Ward et al., 2005) and the reaction checked for specificity using agarose gel electrophoresis. The primers used were 5'-TCAACCAACCACAAAGACATTGGCAC-3' (Fish-F1) and 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3' (Fish-R1), from Ward et al. (2005). The fragment was purified using a spin-column kit (Promega) and sequenced (Australian Genome Research Facility) using the same primers as used for PCR. After visual inspection of the sequencing chromatogram to check for base calling errors, the resulting DNA sequence was compared to sequences present in publicly available databases using BLAST (Altschul et al., 1990).

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 (0.01 mg/ml final concentration) for 1-2 hours. Trypsinised 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).

Neutral red uptake cell viability assay

Cell viability was determined using a modified neutral red uptake assay based on standard methods (Borenfreund et al., 1988, Nemes et al., 1979, Winckler, 1974, Cavanaugh, 1990, Babich and Borenfreund, 1990, Repetto et al., 2008). A stock solution of 1 mg/mL neutral red (Sigma) was prepared in ethanol and diluted in standard growth medium (Liebovitz' L-15 supplemented as above) to a concentration of 10 µg/mL. Cells were incubated in this medium for 2 hours at 25°C, and a short washing/fixation step was performed using 10% (w/v) $CaCl_2$ and 4% formaldehyde. Cell-associated neutral red was then solubilised in an aqueous solution of 50% ethanol and 10% acetic acid for 5 minutes on an orbital shaker. Absorbance was read at 550 nm using a microplate spectrofluorometer (BMG Fluostar).

DCFH assay

Detection of cytoplasmic reactive oxygen species was performed using 2',7'-dichlorohihydrofluoescein diacetate (H_2DCFDA; Molecular Probes/Life Technologies). After completely removing growth media, cells were washed twice with 0.2 mL PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (designated PBS^+). Cells were loaded with 10 µM H_2DCFDA in PBS^+ at 25°C for 30 minutes in darkness. The probe solution was removed and the wells were washed twice briefly with PBS^+ before adding assay medium. Probe-free controls were included for cells treated with vehicle, cells treated with the highest concentration of antioxidant, and cell-free wells (to control for background fluorescence attributable to antioxidant-treated cells, solvent-treated cells and empty wells, respectively). To induce cellular oxidative stress, cells were incubated in serum-free L-15 medium (otherwise supplemented as described above). For non-stressed controls, cells were incubated in L-15 containing 10% FBS (and supplemented as above). After 4 hours at 25°C, fluorescence was measured from the underside of the wells using a plate fluorometer (BMG Fluostar) fitted with 485 nm excitation and 520 nm emission filters.

TBARS assay

Quantification of thiobarbituric acid-reactive substances (TBARS) in cultured cells was carried out using a procedure based on previously described methods (Reilly and Aust, 1999, Bird and Draper, 1984). Cells were washed with 1 mL PBS^+ before incubating for 1 hour at 25°C in 2 mL PBS^+ containing 0.5 mM cumene hydroperoxide and 1 µM hemin. After washing twice with 1 mL PBS^+, cells were lysed in 0.3 mL 1% (w/v) SDS for 5 min. Lysates were transferred to 1.5 mL centrifuge tubes. To each tube was added an equal volume of 1 M HCl containing 0.375% (w/v) thiobarbituric acid (TBA), 0.005% (w/v) butylated hydroxytoluene (Sigma) and 15 % (w/v) trichloroacetic acid. Standards were prepared by serially diluting malonaldeyde bis(dimethyl acetal) in an aqueous solution of 1% (w/v) SDS and adding an equal volume of TBARS assay reagent. The tubes were vortexed vigorously and incubated for 2 hours at 75°C. Insoluble material was removed by centrifugation at 16,000 x *g* for 10 minutes and supernatants transferred to a 96-well microplate in triplicate for fluorescence determination. MDA-TBA complexes were detected by reading fluorescence from the top of the wells using 540 nm excitation and 590 nm emission filters (BMG Fluostar).

C_{11}-BODIPY$^{581/591}$ oxidation assay

To detect reactive species in cellular membranes and lipids, we measured the oxidation of 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C_{11}-BODIPY$^{581/591}$; Invitrogen Molecular Probes), a lipid-soluble dye sensitive to oxidation by organic peroxides in the presence of transition metal ions (Drummen et al., 2002). Cells were seeded into wells of black opaque 96 well plates with optically clear bases (Corning) and grown for 24 h. After incubating for a further 24 h in the presence or absence of various antioxidant compounds, cells were washed twice with PBS$^+$ and loaded with C_{11}-BODIPY$^{581/591}$ at a concentration of 2 µM in PBS$^+$. After 30 minutes, unincorporated dye was removed by washing three times with PBS$^+$.

To initiate lipid peroxidation, cells loaded with the C_{11}-BODIPY$^{581/591}$ dye were treated with peroxides and hemin as described in the relevant sections below.

High-throughput microscopy

Oxidation of cell-associated C_{11}-BODIPY$^{581/591}$ was measured by high-throughput microscopy using the InCell Analyzer 1000 (GE Life Sciences). Four fields per well were captured using two filter sets – 480 nm excitation / 535 nm emission for the green channel (1500 milliseconds exposure time), and 565 nm excitation / 620 nm emission for the red channel (150 milliseconds exposure time). Pixel intensities were measured using ImageJ (Abramoff et al., 2004). Background fluorescence was omitted from the analysis of each set of images by setting thresholds such that only cell-associated fluorescence was included in intensity measurements. Threshold levels were identical for all images analysed in a given set. Images were captured prior to treatment with peroxide (cells were incubated in PBS$^+$ during imaging), and then after 30 minutes incubation in the presence of 0.5 mM cumene hydroperoxide and 100 nM hemin in PBS$^+$. Wells were treated in triplicate and experiments performed at least three times on different days.

Fluorescence microplate readings

For purposes of assay optimisation, we undertook preliminary experiments to quantify C_{11}-BODIPY$^{581/591}$ fluorescence in cell cultures using a fluorescence plate reader (BMG Fluostar). In this case, the seeding density was increased to $5x10^4$ cells per well to ensure detectable signal in the green channel, otherwise the procedure was identical. Readings were determined using 485 nm excitation / 520 nm emission filters for the green channel (using a gain setting of 1500), and 540 nm excitation / 590 nm emission filters for the red channel (with the gain set to 2500).

qRT-PCR analysis of antioxidant gene expression in cultured cells

Messenger RNA (mRNA) levels for various genes of interest and control genes were determined using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) using SYBR Green I chemistry as follows.

RNA extraction

Cells were grown in 6-well plates and subjected to various experimental conditions as described in the relevant results section. Total RNA was extracted from approximately 1×10^6 cells using spin column purification (RNeasy kit) according to the manufacturer's instructions, with on-column DNase treatments included to prevent contamination by genomic DNA. Yields and purity were

determined by UV absorbance (NanoDrop), and RNA integrity checked using agarose gel electrophoresis after denaturation by heating to 70°C for 5 minutes and rapid cooling on ice.

cDNA synthesis

Complementary DNA (cDNA) was synthesised from 1 to 2 µg total RNA using Moloney Murine Leukaemia Virus (M-MuLV) reverse transcriptase (RT) according to the manufacturer's instructions, with some modifications. Briefly, total RNA was combined with 1 mM each dNTP and 10 µM anchored oligo-dT primer (dT_{28}-VN) in a total volume of 10 µL RNase-free water, denatured for 5 minutes at 70°C and placed immediately on ice. After adding 4 µL 5X M-MuLV RT buffer (New England Biolabs; NEB), 1 µL RNase inhibitor (Promega) and 200 units M-MuLV RT (NEB), the volume was adjusted to 20 µL with RNase-free water. Reactions were carried out at 50°C for 1 hour or 42°C for 90 minutes and the RT heat-inactivated at 70°C for 15 minutes. In general, cDNA was diluted ten-fold and 5 µL diluted cDNA was used in each qPCR reaction.

Primer design

Unless otherwise specified, each primer pair utilised in this project (**Table 1**) was designed to amplify cDNA from all fish species, i.e. primers were designed against highly conserved regions of mRNA from representative sequences present in available databases. In some cases, one or both primers in a given pair were degenerate – some nucleotide positions were randomised during synthesis, in order to allow the detection of a given mRNA from any fish species. This approach was taken because in many cases, sequence data for the species of interest were not available. In some cases where sequence data were available for a particular species, specific primers were designed in addition to a conserved primer pair (this is stated where applicable).

Table 1. Oligonucleotide primers used for qRT-PCR. (N.B. IUB mixed base codes are used)

Gene (orientation relative to coding sequence)	Sequence (5'-3')	Optimal annealing temperature (°C)	Product length (base pairs)
β-actin (forward)	TCCCTGGAGAAGAGCTACGA	58	98
β-actin (reverse)	AGGAAGGAAGGCTGGAAGAG	58	
GAPDH[a] (forward)	GCCACCCAGAAGACNGTNGA	60	85
GAPDH (reverse)	GTGGANGCDGGRATGATGTT	60	
GPx1[b] (forward)	GATTACACCCAGATGAACGA	58	104
GPx1 (reverse)	TTCTTGCAGTTCTCCTGATG	58	
Prx1[c] (forward)	TGAGGCAGATCACCATCAA	58	86
Prx1 (reverse)	GTCAGTGTGCTGGAAGG	58	
GPx4[d] (forward)	CCCCAGTAAACTACTCTCAGTT	58	174
GPx4 (reverse)	TCRATCTTRCTGAACATGTCRAACT	58	

[a]Glyceraldehyde-3-phosphate dehydrogenase; [b]Glutathione peroxidase 1; [c]Peroxiredoxin 1. [d]Glutathione peroxidase 4.

For primers designed against conserved regions, sequence data for all fish species present in publicly available databases were collected. Multiple sequence alignments were created using AlignX (part of the Invitrogen Vector NTI software package) and highly conserved regions identified by visual inspection. Primers were manually designed against highly conserved regions and checked for self- and inter-complementary sequences using SciTools (Integrated DNA technologies). Primers were

designed such that the melting temperature of both primers was approximately 60°C, and product length was between 80 to 200 base pairs to ensure efficient amplification. Primers that met the criteria were synthesised at 40 nmole scale and sequencing/PCR purity (Geneworks).

For primer pairs specific for a particular species, the cDNA sequence of interest was entered into the input for the online version of the Primer3 primer design software package (Rozen and Skaletsky, 2000), and primer pairs chosen according to a set of criteria as follows. The product size range was restricted to 80-200 base pairs; optimal primer length was set to 20 bases; optimal primer melting temperatures were centred around 60°C (with minimum of 57°C and a maximum of 63°C); maximum self-complementarity was set to 5 base pairs; maximum 3' self-complementarity was set to 3 base pairs; the remaining parameters were left at the default settings. From the resulting primer pairs, one pair was selected for synthesis (Geneworks) according to proximity to the 3' end of the transcript (pairs closer to the 3' end were favoured) and lowest number of self- and inter-complementary bases.

Upon receipt of newly synthesised primers, stock solutions were prepared by dissolving pellets in 10 mM Tris-Cl, pH 8.5, to a concentration of 100 µM. Complete dissolution was achieved by heating to 50°C for 10-15 minutes, or by incubating overnight at 4°C, followed by vigorous vortexing. The resulting 100 µM stock solutions were stored at -20°C and diluted to 10 µM in molecular biology-grade water before use.

Standard PCR for specificity testing

To check for primer specificity before proceeding to qRT-PCR, standard PCR was carried out using cDNA generated from an appropriate sample (depending on the primer pair) and the products checked on agarose gels. Reactions contained 1X Platinum Taq buffer, 0.4 mM each dNTP, 2 mM $MgSO_4$, 0.4 µM forward primer, 0.4 µM reverse primer, and 1 unit Platinum Taq DNA polymerase. After an initial denaturation step for 2 minutes at 95°C, reactions were cycled 30 times at 95°C for 10 seconds, 58-60°C for 20 seconds and 72°C for 30 seconds.

Agarose gel electrophoresis was used to check PCR products for specificity. Products were separated on 2.5-3% agarose gels in Tris-acetate-EDTA (TAE) buffer using standard procedures (Sambrook, 2001). Gels were stained with ethidium bromide or SYBR Safe dye and imaged digitally (Bio-Rad Gel Doc).

Amplification efficiency testing of primer pairs for qRT-PCR

PCR products generated during specificity testing (above) were purified using spin columns (QIAquick) according to the manufacturer's protocol. The purified product was quantified using UV absorbance (NanoDrop) and a series of ten-fold dilutions prepared in sterile Milli-Q water. Quantitative SYBR Green I-based PCR was carried out using 5 to 6 steps of this dilution series, starting from an approximate PCR product concentration of 1 nM. Reactions were prepared in a total volume of 20 µL, containing 5 µL diluted PCR product, 0.5 µM each primer and 10 µL Platinum® SYBR® Green qPCR SuperMix UDG (2X reaction mix). Reactions were performed in a RotorGene 2000 (Corbett/Eppendorf) using the 72-tube rotor. Cycling conditions consisted of 50°C for 2 minutes (designed to degrade dU-containing templates from possible PCR product carryover contamination), 95°C for 2 minutes, then 40 cycles of 95°C for 10 seconds, 58°C for 15 seconds and 72°C for 20 seconds (fluorescence in the green channel was measured at the end of this step). A melting curve

was then generated by heating reaction products from 72 to 95°C in 0.5°C increments, holding for 5 seconds and collecting fluorescence data at each increment.

The Rotor-Gene version 6.1 software package (Eppendorf) was used to calculate reaction amplification efficiencies for each primer set. Efficiencies are calculated by comparing the slope of the curve resulting from plotting threshold cycle (the PCR cycle at which the fluorescence reaches a value significantly above the background signal) against template concentration and comparing this to a theoretical curve resulting from a 100% efficient reaction (100% efficiency gives a slope of -3.32). Primer pairs with amplification efficiency less than 90% were discarded and new primers designed for the mRNA/cDNA. In addition to efficiency testing, melt curves were visually inspected to check for the presence of a single reaction product and to confirm the absence of artefacts such as primer dimers in template-containing reactions.

Quantitative PCR

Quantitative PCR reactions were carried out using a commercial SYBR Green I-based reaction mixture, Platinum® SYBR® Green qPCR SuperMix UDG, containing uracil DNA glycosylase (UDG) and deoxy-uracil (dU).

Reactions were prepared in a total volume of 20 µL. A master mix containing primers, Platinum® SYBR® Green qPCR SuperMix UDG and molecular biology-grade water was prepared for each gene of interest plus the normalising gene. Fifteen µL of this master mix was distributed between the appropriate number of reaction tubes, and 5 µL of a ten-fold dilution of cDNA was added. Reactions were prepared in duplicate. No-template controls (NTC) were included in each run. Reactions were performed in a RotorGene 3000 (Corbett/Eppendorf) using the 72-tube rotor. Reaction conditions consisted of a 50°C hold for 2 minutes (designed to degrade dU-containing templates from possible PCR product carryover contamination), a 95°C hold for 2 minutes, then 40 cycles of 95°C for 10 seconds, 58°C for 15 seconds and 72°C for 20 seconds (fluorescence in the green channel was measured at the end of this step in each cycle). A melting curve was then generated by heating from 72 to 95°C in 0.5°C increments, collecting fluorescence data during a hold for 5 seconds at each increment.

A normalised relative quantification approach was used to determine changes in mRNA levels in cell cultures subjected to experimental treatments. In this approach, changes in mRNA quantity are expressed as a ratio relative to a control culture, and this ratio is normalised to the ratio of another gene, the expression of which is assumed to be stable under the experimental conditions used. The chosen normalising gene is often a 'housekeeping' gene, or one which is required for normal cellular functions. Such a gene is usually under the control of a promoter which is always functional and is not considered inducible or repressible under a wide range of conditions. Common choices for a normalising gene are beta-actin (β-actin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-2-microglobulin (β2-MG), elongation factor 2A (ELF2A), or 18S ribosomal RNA (18S rRNA; only applicable if random primers are used instead of oligo-dT for cDNA synthesis), amongst others. We chose β-actin, primarily due to the wide availability of sequence data from fish. In preliminary experiments using constant total RNA input into cDNA reactions, β-actin mRNA quantity was stable in treated cultures relative to control cultures. The normalised relative quantity for a given target gene was determined as described below.

Data processing and statistical analysis

Quantitative PCR data were analysed using the Rotor-Gene version 6.1 software package to process raw data, Microsoft Excel 2007 to compile data, and the REST 2009 software package (Pfaffl et al., 2002) to determine normalised relative expression ratios. To obtain cycle threshold data for each reaction, a comparative quantification analysis was performed on the data set with the calibrator sample left at the default setting of sample 1. This allowed the software to choose an appropriate threshold at which cycle threshold (Ct) values were generated. The resulting Ct values were imported into REST 2009 along with amplification efficiencies for each individual reaction. For each gene of interest, expression ratios relative to control samples were normalised to ratios for β-actin.

Expression ratios obtained using the above procedure were analysed for statistical significance by comparing the expression ratio to that of a hypothetical ratio of 'no change', which is equal to a value of 1. This was achieved by implementing Student's *one-sample t test*, which allows the comparison of a mean to a hypothetical value using GraphPad Prism software, version 5.

DPPH radical scavenging assay

Antioxidants were serially diluted in 50 µL of an appropriate solvent in the wells of a 96-well microplate before adding an equal amount of 50 mM DPPH in ethanol. After allowing quenching to proceed to completion (approximately 5 minutes), absorbance at 517 nm was determined using a plate spectrophotometer (BMG Fluostar). Three independent experiments were performed, each with wells prepared in duplicate. Data were analysed using Prism 5 (GraphPad).

Results and discussion

Development of cell-based assays for antioxidant activity in a fish cell line

Confirmation of the species of origin of the EPC cell line

The cell line used in the present study was derived from the American Type Culture Collection cell line CRL-2872, designated 'EPC' (for Epithelioma Papulosum Cyprini), and was kindly provided by Dr Mark Crane (Australian Animal Health Laboratories, CSIRO Livestock Industries) in 2004. This cell line was first reported as having been isolated from a skin lesion on carp (*Cyprinus carpio*) (Fijan et al., 1983), but the line stored and distributed by the ATCC is now known to have originated from fathead minnow (*Pimephales promelas*), as is acknowledged by the ATCC (http://www.atcc.org). It is not known whether the original carp-derived cell line was misidentified, or cultures maintained at the ATCC were contaminated with a *Pimephales promelas* cell line at some point prior to deposition. A *Pimephales promelas* continuous cell line is also available from the ATCC, designated FHM (ATCC Number CCL-42). In order to avoid confusion with the FHM cell line, we continue to refer to the *Pimephales promelas* cell line in use in our laboratory at Flinders University 'EPC' or 'EPC cells', both of which terms are used interchangeably throughout this report.

Cross-contamination of cultured cells is a widespread issue, particularly in human cell biology research (Markovic and Markovic, 1998, Yoshino et al., 2006). A recent comprehensive review of reported instances of cell line contamination states that approximately 360 cell lines deposited in culture collections worldwide are contaminated with cells of source different from that originally stated by the depositor, of which over 100 were of a single cell type, HeLa (Capes-Davis et al., 2010). In the project reported here, we experienced such an issue with a continuous cell line which was in regular usage in our laboratory. It was determined that during or shortly after the isolation of cells from various tissues of SBT, the primary cell cultures were contaminated with the EPC cell line. This was confirmed using two techniques as described below. Subsequent to this discovery, we focussed on the use of the EPC cell line to optimise assays and perform detailed high-throughput antioxidant screening, and performed species-specific assays using primary hepatocyte cultures and fibroblast-like cells.

The original EPC cell line derived from carp (*Cyprinus carpio*) had a reported chromosome number ranging from 84 to 107 (Fijan et al., 1983). The expected chromosome number for carp is 100 (Fijan et al., 1983) and for fathead minnow it is 50 (Levan et al., 1966). (Note: Variation in chromosome number is common in immortal cell lines). After counting a minimum of 30 metaphase spreads, total chromosome number for the EPC-derived cell line used in the current project was determined to fall within the range 31 to 58 per cell. Representative metaphase spreads and chromosome counts are shown below for SBT (**Figure 1A**) and the EPC cell line maintained at Flinders University (**Figure 1B**). The SBT chromosomes can be arranged into 24 pairs of identical chromosomes, while chromosomes in the EPC cell line appear to have undergone extensive rearrangement and could not be paired visually. The EPC cell line has approximately half as many chromosomes as reported for the original EPC cell line, and half as many chromosomes as the normal chromosome number for carp. While tuna and related fish have a similar number of chromosomes to fathead minnow (48), the size and centromere location is obviously different according to the karyotype analyses presented here.

Figure 1. A comparison of the karyotype of cultured SBT cells and the EPC cell line. A representative metaphase spread (left) is shown alongside chromosome pairs arranged according to morphology (right). (A) SBT primary cell culture and (B) the EPC cell line.

To confirm species identity, genomic DNA was extracted from EPC cells and a fragment of the mitochondrial gene for cytochrome c oxidase subunit I (*cox1*) was amplified using published methods (Ward et al., 2005). Both strands were sequenced and similarity searches were performed against publicly available nucleotide databases using the BLAST algorithm (Altschul et al., 1990). These searches returned exact matches with *cox1* sequences from *Pimephales promelas* (fathead minnow). A pairwise alignment was constructed, showing 100% sequence identity between the *cox1* sequence obtained from the EPC cells maintained at Flinders University and a *cox1* sequence extracted from GenBank (**Figure 2**). These results were independently confirmed by Dr Mark Crane using cell stocks maintained at AAHL which is where the Flinders cell stocks originally came from.

The EPC cell line displays a transformed (immortal) phenotype, meaning that it can be propagated in the laboratory indefinitely and revived from cryostorage without significant loss of viability. These traits are highly suitable for the development of cell-based assays that are reproducible over an extended period of time. In the absence of a cell line from the species of interest to the South Australian marine aquaculture industry (primarily *Thunnus maccoyii* and *Seriola lalandi lalandi*), we used the EPC cell line for the development of methods for the characterisation of antioxidants, and then applied those methods to primary cells isolated from the species of interest.

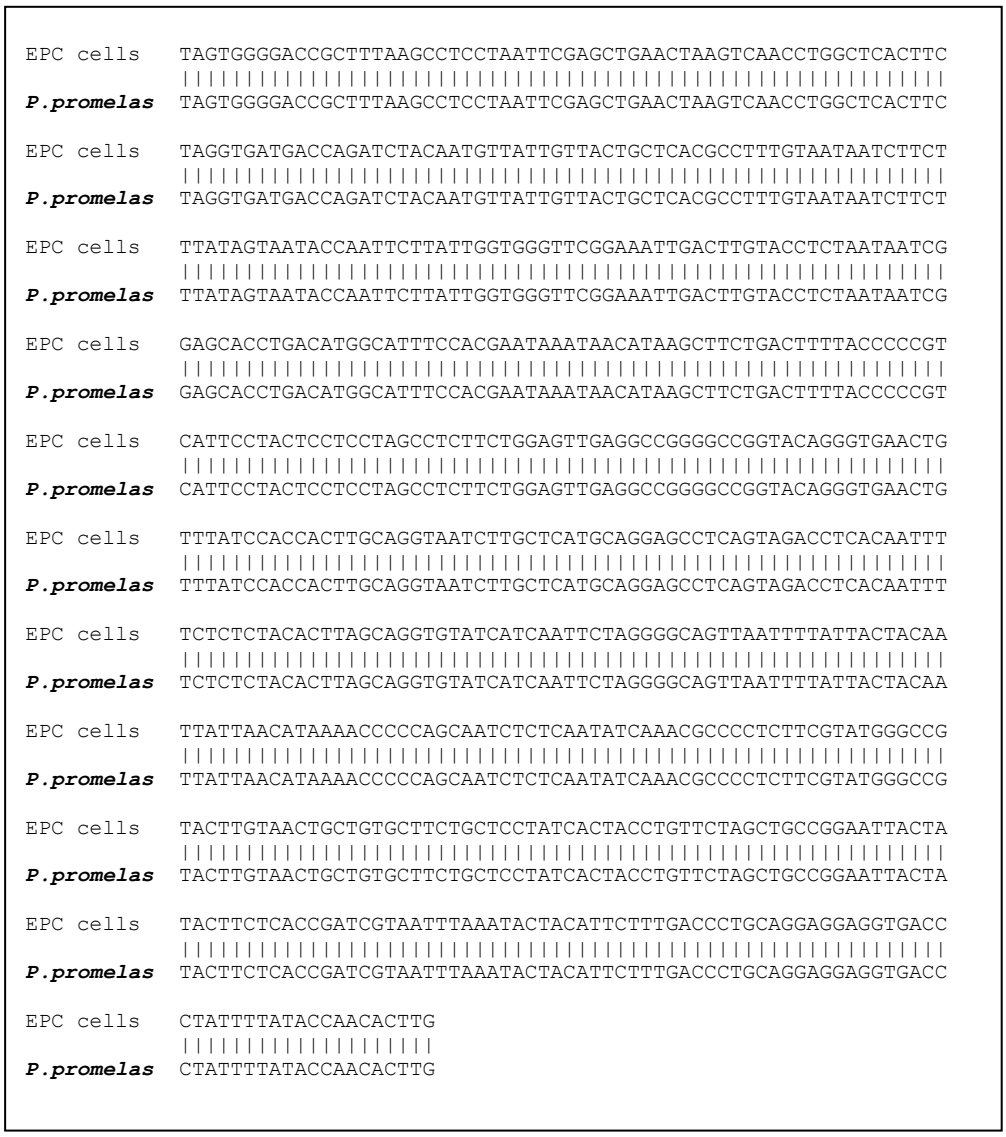


Figure 2. Confirmation of the species of origin of the EPC cell line by sequence analysis of a fragment of the mitochondrial gene for cox1. DNA was extracted from the EPC cell line and a fragment of the *cox1* gene was amplified and sequenced. The *cox1* sequence from fathead minnow (*Pimephales promelas*) was obtained from GenBank (accession number EU525089.1) and aligned with the EPC *cox1* sequence, showing 100% identity.

Development of cell-based methods for antioxidant activity

Neutral red uptake cell viability assay development

To establish concentration-response relationships between peroxide concentration and cell viability in a high-throughput format, we implemented a neutral red uptake assay based on previously published methods (Cavanaugh, 1990, Babich and Borenfreund, 1990, Repetto et al., 2008). EPC cells were seeded into the wells of 96-well tissue culture plates at a density of 1.5×10^4 cells per well and grown for 24, 48 or 72 hours in L-15 medium containing 10% (v/v) FBS. The medium was exchanged for serum-free L-15 containing various concentrations of hydrogen peroxide. After 1 hour at 25°C, media were exchanged with fresh medium containing neutral red and the plates incubated for 2 hours at 25°C. Uptake of neutral red by viable cells was determined by absorbance of light at a

wavelength corresponding to neutral red. Mean neutral red uptake and standard error for eight replicate wells were plotted against hydrogen peroxide concentration, and sigmoidal concentration-response curves fitted to the data (**Figure 3**). As expected, higher cell densities resulted in increased neutral red uptake, and uptake was diminished in cells exposed to high concentrations of hydrogen peroxide. This experiment shows that the neutral red uptake assay is a good indicator of cell number and viability and is well suited to experiments involving the exposure of cells to peroxides and other treatments promoting oxidative stress.

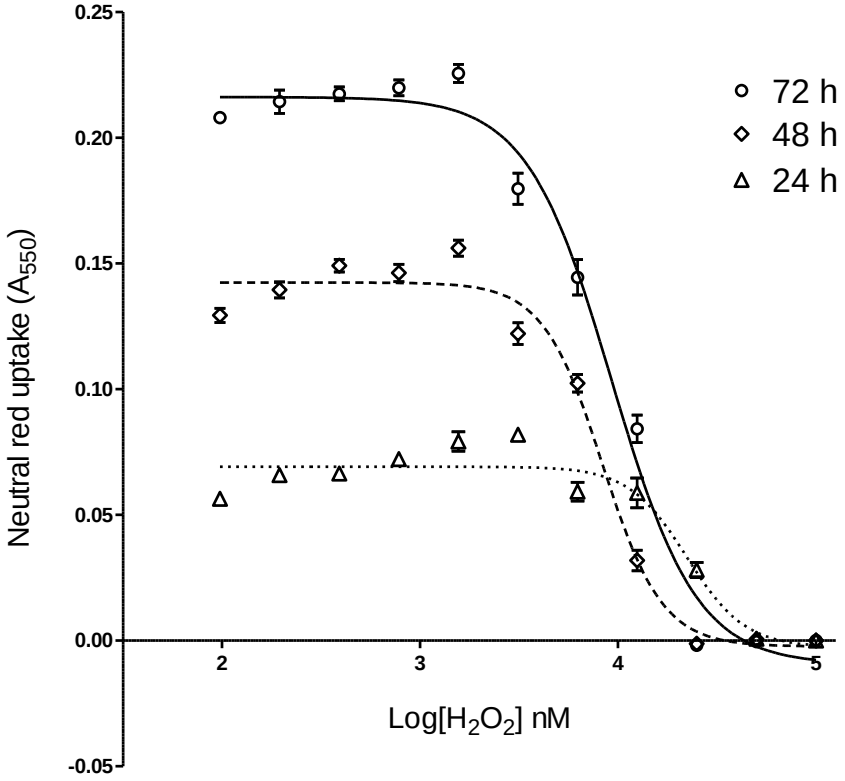


Figure 3. A representative example of the neutral red uptake assay in EPC cells grown in 96-well plates. Data shown are background subtracted means ± standard error from 6 replicate wells. Cells were seeded into three plates at a density of 1.5 x 10^4 cells per well and allowed to proliferate for 24, 48 or 72 hours before treatment with varying concentrations of hydrogen peroxide. Increased cell density resulted in increased neutral red uptake, and cell death in response to high concentrations of hydrogen peroxide resulted in diminished uptake of neutral red.

DCFH assay development

To determine the most appropriate conditions for inducing cellular oxidative stress, we undertook a series of preliminary experiments involving the treatment of DCFH-loaded EPC cells with exogenous peroxides, and observed changes in DCF fluorescence and cell viability. While peroxides induced DCF fluorescence rapidly, there was a significant impact on cell viability during relative short incubations (e.g. 1 hour or less). Furthermore, DCFH is known to leach from certain cell types, enabling oxidation by exogenously applied ROS (Royall and Ischiropoulos, 1993). Since we are interested in cellular oxidative stress and the prevention of such stress by antioxidant compounds, we investigated methods of inducing intracellular ROS rather than attempting to mimic oxidative stress conditions by applying peroxides exogenously. Others have reported that ROS are generated in cultured cells

when cells are deprived of certain medium components such as FBS (Andoh et al., 2002, Atabay et al., 1996, Galli and Fratelli, 1993, Lee et al., 2001, Nakajima et al., 2008). We therefore investigated whether incubation in serum-free growth medium could induce DCFH oxidation in EPC cells.

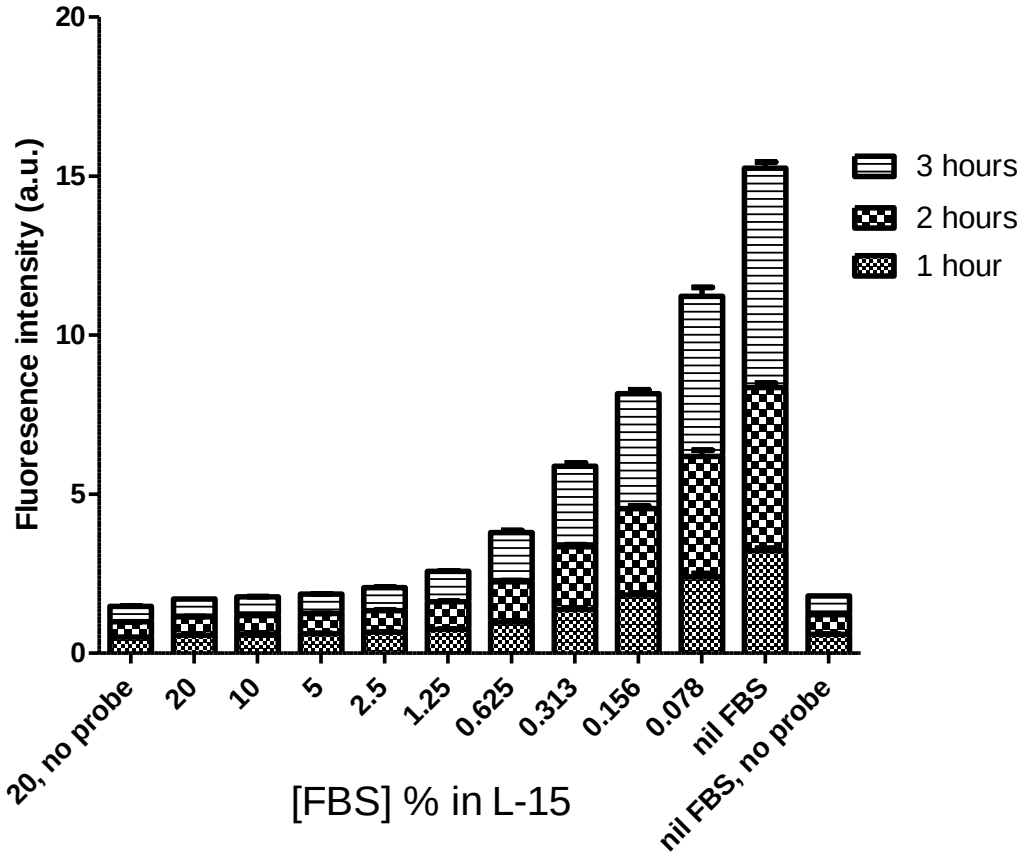


Figure 4. Induction of ROS formation in EPC cells by serum deprivation as indicated by DCF fluorescence. Cells were loaded with the probe (H_2DCFDA), washed thoroughly and incubated in L-15 medium containing various concentrations of FBS for 3 hours, taking fluorescence readings every hour using filters appropriate for DCF (485 nm excitation, 520 nm emission; reported above as arbitrary units, a.u.).

After loading EPC cells with H_2DCFDA for 30 minutes, the cells were washed extensively to remove extracellular H_2DCFDA and incubated in L-15 medium for 3 hours, with fluorescence determined every hour (**Figure 4**). Decreasing FBS concentration resulted in a corresponding increase in DCF formation, indicating that serum deprivation induces ROS formation in cultured EPC cells. This approach formed the basis of subsequent antioxidant activity assays, which involved pre-treatment with antioxidant compounds followed by serum starvation and determination of DCF formation.

A pilot experiment was carried out to optimise the serum starvation period with respect to obtaining an adequate dynamic range of DCF fluorescence signal while maintaining cell viability. We found that DCF fluorescence in serum-deprived EPC cells increased with time up to 4 hours with no appreciable loss of cell viability (**Figure 5**). Ascorbate pre-treatment inhibited the formation of DCF in serum-deprived EPC cells in a concentration-dependent manner (this is discussed further below in the section entitled '*Effects of antioxidant pre-treatment on cellular ROS during serum starvation*').

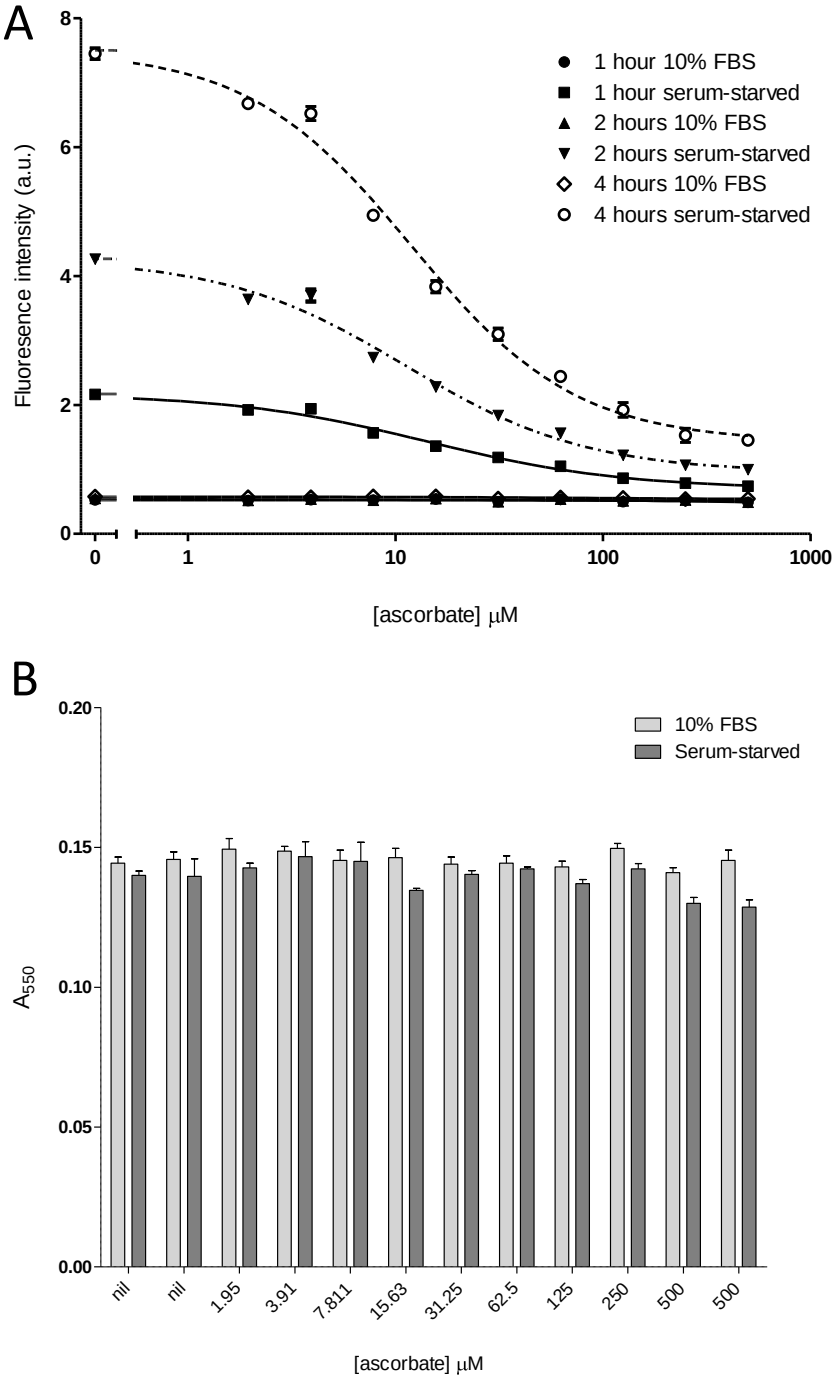


Figure 5. Optimisation of serum starvation period for the DCFH assay in EPC cells pre-treated with sodium ascorbate for 24 hours. (A) DCF fluorescence in EPC cells over time after pre-incubation with various concentrations of ascorbate. Data shown are means ±SE of fluorescence intensity (arbitrary units) in six replicate wells. (B) Neutral red uptake in EPC cells pre-treated with ascorbate for 24 hours, loaded with H_2DCFDA and serum-starved for 4 hours. Data shown are means ±SE of absorbance at 550 nm for three replicate wells.

TBARS assay development

The thiobarbituric acid-reactive substances (TBARS) assay is widely used as an indicator of lipid peroxidation in serum, body tissues and cultured cells. The assay detects aldehydes, primarily malondialdehyde, that form during the spontaneous breakdown of lipid hydroperoxides. The TBA-malondialdehyde adduct formed during the assay is coloured and fluorescent, allowing quantification using a spectrophotometer or fluorometer.

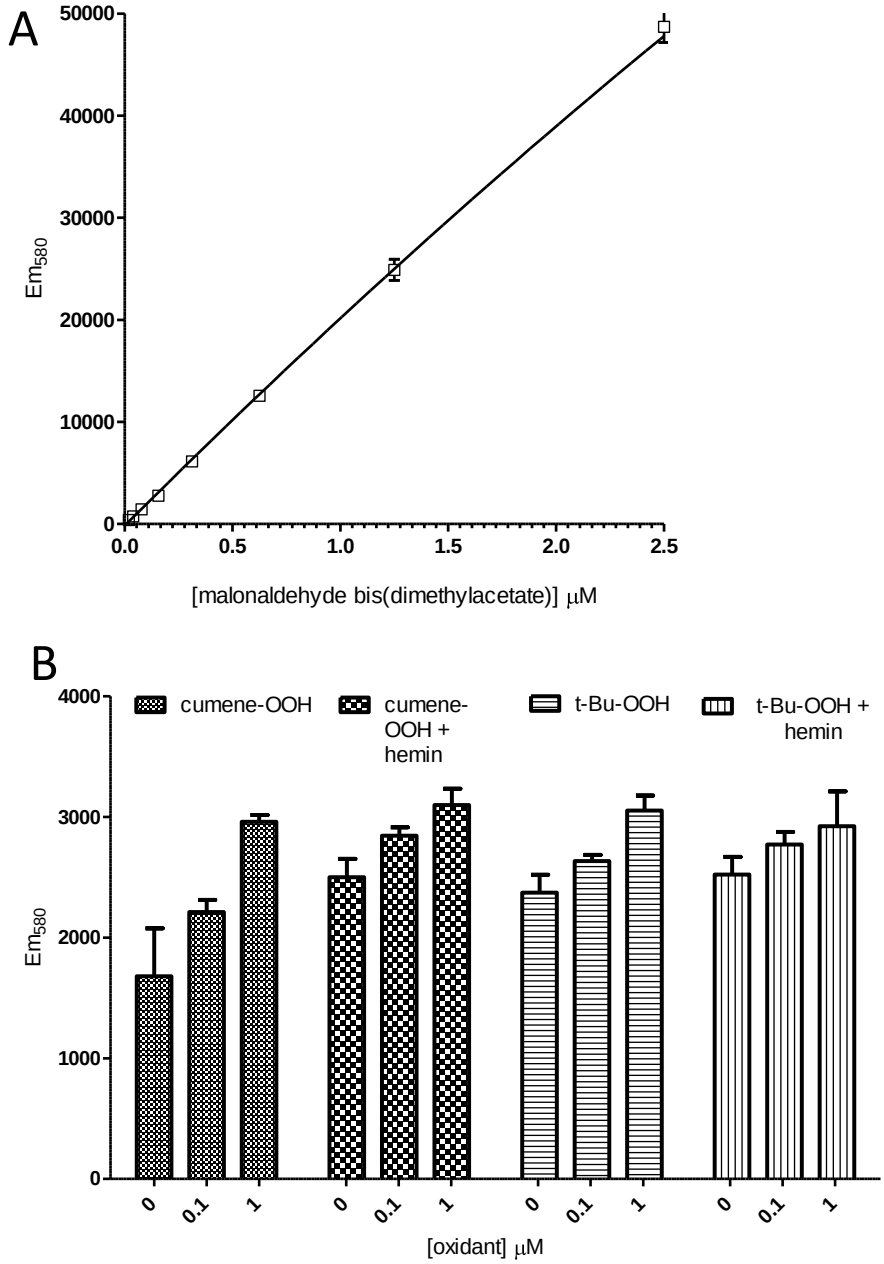


Figure 6. TBARS assay optimisation for use with cultured cells (A) Determination of the concentration-response curve and detection limit for the TBARS assay, using malonaldehyde bis(dimethylacetal) as standard. Data shown are means ±SE of fluorescence intensity (arbitrary units) readings from three replicate wells (*n* = 3). (B) TBARS formation in EPC cells treated with organic peroxides (0.1 or 1 μM) and hemin iron (1 μM) for 1 hour at 25°C. Data shown are means ±SE of fluorescence intensity (arbitrary units) readings from three replicate wells.

To obtain detectable MDA formation in EPC cells treated with organic peroxides in the presence of iron and analysed using the TBARS assay, we found that a minimal fully-confluent cell culture area of around 10 cm^2 (equivalent to one well of a 6-well tissue culture plate) was required, corresponding to around 1 x 10^6 EPC cells. Despite adequate signal from this number of cells, there was a significant contribution to background absorbance/fluorescence from components in the cell lysates, such that the useful dynamic range of the assay was not large. Nevertheless, in cells treated with organic peroxides in the presence or absence of iron in the form of hemin, modest increases in TBARS were observed (**Figure 6**). Fluorescence detection of TBA-MDA adducts gave more sensitive readings than absorbance. Standard curves prepared in cell lysis buffer revealed a detection limit of around 20 nM using the fluorescence method (**Figure 6**). The background fluorescence resulting from untreated cells was above this limit, suggesting the presence of interfering substances. Despite the relatively high background, apparent MDA-TBA content in peroxide-treated cells increased with peroxide concentration, indicating the formation of lipid hydroperoxides detectable by this method.

We chose to use cumene hydroperoxide and hemin co-treatment as a means of inducing lipid peroxidation in cultured EPC cells. The ability of various antioxidants to prevent lipid peroxidation under such conditions was investigated and is reported in the section below ('*Effects of antioxidant pre-treatments on lipid peroxidation in response to organic peroxides*').

C_{11}-BODIPY$^{581/591}$ assay development

Due to the poor dynamic range of the TBARS assay (see above), we pursued alternative methods for the estimation of lipid peroxidation. For application in the context of antioxidant characterisation and screening, it is advantageous to use a method that allows the simultaneous testing of many different compounds (or different concentrations of the same compound). Because the TBARS assay required a relatively large number of cells to obtain a signal detectable above background levels, it is not suited to high-throughput assays. We therefore investigated the suitability of the dye-conjugated fatty acid C_{11}-BODIPY$^{581/591}$ as an indicator of lipid peroxidation in EPC cells. This probe has been widely reported as a possible replacement for the TBARS assay in certain conditions (Drummen et al., 2002, Drummen et al., 2004, Itoh et al., 2007, Naguib, 1998, Pap et al., 1999). The C_{11}-BODIPY$^{581/591}$ compound is fluorescent in the red region of the visible spectrum (emission above 590 nm with excitation below 580 nm) until conjugated double bonds in the structure are oxidised, after which the compound exhibits a green fluorescence (excitation maximum around 480 nm and emission maximum around 520 nm). This has enabled researchers to determine the amount of oxidised probe present in cells at a given time as a proportion of the total amount of probe present (Pap et al., 1999).

To establish a suitable treatment for use in antioxidant screening experiments, we investigated the efficiency of C_{11}-BODIPY$^{581/591}$ oxidation in EPC cells treated with various peroxides in combination with iron in the form of hemin. As has been reported by researchers using mammalian cells (Drummen et al., 2004; Drummen et al., 2002), we found that hemin enhanced oxidation of the probe in the presence of a constant amount of cumene hydroperoxide (**Figure 7A**). We also observed that in the presence of hemin, the treatment of cells with cumene hydroperoxide (cumene-OOH) or *tert*-butyl hydroperoxide (*t*-butyl-OOH) induced the oxidation of C_{11}-BODIPY$^{581/591}$, whereas hydrogen peroxide (HOOH) did not (**Figure 7B**). Based on this data, we chose treatment with cumene hydroperoxide and hemin as a standard treatment to induce the oxidation of C_{11}-BODIPY$^{581/591}$ in EPC cells, and investigated the protective effects of various antioxidants (reported in

the section below entitled '*Effects of antioxidant pre-treatments on lipid peroxidation in response to organic peroxides*').

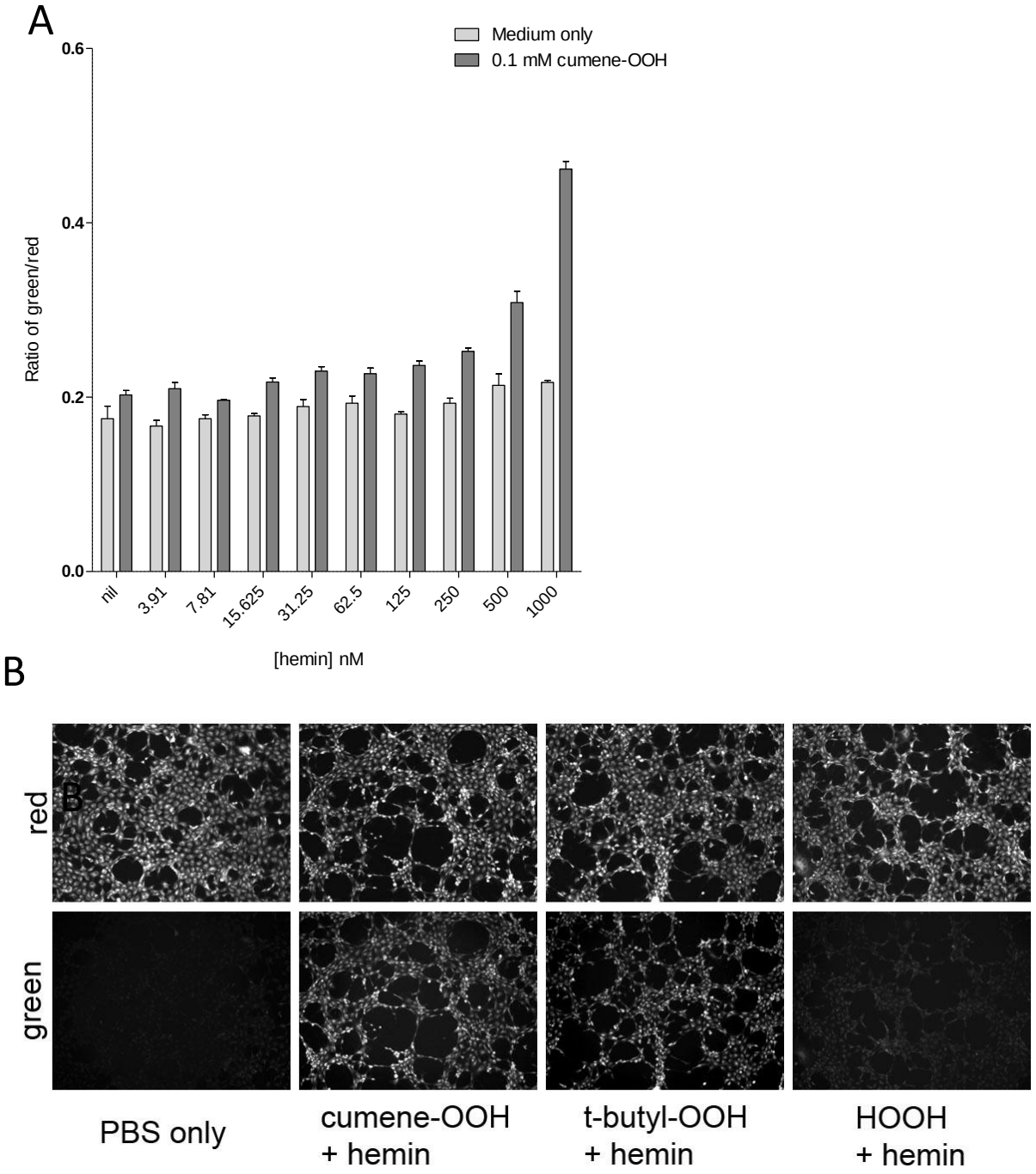


Figure 7. Determination of conditions for the efficient oxidation of C11-BODIPY581/591 in EPC cells. (**A**) Effect of increasing hemin concentration in the presence of cumene hydroperoxide. Data shown are ratios of green to red signal after determining intensity values using a fluorescence plate reader. Data shown are means ±SE from a minimum of three replicate wells (*n* = 3). (**B**) Fluorescence microscopy of EPC cells loaded with C_{11}-BODIPY$^{581/591}$ and treated with various peroxides in the presence of 0.1 mM hemin. Filter sets used were 480 nm excitation / 535 nm emission for the green channel and 565 nm excitation / 620 nm emission for the red channel.

Effects of antioxidant pre-treatment on cellular ROS formation during serum starvation

We implemented the DCFH assay in a cell-based model of cellular oxidative stress in fish, and applied the assay for the characterisation of a range of antioxidant compounds representing the classes of antioxidants used as dietary supplements or feed preservatives. We determined antioxidant activity in EPC cells in terms of the ability of various antioxidants to prevent cytoplasmic ROS formation, as measured by DCFH oxidation, in EPC cells subjected to conditions that promote oxidative stress.

The model antioxidants used in this study — sodium ascorbate, α-tocopherol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) and *tert*-butyl hydroquinone (*t*-BHQ) — were chosen as representatives of different classes of antioxidant compounds. Sodium ascorbate is a good example of the 'vitamin C' class of antioxidants – when dissolved in aqueous solution with neutral pH, the ascorbate moiety is identical to that obtained by dissolving ascorbic acid in neutral pH solutions. The term 'vitamin E' refers to any isoform of tocopherol or tocotrienol that displays biological activity as a fat-soluble antioxidant. We chose α-tocopherol as a representative compound due to its widespread use as a dietary additive and its common natural occurrence in biological systems. Trolox® was included in our analyses as it is a tocopherol derivative with a modified structure that enhances its aqueous solubility, which we predicted may result in different biological activity in cell-based assays than the highly lipid-soluble α-tocopherol. As a representative of phenolic antioxidants, we chose *t*-BHQ, which is a common food preservative and is also a metabolite of butylated hydroxyanisole (BHA) (Hirose et al., 1988, Cummings et al., 1985), another preservative often used to prevent lipid peroxidation and rancidity in foods.

We found that sodium ascorbate, Trolox®, and to a lesser extent, *t*-BHQ, inhibited DCFH oxidation in serum-starved EPC cells in a concentration-dependent manner (**Figure 8**). Conversely, α-tocopherol did not inhibit DCF formation, even at extremely high concentrations. This is probably related to differential compartmentalisation of the probe (i.e. DCFH) and the antioxidant. The results suggest that ROS generation in this assay occurs primarily in the aqueous phase, either in the cytoplasm or possibly in the extracellular medium in the case that DCFH leakage from the cell is considered. Because α-tocopherol accumulates in cellular membranes, the opportunity for physical interaction with the ROS responsible for DCFH oxidation is likely to be limited, allowing any ROS in the aqueous phase to oxidise the probe. The fact that Trolox® inhibits DCFH oxidation under similar conditions supports this hypothesis, since the antioxidant moiety in Trolox® and α-tocopherol is the same (the difference in chemical structure lies in the side chain, which is polar in Trolox® and non-polar in α-tocopherol, making Trolox® considerably more water-soluble than α-tocopherol).

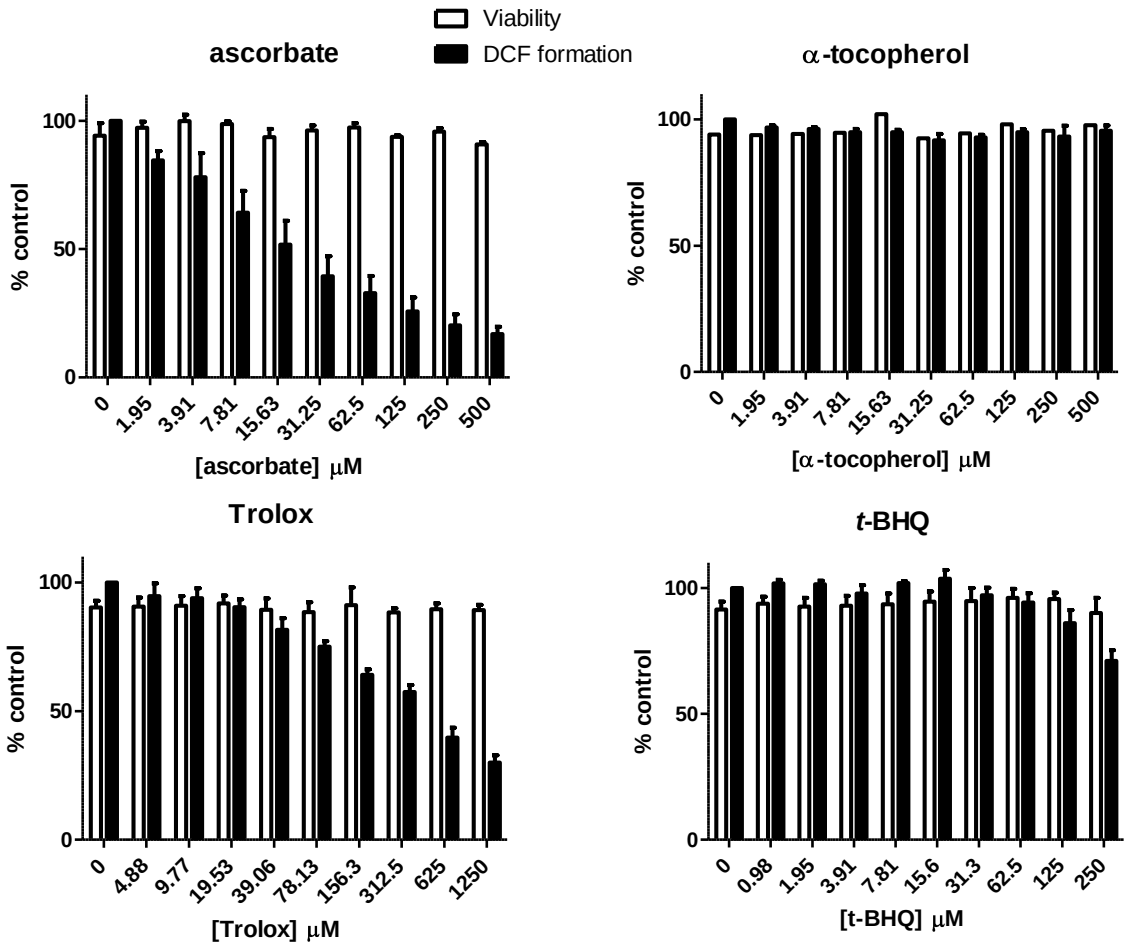


Figure 8. Effects of antioxidant pre-treatment on ROS content in serum-starved EPC cells, as measured by the DCFH assay. Cells were incubated in the presence of various concentrations of different antioxidants as shown, before loading with H_2DCFDA, washing and subjecting the cells to serum starvation. Viability is expressed as a proportion of controls incubated in medium containing FBS without added antioxidants. DCF formation is expressed as a percentage of serum-starved, solvent-only controls. Data shown are means ±SE from a minimum of three independent experiments (*n* = 3).

The nature of the ROS responsible for DCF formation in serum-starved EPC cells is unclear, although DCFH is known to react well with the hydroxyl radical as well as numerous reactive nitrogen species (Ischiropoulos et al., 1999), but is not efficiently oxidised by hydrogen peroxide (in the absence of ferrous iron) or superoxide (LeBel et al., 1992). The reduced form of DCF (referred to here as DCFH but also known as H_2DCF), the redox-sensitive cleavage product of H_2DCFDA resulting from cellular esterase activity, is polar in nature and is thus a good indicator of cytoplasmic reactive species. Because DCFH is charged, it is not likely to traverse the membranes of compartments such as lysosomes and peroxisomes, two possible sources of reactive species (in addition to mitochondria). Indeed, lysosomal disruption in the absence of oxidative stress has been shown to result in very high levels of DCF formation (Karlsson et al., 2010). Increased cytoplasmic oxidation of H_2DCF has been shown to occur in the presence of either cytochrome *c* liberated from mitochondria, or iron released from lysosomal storage (Karlsson et al., 2010). With this in consideration, it is possible that the reactive species responsible for DCF formation in EPC cells in serum-free medium derive from

Fenton reactions after the release of ferrous iron from lysosomes or mitochondria. It must be noted, however, that the uptake of Neutral Red dye in serum-starved cells was not markedly different from that observed in cells incubated in the presence of 10% FBS (**Figure 5B**), suggesting that lysosomal integrity and function was not adversely affected by serum deprivation during the assay period. Despite the nature of reactive species responsible for DCFH oxidation in the present study, it is clear that cellular uptake of soluble antioxidant compounds such as ascorbate and Trolox® prevents DCF formation, suggesting that this assay is well suited to the characterisation of soluble antioxidants in fish cells.

Effects of antioxidant pre-treatments on lipid peroxidation in response to organic peroxides

Lipid instability is a major issue in fish aquaculture due to the presence of high levels of polyunsaturated long-chain fatty acids present in fish flesh, which are prone to oxidation after harvest. Research into the molecular aspects of lipid stability and the prevention of lipid peroxidation by antioxidants will benefit from the establishment of a laboratory model system involving live fish cells. To further develop our EPC cell-based model system, we investigated whether pre-treatment of cells with antioxidants could prevent lipid peroxidation as estimated by TBARS content and oxidation of the dye-conjugated fatty acid, C_{11}-BODIPY$^{581/591}$.

To compare the results of TBARS assays with those obtained from the DCFH assay described above, we examined the same panel of antioxidants. We observed that TBARS formation in EPC cells treated with cumene hydroperoxide and hemin was strongly inhibited by pre-treatment with α-tocopherol, while Trolox® and *t*-BHQ inhibited TBARS formation only slightly (**Figure 9**). Although the background levels were relatively high, α-tocopherol potently inhibited TBARS formation at concentrations above 1.25 μM. This clearly shows that the lipid-soluble α-tocopherol is a good inhibitor of lipid peroxidation in the context of the assay conditions used here.

Interestingly, ascorbate caused a concentration-dependent increase in cellular TBARS content, with cell viability also diminishing as ascorbate concentration increased. This is not completely unexpected, as ascorbate is known to undergo redox cycling and form radicals in the presence of iron (Floyd and Lewis, 1983, Schneider et al., 1988). It has been shown that lipid peroxidation in tissue homogenates, as estimated by TBARS formation, is exacerbated in the presence of ascorbate but that this is prevented by strong chelators (Heikkila and Manzino, 1987), suggesting that metal ions catalyse the formation of reactive species in the presence of ascorbate. There are reports that high-dose vitamin C supplementation in humans does not lower serum TBARS levels when compared to tocopherol or combined ascorbate/tocopherol supplementation (Jialal and Grundy, 1993, Porkkala-Sarataho et al., 2000). It would be interesting to determine whether ascorbate supplementation in fish without simultaneous α-tocopherol supplementation exacerbates lipid peroxidation, and whether ascorbate supplementation diminishes the protective effects of α-tocopherol against lipid peroxidation in fish.

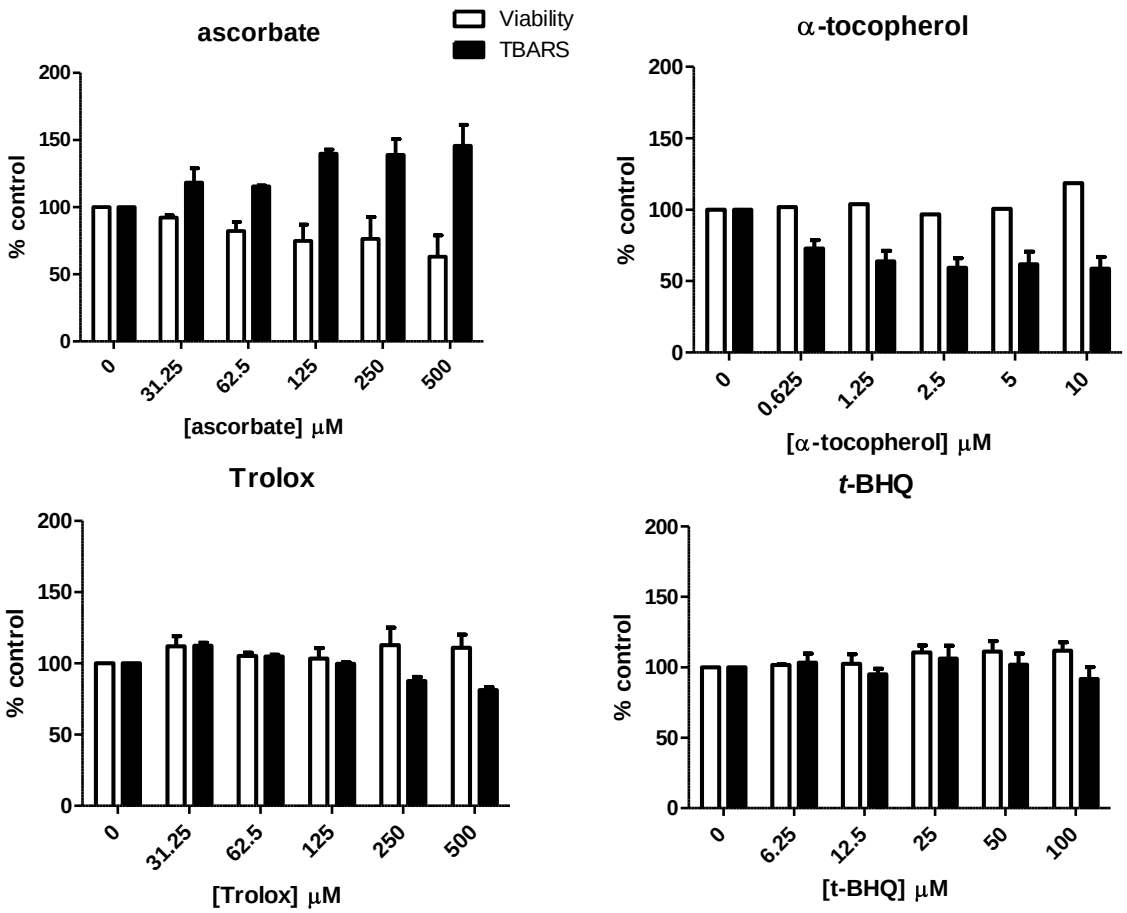


Figure 9. Effects of pre-treatment with various antioxidants on lipid stability in EPC cells as estimated using the TBARS assay. Cells were incubated with cumene hydroperoxide and hemin for 1 hour to initiate lipid peroxidation. Viability and TBARS content are expressed as a percentage of solvent-only controls (without antioxidant pre-treatment, labelled '0'). Data shown are means ±SE from a minimum of three independent experiments (*n* = 3 to 5).

The C_{11}-BODIPY$^{581/591}$ assay was also implemented in order to investigate the same panel of model antioxidant compounds in a high-throughput format (96-well plates). The results were similar to the TBARS assay, with α-tocopherol potently inhibiting oxidation of the probe, and no protective effect displayed by the other antioxidants (**Figure 10A**). The apparent protection by *t*-BHQ in wells treated with high concentrations of this antioxidant was probably due to toxicity, as evidenced from the loss of cell adhesion to the plate surface, which was clearly visible in microscope images in wells treated with higher concentrations of this compound (**Figure 10B**). However, a possible protective effect of *t*-BHQ was evident from the diminished oxidation of the probe in cells treated with 50 μM *t*-BHQ without obvious toxicity. One advantage of performing microscopic analysis of C_{11}-BODIPY$^{581/591}$ oxidation rather than determining fluorescence readings with a plate reader is that loss of cell adhesion due to cytotoxic effects of treatments is clearly visible without the need for performing cell viability assays. It was interesting to note the difference in apparent toxicity of *t*-BHQ compared to the results obtained with the TBARS assay. This is probably related to differences in incubation and washing protocols.

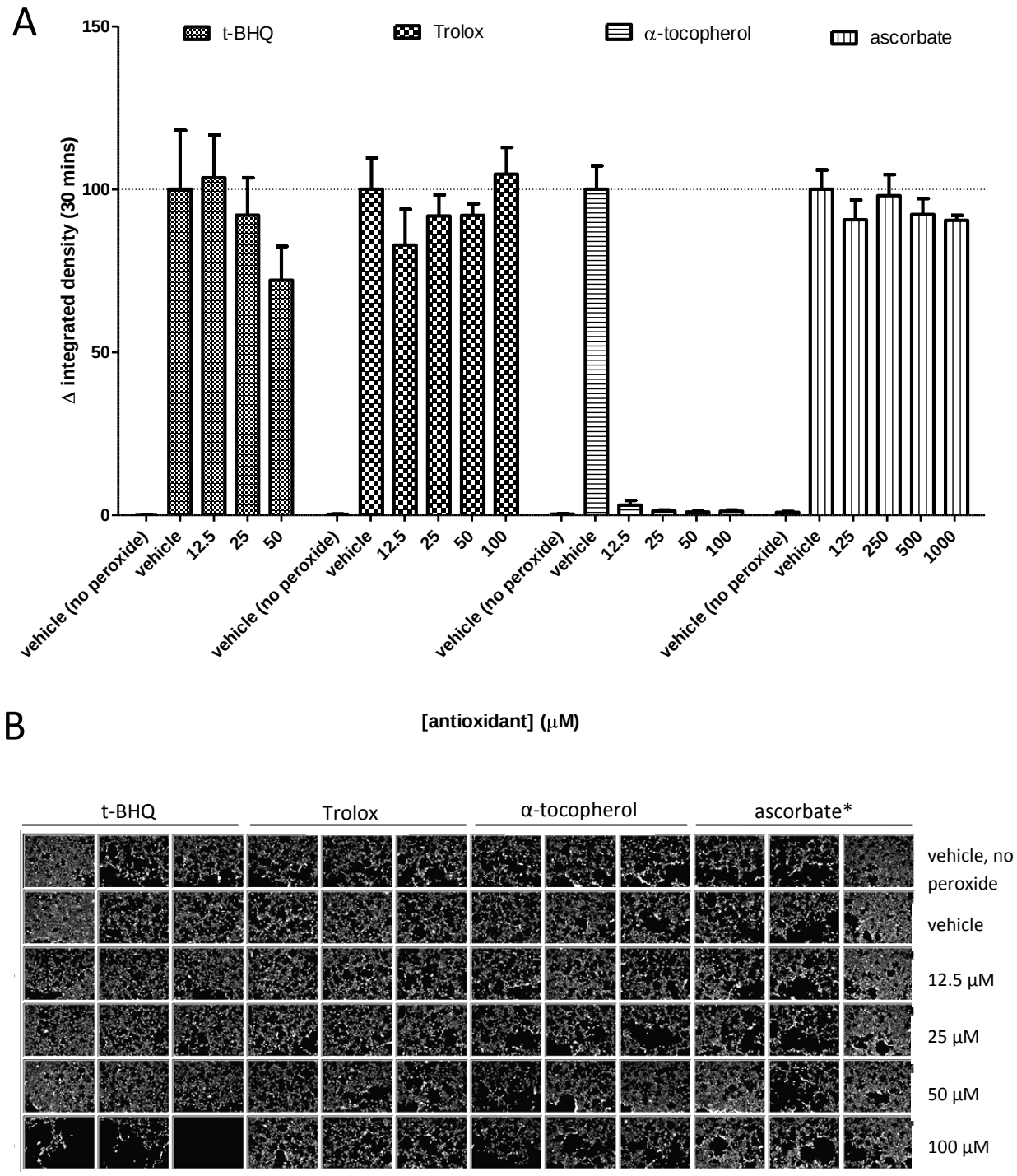


Figure 10. Effects of antioxidant pre-treatment on C_{11}-BODIPY$^{581/591}$ oxidation in EPC cells. (**A**) Lipid stability in EPC cells as estimated by C_{11}-BODIPY$^{581/591}$ oxidation after 30 minutes treatment with cumene hydroperoxide and hemin. Data shown are background-corrected change in integrated density (area x intensity) of the means ± SE of three replicate wells (*n* = 3). Four image fields were measured from each well using filters corresponding to oxidised C_{11}-BODIPY$^{581/591}$. (**B**) A representative set of images of EPC cells loaded with C_{11}-BODIPY$^{581/591}$ showing data from a single field captured in the red channel prior to treatment with cumene hydroperoxide and hemin. Note the lack of cells in the 100 μM *t*-BHQ treatment, indicating cytotoxicity. *In the case of ascorbate treatments, concentration values used in the assay were 10-fold higher than those shown here.

In contrast to the TBARS assay, no increase in apparent C_{11}-BODIPY$^{581/591}$ oxidation was observed in cells treated with high concentrations of ascorbate. This suggests that the C_{11}-BODIPY$^{581/591}$ assay may not be as sensitive as the TBARS assay for the measurement of lipid peroxidation, or alternatively that the TBARS assay may be detecting artefacts not related to lipid peroxidation in cells treated with ascorbate and hemin. We believe this should be investigated further, since although the ascorbate concentrations applied here are very high, it is possible the inclusion of excessive amounts of ascorbate in aquaculture feeds may have a detrimental effect on lipid stability. To address this further, we performed experiments in which cells were pre-treated for 24 hours with α-tocopherol alone, or α-tocopherol combined with ascorbate, and then subjected to treatment with cumene hydroperoxide and hemin. Estimates of cellular lipid peroxidation as indicated by the TBARS assay revealed that ascorbate abrogated the protective effect of α-tocopherol under these conditions (**Figure 11**). This is an interesting observation since these two antioxidants are often used in combination in aquaculture practices.

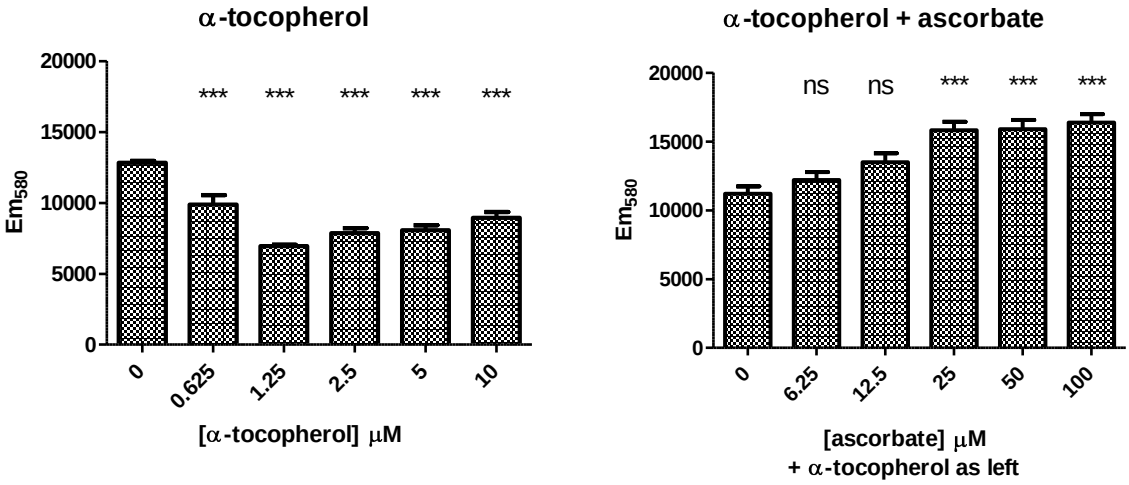


Figure 11. Lipid peroxidation in EPC cells pre-treated with α-tocopherol alone or α-tocopherol combined with ascorbate, as measured using the TBARS assay. Data shown are raw fluorescence data (means of arbitrary units ±SE) from three replicate wells, representative of three independent experiments (n = 3). *** p < 0.001 compared to control cultures according to one-way ANOVA followed by Dunnett's test (ns = not significant).

The results presented in this section show the value of investigating antioxidant efficacy using cell-based assays – cellular uptake is taken into account, cytotoxicity is apparent, and detailed concentration-response relationships can be established due to the ability to test a wide range of conditions simultaneously. With regard to testing antioxidant efficacy in cell-based assays, we observed that the results of a given assay depend on the aqueous solubility of both the antioxidant and the indicator used in the assay. We have shown that a representative vitamin E compound protects cells against lipid peroxidation, which reflects the known effects of this class of antioxidants *in vivo*. We have also determined that ascorbate exacerbates lipid peroxidation in the presence of iron.

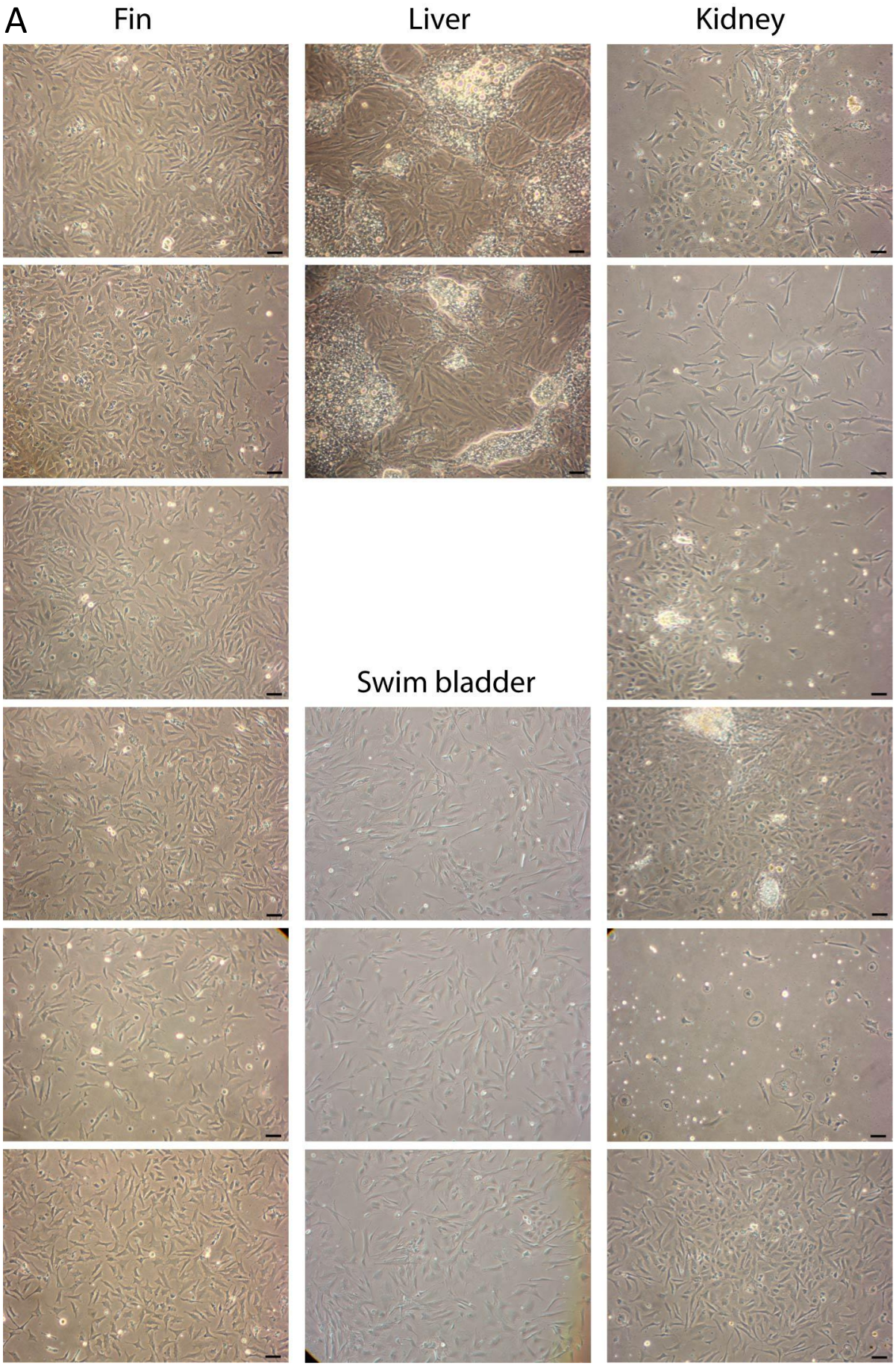
Effects of antioxidants on cellular oxidative stress in primary hepatocyte cultures and fibroblast-like cells from large marine teleosts

Isolation and culture of hepatocytes and fibroblast-like cells from Seriola lalandi

Primary hepatocytes were isolated from the livers of small (less than 1 kg) Yellowtail Kingfish (*Seriola lalandi lalandi*) specimens and seeded directly into the wells of microplates for use in antioxidant assays. Based on mammalian research, the use of primary hepatocytes as a model system for liver function in the whole organism relies on immediate usage, as the expression of liver-specific genes in cultured hepatocytes declines rapidly in culture (Harris et al., 2004, Boess et al., 2003). We successfully isolated YTK primary hepatocytes and investigated antioxidant efficacy against cellular ROS formation under serum starvation using the DCFH assay (see the following section).

In an attempt to establish a population of replicative cells for various applications, cells were isolated and cultured from a range of different *Seriola lalandi* tissues (**Figure 12A**). Fibroblast-like cells proliferated from kidney, liver, pectoral fin connective tissue and swim bladder tissue. Cells isolated from kidney tissue proliferated for 2-3 passages before growth halted (referred to as replicative senescence). Liver isolates appeared to proliferate for a number of weeks in a single flask but did not survive passage into new flasks. Swim bladder connective tissue produced cells that survived for more than 8 weeks but differentiated into a tightly packed monolayer and could not be passaged. Cells isolated from pectoral fin connective tissue were passaged successfully 12 times over a period of more than 12 weeks before entering replicative senescence. This could be repeated a number of times, suggesting that connective tissue associated with the pectoral fin is a reliable source of proliferative fibroblast-like cells for use in cell-based research. It must be noted, however, that replication was quite slow and while the cells could be successfully revived from cryostorage, viability after storage was markedly reduced, limiting the opportunity for undertaking experimentation using this cell type (in comparison to a cell line such as EPC). Nevertheless, we did undertake a number of antioxidant activity assays and gene expression analyses using this cell type (described below).

The species of origin of the YTK fibroblast-like cells (designated YTKF) was confirmed by amplifying and sequencing a fragment of the mitochondrial *cox1* gene. The sequence obtained was compared to sequences in publicly available databases, revealing 100% sequence identity with a *cox1* sequence from *Seriola lalandi*, GenBank accession number EF609460.1 (**Figure 12B**).


A
Fin
Liver
Kidney
Swim bladder

B

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YTKF:       6   ccctcctccagctgggtcaaagaaggcagtatttaagtttcggtctgtcagaagcattgt 65
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S.lalandi: 628 ccctcctccagctgggtcaaagaaggcagtatttaagtttcggtctgtcagaagcattgt 569

YTKF:       66  aatgccggcggctaaaactggaagtgataggagcaggagcacggccgtgattagtacagc 125
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S.lalandi: 568 aatgccggcggctaaaactggaagtgataggagcaggagcacggccgtgattagtacagc 509

YTKF:       126 tcaaacgaataggggaatttggtacatggaaacggcatggggtttcatgttgatgatggt 185
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S.lalandi: 508 tcaaacgaataggggaatttggtacatggaaacggcatggggtttcatgttgatgatggt 449

YTKF:       186 cgtaataaagttaatagctcctagaattgaggagattccagctaaatgaagggagaaaat 245
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S.lalandi: 448 cgtaataaagttaatagctcctagaattgaggagattccagctaaatgaagggagaaaat 389

YTKF:       246 tgttaagtctacggatgctcctgcgtgagcgaggttgccggctagaggcgggtagactgt 305
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S.lalandi: 388 tgttaagtctacggatgctcctgcgtgagcgaggttgccggctagaggcgggtagactgt 329

YTKF:       306 tcaacccgttccggccccggcttcaacgcctgaagaggctaaaagtaggaggaacgaagg 365
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S.lalandi: 328 tcaacccgttccggccccggcttcaacgcctgaagaggctaaaagtaggaggaacgaagg 269

YTKF:       366 gggaaggagtcagaagctcatattgtttattcgggggaatgctatatcgggagccccaat 425
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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YTKF:       426 catcaaggggatgagtcagttcccgaaccctccaatcataattggcattactataaagaa 485
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YTKF:       486 aattattacaaacgcgtgcgctgtaacgattacgttataaatttgatcgtctcccagaag 545
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YTKF:       546 agcaccgggttggctcagttctgctcggatgagcaaacttaaggctgtgccgaccatgcc 605
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S.lalandi: 88  agcaccgggttggctcagttctgctcggatgagcaaacttaaggctgtgccgaccatgcc 29

YTKF:       606 ggctcaggcaccaaatactagatagagg 633
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S.lalandi: 28  ggctcaggcaccaaatactagatagagg 1
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Figure 12. YTK primary cell culture (**A** [previous page]) Isolation and growth of fibroblast-like cells from pectoral fin connective tissue, liver, kidney and swim bladder tissues of Yellowtail Kingfish (*Seriola lalandi*). The images shown are from cultures grown for 5-7 days after isolation. Images were captured using an inverted phase contrast microscope and digital camera. Bar = 50 µm. (**B**) Confirmation of the species of origin of YTKF cells by *cox1* sequencing. The YTKF sequence shows 100% identity with a *Seriola lalandi cox1* sequence (GenBack accession number EF609460.1). Note that the sequence shown for YTKF was obtained using the reverse primer, such that the complementary sequence was aligned with EF609460.1 (meaning that the nucleotide numbers are reversed).

Effects of pre-treatment with antioxidants on cellular ROS formation in YTK primary hepatocytes

For antioxidant assays, we focused on the use of primary hepatocytes as a means of determining efficacy and cytotoxicity. This approach was based on the concept that mammalian primary hepatocytes utilised within a short time after isolation are known to express many genes that are

expressed in liver tissue *in vivo* and hence are good models of liver function (Segner and Cravedi, 2001, Tuschl et al., 2008). Primary hepatocytes isolated from mice and rats are used extensively in toxicology research (extensively reviewed elsewhere (Tuschl et al., 2008)). Fish primary hepatocytes from a number of species are also used for predicting the toxicity of environmental pollutants (Scholz and Segner, 1999, Segner and Cravedi, 2001, Laville et al., 2004) and for research into fatty acid metabolism (Mikkelsen et al., 1994, Mikkelsen et al., 1993, Rosjo et al., 1994).

YTK primary hepatocytes pre-treated for 24 hours with various concentrations of ascorbate or Trolox and then subjected to serum starvation showed a reduction in the formation of cytoplasmic ROS according to the DCFH assay (**Figure 13A**). For both of these antioxidants, the concentrations required for a given reduction in DCF formation in YTK primary hepatocytes were markedly higher than those required for the same reduction in DCF formation in EPC cells. This may be due to a higher basal level of ROS in cultured primary hepatocytes, which would result in the depletion of antioxidants during the pre-incubation period, resulting in elevated apparent ROS levels and increased DCF formation.

While a small degree of ROS inhibition was observed in cells treated with low concentrations of *t*-BHQ, toxicity was apparent in cells treated with *t*-BHQ concentrations above 50 µM. Compared to the EPC cell line, YTK primary hepatocytes were considerably more sensitive to the cytotoxic effects of *t*-BHQ. This may be related to differences in the expression of antioxidant enzyme systems in the two cell types. Alternatively, EPC cells may be resistant to apoptosis due to the disruption of one or more cell cycle control pathways, a phenomenon which is common in immortal cell lines. We also observed a cytotoxic effect in YTK primary hepatocytes treated with high concentrations of Trolox®, although the concentrations used were ten-fold higher than those use in assays involving EPC cells. It was interesting to note that cell viability did not appear to be adversely affected by extremely high concentrations of ascorbate in this assay (up to 10 mM). Alpha-tocopherol treatment was also not cytotoxic to YTK primary hepatocytes, however, similarly to the case with EPC cells, it did not inhibit the formation of cytoplasmic ROS according to the DCFH assay.

We also determined C_{11}-BODIPY$^{581/591}$ oxidation in YTK primary hepatocytes exposed to cumene hydroperoxide or *t*-butyl hydroperoxide in the presence of hemin (**Figure 13B**). Like EPC cells, cumene hydroperoxide appeared to induce oxidation of the probe slightly more efficiently than *t*-butyl hydroperoxide, but was also more toxic. We undertook experiments to determine antioxidant protection against C_{11}-BODIPY$^{581/591}$ oxidation, but these were inconclusive (data not shown). The main reason for this was that at the time YTK hepatocytes were available, the C_{11}-BODIPY$^{581/591}$ assay had not been optimised (we undertook extensive optimisation using a plate reader, which was largely unsuccessful – this assay appears to perform markedly better in a high-throughput microscopy format as was eventually utilised for experiments involving EPC cells). It would be interesting to examine whether the apparent potency of α-tocopherol in YTK hepatocytes is different to that observed in EPC cells using either the C_{11}-BODIPY$^{581/591}$ assay (or indeed, using the TBARS assay). It would also be of interest to establish whether other cell types from different species respond to oxidizing treatments differently, which may reflect differences in basal levels of enzyme systems responsible for protecting cellular fatty acids from oxidative damage.

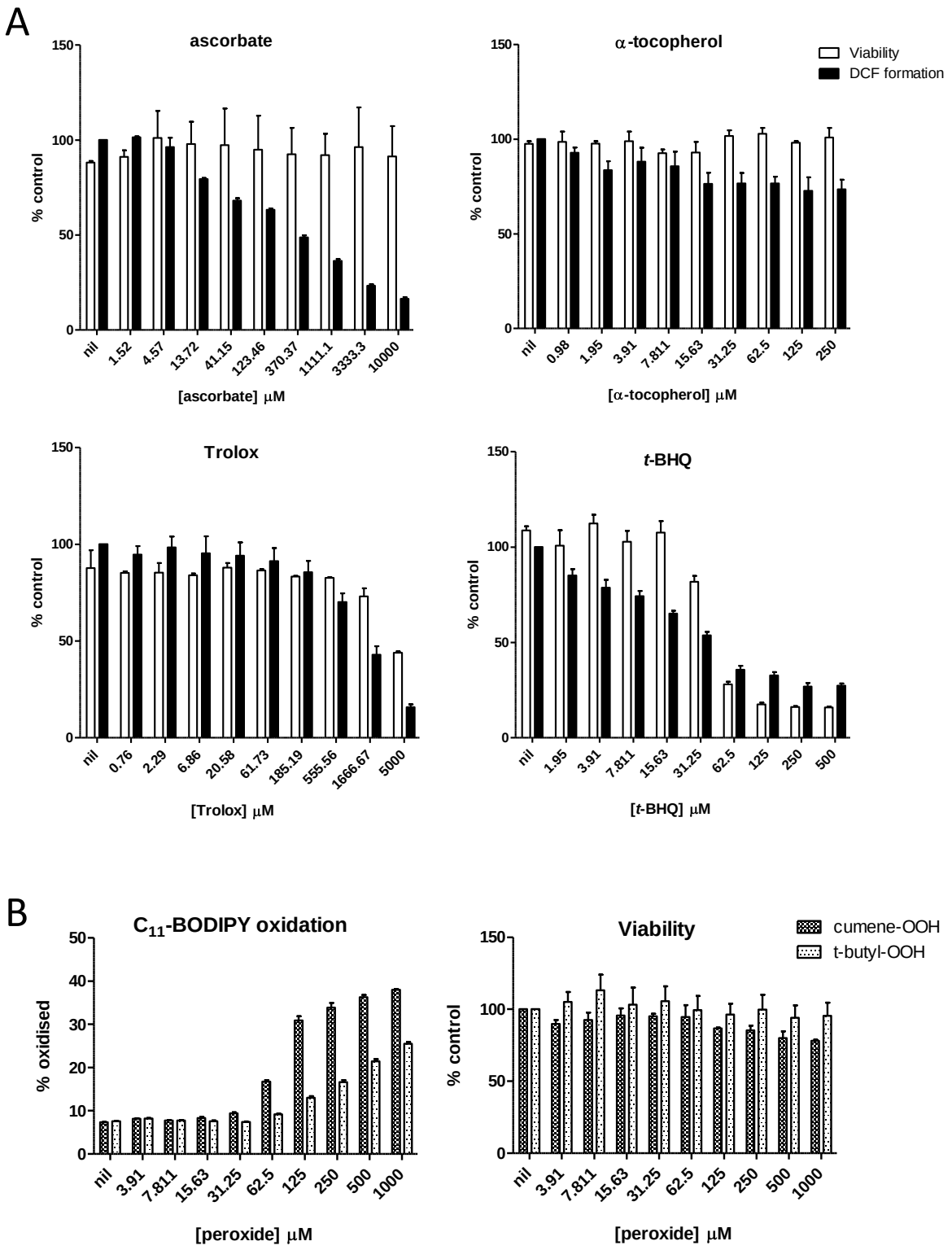


Figure 13. Effects of antioxidant pre-treatment on oxidative stress and lipid peroxidation in YTK primary hepatocytes (**A**) Effect of antioxidant pre-treatments on cytoplasmic ROS content in YTK primary hepatocytes as measured using the DCFH assay. Primary hepatocytes were isolated from YTK liver tissue, grown for 24 hours, treated with antioxidants (as shown) for 24 hours, loaded with H_2DCFDA and subjected to serum starvation. Viability is expressed as a proportion of controls incubated in medium containing FBS without added antioxidants. DCF formation is expressed as a proportion of cells incubated in serum-free medium

without added antioxidants. Data shown are means ±SE from three independent experiments (*n* = 3). (**B**) C_{11}-BODIPY$^{581/591}$ oxidation in YTK primary hepatocytes exposed to cumene hydroperoxide or *t*-butyl hydroperoxide in the presence of 1 μM hemin. Data shown are means ±SE from three replicates (*n* = 3).

Effects of antioxidants on cellular ROS in YTK fibroblast-like cells

YTK fibroblast-like cells (designated YTKF) displayed good potential for use in cell-based assays – the cells proliferated over an extended period of time, could be subcultured or seeded into microplates without loss of cell viability, and because the cells have not been transformed into a cancerous phenotype, cell cycle control mechanisms are intact (in contrast to established cell lines, which proliferate indefinitely due to genetic mutations in one or more cell cycle control pathways, making them less prone to undergoing apoptosis in response to toxic compounds). To compare antioxidant efficacy in YTKF cells to that observed using EPC cells and YTK fibroblasts, YTKF cells were pre-treated with various antioxidants before inducing oxidative stress by serum starvation and determining ROS using the DCFH assay.

Ascorbate and to a lesser extent, Trolox®, inhibited cellular ROS formation in YTKF cells in a concentration dependent manner (**Figure 14**). As was observed with YTK primary hepatocytes and YTKF cells required increased concentrations of ascorbate or Trolox® to inhibit ROS formation by a given proportion compared to that required in EPC cells. This difference in apparent potency is likely to be related to differences in background ROS levels between the cell types, which could be due to varied expression of innate antioxidant enzymes. In many cell lines that display a transformed phenotype, the expression of one or more antioxidant enzyme systems is elevated. It has been proposed that this contributes to strong growth and enhanced survival in culture conditions, since cell culture conditions inherently promote oxidative stress. It would be interesting to determine whether antioxidant supplementation could enhance the survival and delay replicative senescence in a primary cell type such as YTKF.

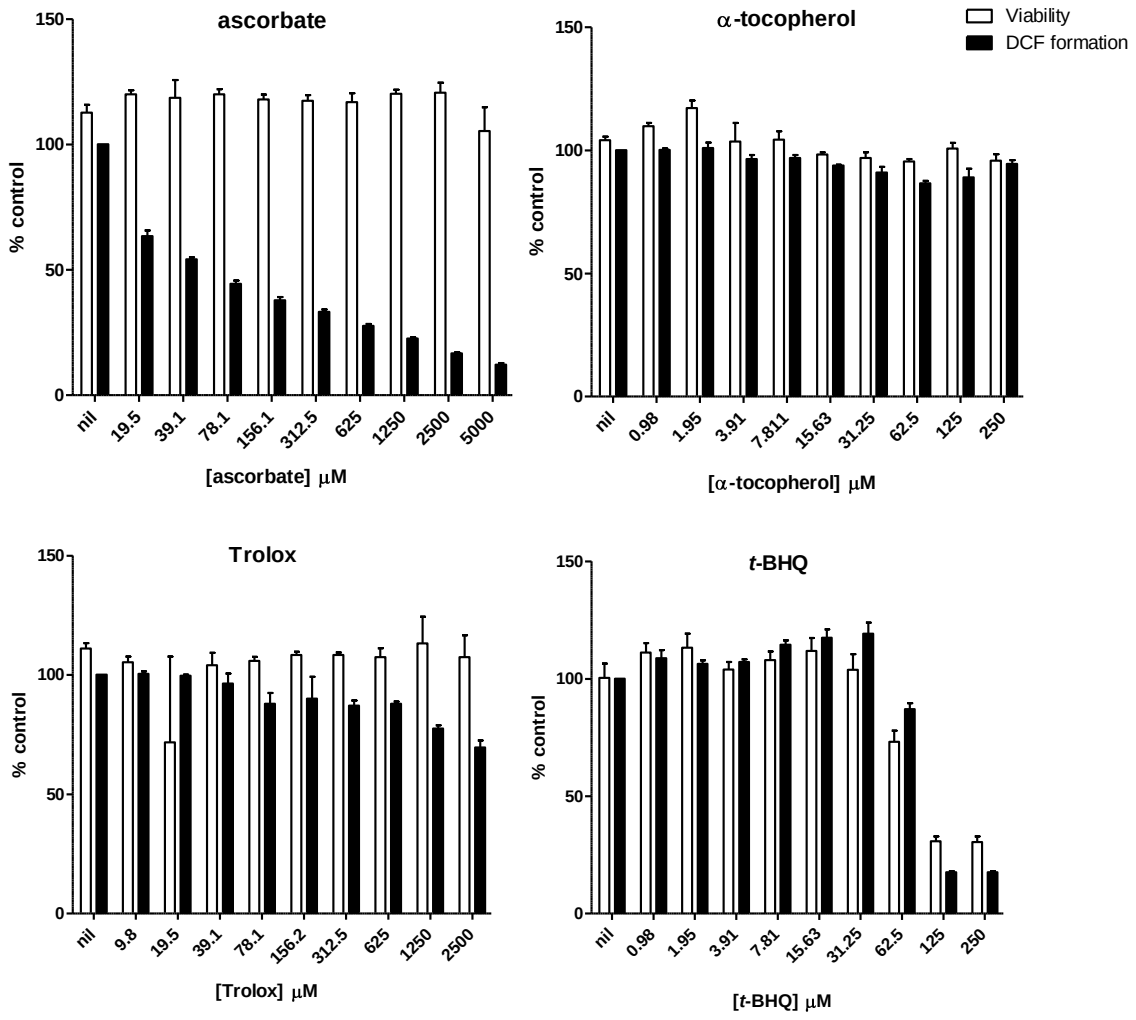


Figure 14. Effects of antioxidant pre-treatment on ROS formation in serum-starved YTK fibroblast-like cells according to the DCFH assay. Cells were incubated with various antioxidants as shown before loading with H_2DCFDA and inducing oxidative stress by serum starvation. DCF formation is expressed as a proportion of cells incubated in serum-free medium without added antioxidants. Data shown are means ±SE of three replicated wells (*n* = 3) from a representative experiment.

Isolation and culture of hepatocytes from Thunnus maccoyii

Hepatocytes were isolated from liver tissue taken from freshly harvested 15-25 kg Southern Bluefin Tuna (SBT; *Thunnus maccoyii*) specimens ranched in the southern Spencer Gulf, South Australia. Survival of hepatocytes after isolation was relatively poor, with low cell numbers obtained after seeding plates for cell-based assays. Cell survival rates may have been compromised by the substantial travel time between sampling and cell isolation, which was 6-8 hours, during which time liver samples were incubated in L-15 growth medium supplemented with FBS and antibiotics. Despite the presence of a rich growth medium, it is likely that significant numbers of red blood cells were present in the liver samples, which may have promoted cellular oxidative stress due to exposure to air in the presence of haem iron. Furthermore, the survival of hepatocytes may be diminished in large SBT specimens compared to small YTK specimens. Despite poor survival rates, we

investigated antioxidant efficacy and toxicity in SBT primary hepatocytes using our panel of representative compounds (see next section).

SBT hepatocytes survived in culture and proliferated for a short period of time (approximately 4 weeks) before reaching a terminally differentiated or senescent state (**Figure 15**). This was encouraging, as it showed that culture conditions were suitable for the short term survival of hepatocytes from this species. In the future, cultures such as these could be treated with mitogens and/or mutagens to form continuous cell lines suitable for the purposes of virus screening.

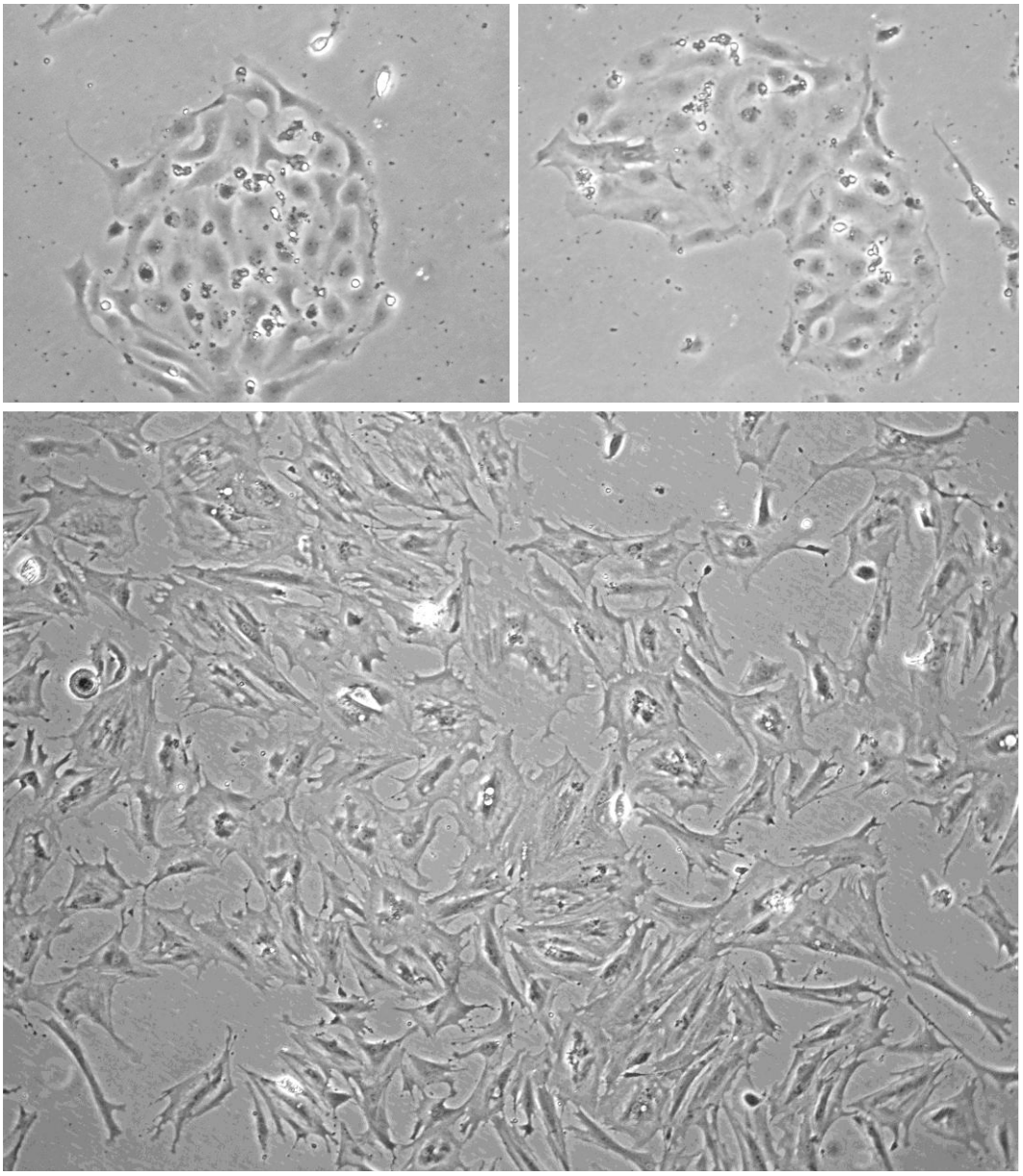


Figure 15. SBT primary cell culture (**A**) Hepatocytes from SBT (*Thunnus maccoyii*) imaged 48 hours after isolation and (**B**) fibroblast-like cells derived from the same hepatocyte cultures after approximately 2 weeks in culture. Images were captured at 100X magnification using an inverted phase contrast microscope.

Effects of antioxidants on cellular ROS in SBT primary hepatocytes

Despite low cell survival rates after isolation of SBT primary hepatocytes, we observed a similar response to the same set of model antioxidant compounds tested in YTK primary hepatocytes, with ascorbate and Trolox® displaying concentration-dependent inhibition of ROS formation, *t*-BHQ displaying a small degree of inhibition, and α-tocopherol displaying no inhibition of ROS formation according to the DCFH assay (**Figure 16**). Because of the low cell numbers present in each well of the microplates utilized for high-throughput DCFH assays, changes in cell viability during the assay were unclear due to insufficient assay sensitivity.

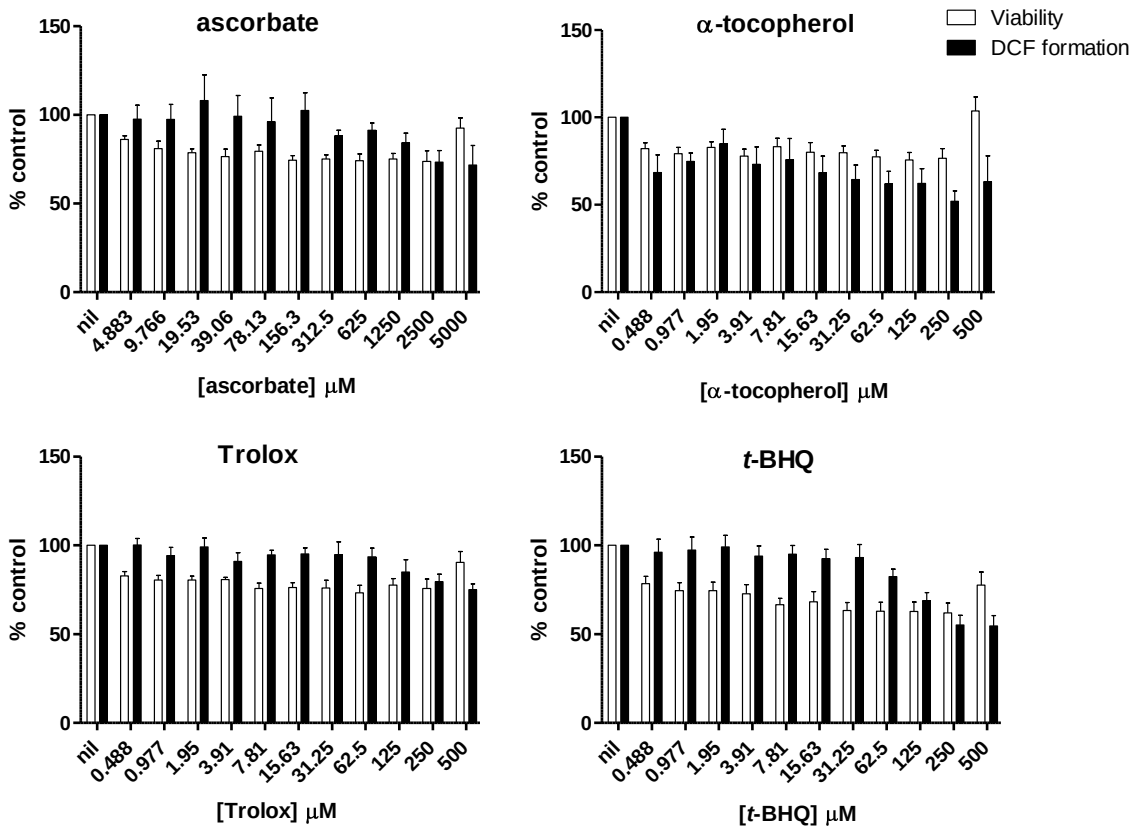


Figure 16. Effect of antioxidant pre-treatments on cytoplasmic ROS content in SBT primary hepatocytes as measured using the DCFH assay. Primary hepatocytes were isolated from SBT liver tissue from 5 fish, grown for 24 hours in 96-well plates, treated with antioxidants (as shown) for 24 hours, loaded with H_2DCFDA and subjected to serum starvation. Viability and DCF formation data are expressed as a proportion of cells incubated in serum-free medium without added antioxidants. Data shown are means ±SE from five fish (*n* = 5).

Clearly, changes in sampling and isolation procedures are required for improved survival of SBT primary hepatocytes in culture conditions. It is likely that reducing the time between sampling and cell isolation will result in improved cell viability. Access to smaller specimens may also improve survival rates due to increased numbers of proliferating cells present in the liver and other tissues of younger fish.

Determination of antioxidant activity in natural extracts using cell-based assays

As the proportion of fish products sourced from aquaculture increases, consumers may begin to examine in detail the practices used in production. One way of targeting particular discerning market demographics, such as consumers looking for 'organic' produce, is to ensure that all feed additives are derived from naturally occurring sources. Furthermore, a number of antioxidant-rich byproducts of agriculture are widely available. With this in mind, we investigated the effects of three natural extracts on cellular ROS formation in serum-starved EPC cells using the DCFH assay, and determined cytotoxicity using the neutral red uptake assay. The extracts used were grape seed extract (containing phenolic antioxidants, tannins and proanthocyanidins), rosemary extract (containing carnosic acid and carnosol) and *Haematococcus* extract (containing the carotenoid astaxanthin).

We found that grape seed extract inhibited ROS formation in a concentration-dependent manner but was also considerably cytotoxic to EPC cells, while rosemary extract appeared to be less toxic than grape seed extract but displayed a higher antioxidant potency (**Figure 17A**). *Haematococcus* extract and the active ingredient, astaxanthin, did not display a potent antioxidant effect in EPC cells according to the DCFH assay despite being reported as a powerful antioxidant compared to other carotenoids (Palozza and Krinsky, 1992, Naguib, 2000). We postulated that this may be related to the poor aqueous solubility of astaxanthin, suggesting that the TBARS assay or C_{11}-BODIPY$^{581/591}$ assay may be more suitable for this compound. To test this, we investigated the protective effect of astaxanthin against lipid peroxidation according to the TBARS assay in EPC cells pre-treated with astaxanthin and exposed to cumene hydroperoxide and hemin (**Figure 17B**). We found that astaxanthin did not inhibit lipid peroxidation at concentrations up to the maximum tested of 10 µM. This is in contrast to reports of astaxanthin displaying antioxidant activity comparable to α-tocopherol in lipid systems (Naguib, 2000). This discrepancy may be due to differences between biochemical approaches in lipid and cell-based methods, and is worthy of further investigation.

The assays developed in the current project are highly suited to the testing of natural product extracts for antioxidant activity and cytotoxicity – ideally the extract should be tested in both the DCFH assay and the C_{11}-BODIPY$^{581/591}$ assay in order to detect compounds with varying aqueous solubility. For the isolation of novel antioxidant compounds, fractionation of natural extracts could be undertaken prior to testing each fraction using the assays presented here, followed by increasingly stringent fractionation to isolate the most potent compound or group of compounds. This approach may enable the separation of beneficial antioxidants from cytotoxic compounds present in natural extracts.

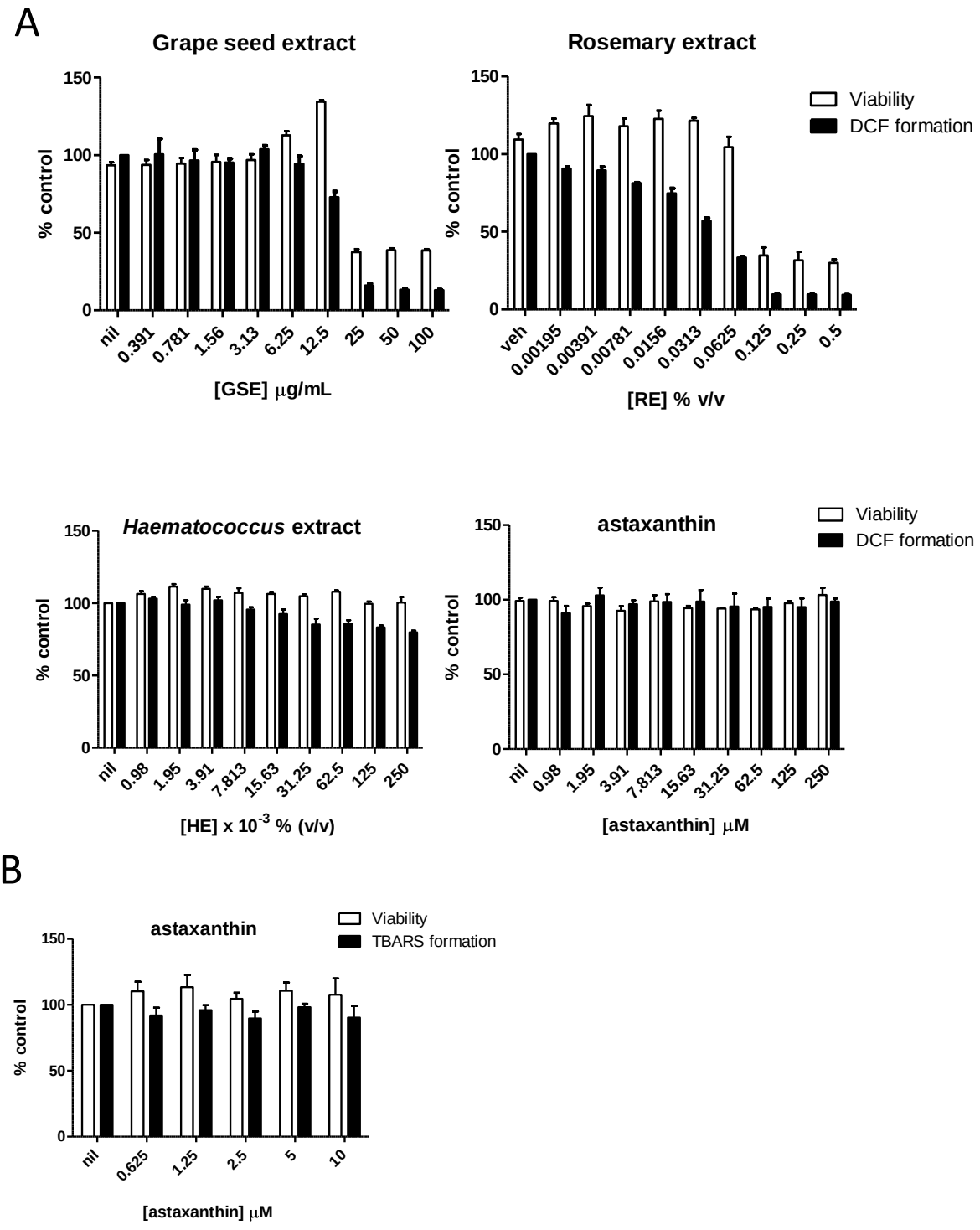


Figure 17. Antioxidant activity and cytotoxicity of natural extracts. (**A**) EPC cells were pre-treated for 24 hours with extracts as shown and cellular ROS estimated using the DCFH assay during serum deprivation for 4 hours. Astaxanthin was included as a control for *Haematococcus* extract. Data shown are means ± SE from three replicate wells (*n* = 3). (**B**) Effect of astaxanthin pre-treatment on lipid peroxidation in EPC cells exposed to cumene hydroperoxide and hemin according to the TBARS assay. Data shown are means ± SE from three replicate wells (*n* = 3)

Investigations into the effects of antioxidant compounds on the expression of genes for endogenous cellular antioxidant enzymes

In addition to direct reduction of reactive species by antioxidants, some antioxidant compounds are known to elicit an 'indirect' antioxidant effect in cells by inducing the expression of innate antioxidant enzyme systems. One example of an indirect mechanism whereby antioxidant compounds are known to function involves a pathway known as the Keap1/Nrf2/ARE system (Maher and Yamamoto, 2010, Kensler et al., 2007, Kobayashi et al., 2009). This pathway detects cellular oxidative stress and activates a network of genes encoding antioxidant enzyme systems. The Keap1/Nrf2/ARE pathway is highly conserved amongst vertebrates and has been well characterised in the zebrafish (Kobayashi et al., 2009). There are two main components involved in the pathway – a redox-sensing cytoplasmic protein, Keap1, and a transcription factor, Nrf2. Under normal conditions, Keap1 associates with and promotes the degradation of Nrf2. Upon oxidation or conjugation of key cysteine residues in the Keap1 structure, the association with Nrf2 is disrupted, allowing the accumulation and nuclear translocation of Nrf2. Nrf2 binds to elements known as 'antioxidant response elements' (ARE) located in the promoter regions of genes involved in the detoxification of reactive species, enhancing their expression.

To investigate the possible role of this system in the expression of selected antioxidant genes in fish, we undertook qRT-PCR analyses of gene expression in response to various treatments, with a focus on a known inducer of the Keap1/Nrf2/ARE pathway, *t*-BHQ.

Optimisation of qRT-PCR assays

To determine mRNA quantity using real-time PCR, SYBR Green I-based assays were developed for genes encoding β-actin, glutathione peroxidase I (GPx1), and peroxidredoxin I (Prx1). For assay optimization and determining reaction efficiency of each primer pair, the target fragment was amplified from a suitable cDNA preparation, checked for specificity using agarose gel electrophoresis, purified, and used to generate a real-time PCR standard curve (**Appendix 4**). In each case, a single band of the predicted size was successfully amplified, and reaction efficiencies for each primer pair were within acceptable limits.

Effects of antioxidant treatments on antioxidant gene expression in EPC cells and cultured YTK fibroblasts

To investigate possible mechanisms underlying the control of the expression of genes encoding GPx1 and Prx1 in response to external factors, we examined changes in the expression of these genes in cultured cells exposed to various antioxidants and treatments. A series of pilot experiments was undertaken to determine conditions that result in the altered expression of the genes of interest. A table of selected results from these pilot experiments is presented (**Appendix 5**). While a number of treatments appeared to induce the expression of either GPx1 or Prx1 (notably serum starvation, which was of interest as this was the means of inducing oxidative stress for the DCFH assay), the treatments also altered the apparent expression of the normaliser gene, β-actin. GAPDH was investigated as an alternative normaliser gene, but was also found to be unstable under a range of experimental conditions. We therefore focused on *t*-BHQ treatment, as this compound did not alter β-actin mRNA levels but induced GPx1, as is discussed further below.

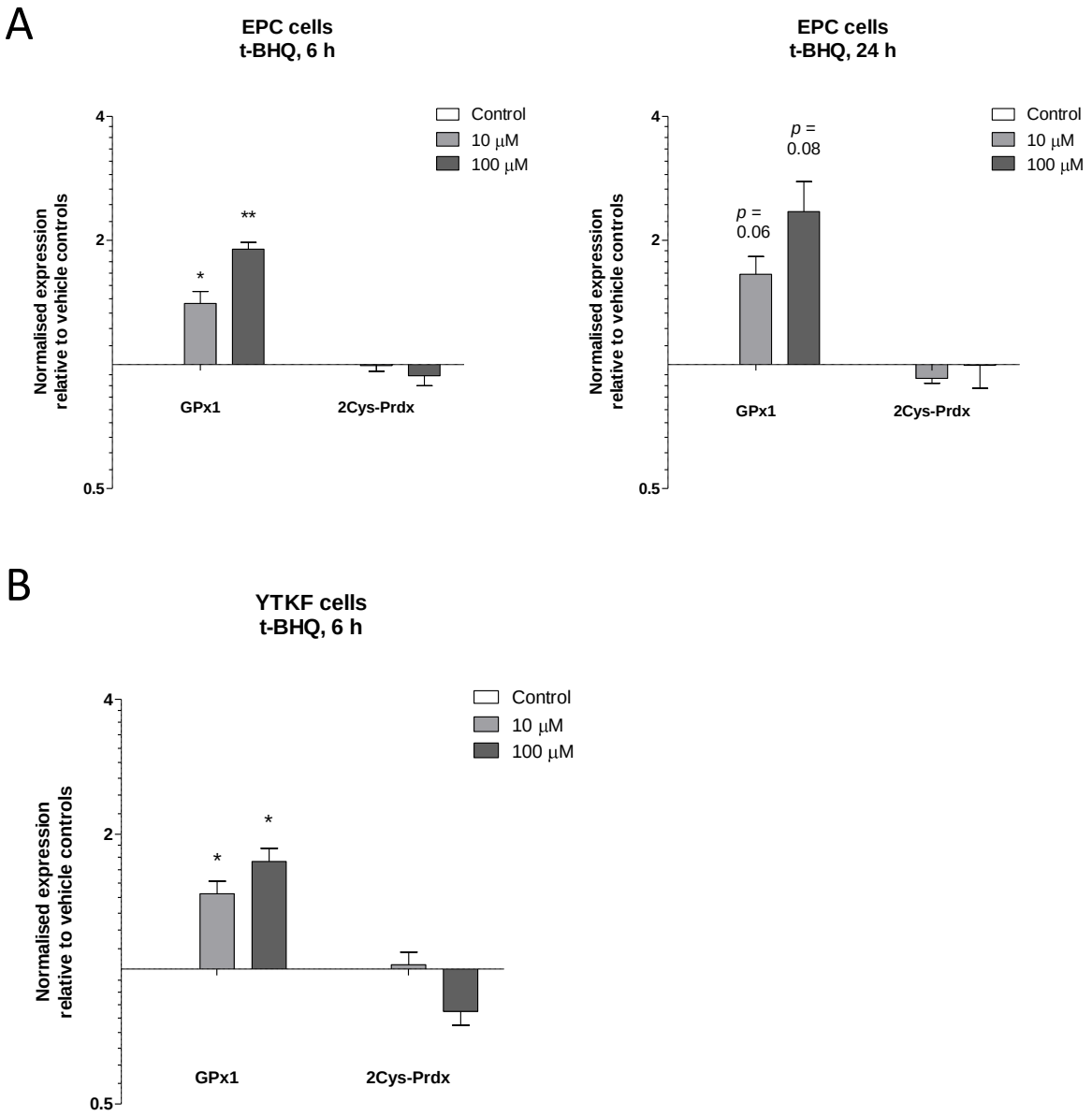


Figure 18. Relative gene expression in cultured cells treated with the model inducer of the Keap1/Nrf2/ARE pathway, t-BHQ. (**A**) EPC cells were exposed to 0 (vehicle control), 10 or 100 μM t-BHQ for 6 or 24 hours and gene expression determined relative to controls and normalised to β-actin. (**B**) YTK fibroblast-like cells were exposed to 0 (vehicle control), 10 or 100 μM t-BHQ for 6 hours and gene expression determined relative to controls and normalised to β-actin. Data shown are means ± SE of three to four independent experiments (*n* = 3-4). * $p < 0.05$, ** $p < 0.01$ according to Student's t-test comparing expression ratios with a hypothetical value of 1 (no change).

We found that the expression of GPx1 increased in response to *t*-BHQ exposure in a concentration- and time-dependent manner, whereas Prx1 expression remained relatively stable. This suggests that GPx1 may be under at least partial control by the Keap1/Nrf2/ARE pathway, indicating that the expression of this enzyme may be controllable in part by indirect antioxidants. This presents a possible opportunity for dietary control of antioxidant systems in fish, which may provide additional health benefits for fish in high-stress situations such as intensive aquaculture in addition to possibly enhancing post-harvest shelf life with respect to the stability of polyunsaturated fatty acids. In

mammals, a compound known to activate Nrf2, sulphoraphane, which is present in high amounts in certain strains of broccoli, has been shown to promote indirect antioxidant effects beneficial to health (Brigelius-Flohe and Banning, 2006, Kim et al., 2008). The present study suggests that the fish GPx1 enzyme may be under the control of Nrf2 and hence may be activated by similar plant-derived indirect antioxidants.

The substrate preference of GPx1 differs between different species – mammalian GPx1 has a preference for hydrogen peroxide and small organic peroxides and cannot reduce phospholipid hydroperoxides in membranes (Toppo et al., 2009), while yeast GPx1 can reduce phospholipid hydroperoxides and is also unusual in that it can be regenerated by thioredoxin in addition to glutathione (Lee et al., 2008). While the fish GPx1 investigated in the current project is most similar to mammalian GPx1 by sequence similarity, at least in SBT according to previous work in our laboratory (Thompson et al., 2010), the range of substrates for the fish version of this enzyme has not yet been determined. It has been noted that in comparison to mammals, hepatic glutathione concentrations in fish are relatively low (Wallace, 1989), suggesting that fish may be more susceptible to a range of xenobiotic compounds that would normally be removed by glutathione-dependent detoxification processes occurring in the liver. The functioning of glutathione-dependent antioxidant enzymes responsible for limiting damage in the event that oxidative stress occurs in the liver may be inhibited in low-glutathione conditions, in which case thioredoxin-dependent enzymes may play a greater role. Furthermore, since the site of fatty acid metabolism is the liver, the high proportion of long-chain polyunsaturated fatty acids present in baitfish and fish oil-based manufactured feeds used in marine aquaculture may exacerbate the need for adequate dietary antioxidant supplementation.

Comparison of apparent antioxidant potency of model antioxidants using chemical assays and cell-based assays

To confirm the antioxidant activity of the model compounds used in the present study, the radical scavenging capacities of each antioxidant were estimated using the DPPH assay (**Figure 19**). As expected, all antioxidants showed potent activity in this assay, with IC_{50} values (**Table 2**) indicating a rank in order of highest to lowest potency of α-tocopherol > *t*-BHQ > Trolox® > ascorbate. In the case of ascorbate, low solubility in alcohols required that the dilutions be performed in water prior to adding DPPH dissolved in methanol, which may have affected the baseline of the assay and the apparent IC_{50}. Nevertheless, all antioxidants used in the present study were capable of potently scavenging DPPH radicals. Conversely, apparent antioxidant potency in cultured cells depended on the assay employed, and was related to the aqueous solubility of the antioxidant. In the case of assays based on fluorogenic probes, the solubility of both the probe and the antioxidant probably affected apparent antioxidant activity. This suggests that a single cell-based assay for antioxidant activity is not likely to be easily developed, and that the best approach for characterising novel antioxidant compounds or screening complex mixtures for antioxidant activity is to employ a combination of cell-based assays.

Table 2. Potency of model antioxidant compounds using *in vitro* versus cell-based approaches.

	α-tocopherol	*t*-BHQ	Trolox	Ascorbate
DPPH (*in vitro*) assay				
IC_{50}	1.167	1.224	1.333	1.533
R^2	0.9972	0.9946	0.9917	0.9097
DCFH (cell-based) assay				
IC_{50}	N/A	248.3	782.6	11.91
R^2	N/A	0.7370	0.9351	0.9002

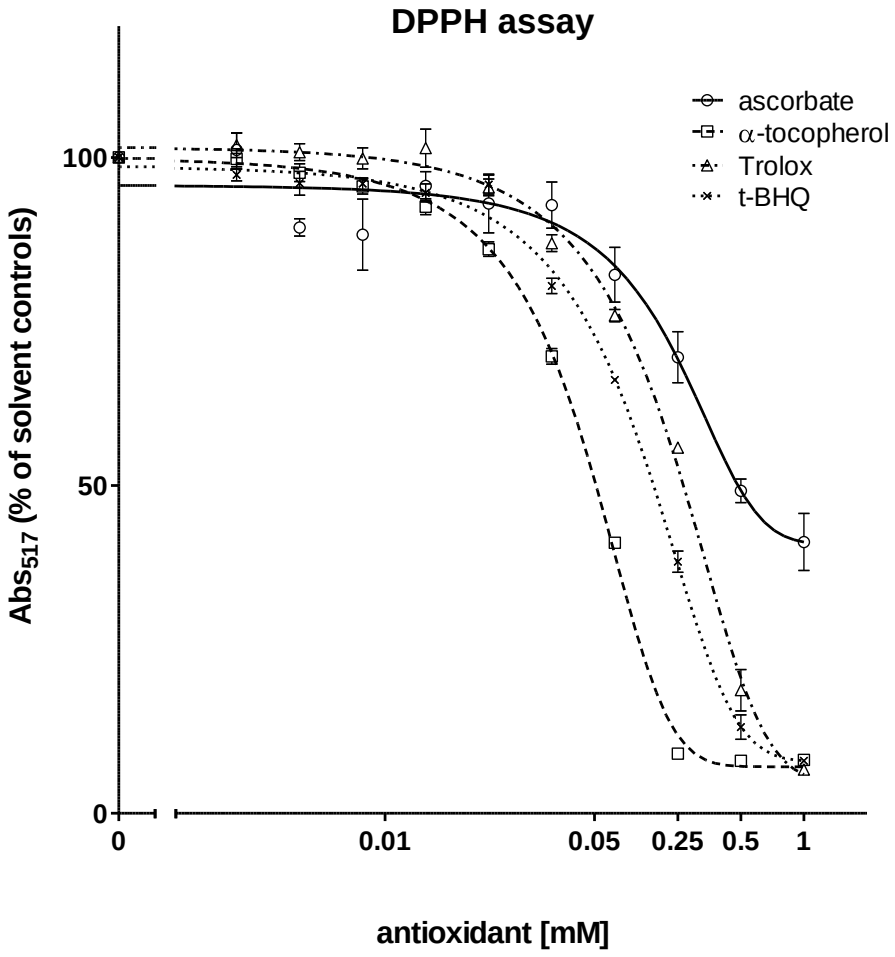


Figure 19. Free radical scavenging capacity of model antioxidants used in the current project as determined by the DPPH radical scavenging assay. Data shown are the means ±SE of three independent experiments (*n* = 3).

An important advantage of using cell-based assays to determine antioxidant activity, in contrast to chemical antioxidant assays, is that factors such as toxicity and cellular uptake are addressed. This is particularly important when characterising novel antioxidant compounds or mixtures. There are two

important applications for antioxidants in aquaculture: 1. To preserve the integrity of fatty acids in manufactured feeds (to prevent rancidity), and 2. To ensure the good health of the animal (often under stressful conditions such as high stocking density). There is also evidence for the added benefit of certain antioxidants that can persist in the harvested product, extending shelf life by preventing lipid oxidation, particularly as is the case with tocopherols (Stéphan et al., 1995, Tocher et al., 2002, Kjaer et al., 2008, Puangkaew et al., 2005).

Commonly used antioxidants for the prevention of feed rancidity are the naturally occurring tocopherols, or the synthetic antioxidants butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA). Interestingly, the antioxidant BHA is metabolised to *t*-BHQ and *tert*-butylquinone (t-BQ), both of which are reported to be genotoxic in a human cell model (Schilderman et al., 1995). The toxicity of *t*-BHQ was apparent in the cell-based assays presented here, in particular in YTK primary hepatocytes, indicating that the cell-based assays developed in the current project are suitable for the prediction of toxicity *in vivo*. Due to the potency of α-tocopherol in the prevention of apparent lipid peroxidation in cell-based assays presented here, we would encourage the use of tocopherols for use as feed preservatives. This would have the advantage of providing simultaneous dietary tocopherol supplementation, which is likely to ensure lipid stability during grow-out and has the potential to persist in the harvested product and enhance shelf-life. Ascorbate and other vitamin-C class compounds should be used at the lower end of the dose spectrum to reduce the possibility of lipid peroxidation, particularly post-harvest in the presence of haem iron.

Benefits

This project has resulted in the development of methods for the isolation and culture of cells from marine finfish species currently in development for intensive aquaculture in South Australia. This provides a platform for laboratory-based research such as virus isolation as well as antioxidant and toxicity screening of compounds with potential for use as feed additives. It is anticipated that the fledgling SBT captive breeding program (undertaken by Clean Seas Tuna in collaboration with numerous research organisations) will benefit from the availability of such cell-based assays.

Cultured cells form the basis of virus screening methods – the YTK fibroblast-like cells described here likely constitute a useful virus screening tool in their existing state (although virus susceptibility testing is yet to be performed), and we have proposed a follow-on project to immortalise these cells and similar cells from SBT to enable routine virus screening.

In summary, this project has highlighted that:

- Different assays are required for water soluble- as compared with lipid soluble-antioxidants
- Cell-based assays (such as those employed here) are required to detect possible cytotoxicity of antioxidants which may compromise their usefulness as antioxidants
- *In vitro* (i.e. cell-free) assays for antioxidant potency may not be good predictors of antioxidant potency in cells or indeed in whole animals
- The addition of antioxidants to cell culture media may increase the chances of producing immortal cell lines from important aquaculture species such as southern bluefin tuna
- The species of origin must be confirmed when working with cell cultures whether from mammals or fish

The tangible outcomes of this project are:

- Reproducible and rapid methods for the testing of antioxidant potency and any possible cytotoxicity
- A reproducible method for the production of primary cultures from the important South Australian aquaculture species yellowtail kingfish (*Seriola lalandi*)
- A method for the production of relatively long-lived cell cultures from yellowtail kingfish (*Seriola lalandi*) and preliminary success with the production of cell cultures from southern bluefin tuna (*Thunnus maccoyii*)
- Standardised methods (karyotyping and PCR) for identifying the species of origin of fish cell cultures
- The karyotype of southern bluefin tuna
- A basis for the development of immortal cell lines from yellowtail kingfish and southern bluefin tuna for the purposes of virus isolation and identification

Further development

In the context of finfish aquaculture, the detection of viruses and routine monitoring of fish stocks is essential for the prevention of disease outbreaks. Confirmation of disease-free status is particularly important when stock is transported interstate. Currently, there are no available SBT or YTK cell lines suitable for the detection of species-specific viruses that may severely impact the industry as a whole in the event of an outbreak. We have proposed a project to immortalise cultured somatic cells described here using standard methods that are well established in mammalian cell biology research. Clean Seas Tuna has provided formal support for this proposal and has granted access to suitable material for sampling. The expertise gained from the current project will be directly applicable to the development of immortalised SBT and YTK cell lines suitable for virus detection and characterisation.

The proposed work includes:

- Isolation and culture of proliferative somatic cells from southern bluefin tuna larvae and fingerlings and the application of a range of established techniques to develop such cells into immortal cell lines
- Immortalisation of both freshly cultured and existing cell cultures of yellowtail kingfish fibroblast-like cells
- Testing of resulting SBT and YTK cell lines for virus susceptibility
- Detection and characterisation of novel viruses of SBT and YTK using immortal cell lines
- Establishing diagnostic procedures for the rapid detection of disease-causing viruses of SBT and YTK

Planned outcomes

The project enabled the development of protocols for the isolation and propagation of somatic cells from SBT and YTK. The methods developed here are applicable to laboratory-based basic research into cell function, gene regulation, protein structural research and investigation of enzyme function. In particular, such cell-based research will provide ongoing benefit to producers involved in the propagation of marine species by providing a means for the investigation of genes associated with particular desirable traits, thus informing any possible future genetic selection strategies.

The cell-based antioxidant and cell viability assays developed for the project are suitable in their current form for use in the preliminary screening of potential feed additives for the prediction of efficacy and the detection of toxicity. It is anticipated that as feed formulations become more species-specific, particularly for SBT for which manufactured feeds are in active development, the cell-based assays presented here will be increasingly utilised by research and development personnel from aquaculture feed companies or research organisations.

Conclusions

The instability of fats in the marketed products of finfish aquaculture is an ongoing concern due to the relatively high levels of long-chain polyunsaturated fatty acids (LC-PUFA) present in the product, that are susceptible to oxidative degradation. In the case of southern bluefin tuna, the discerning target market dictates that rancidity is kept to an absolute minimum. Previous work has shown that including vitamin E and vitamin C in feeds for this species improves the shelf life of the harvested flesh, particularly with respect to colour (Buchanan and Thomas, 2008), although this study was limited in the number of different feeds used (one vitamin-enriched and one control feed). One approach previously proposed for the screening of feed additives for tuna involves the use of Atlantic salmon as a model species (Bransden et al., 2001). We propose that because large-scale diet trials in fish species are costly and time-consuming, the cell-based assays described in this report will be useful for the identification and preliminary characterisation of potential feed additives that enhance lipid stability, in order to better characterise antioxidants and antioxidant combinations prior to any diet trials being undertaken. The research presented here shows that fish cell-based assays are suitable for the detection of antioxidant activity, which can be separated into two classes – antioxidants that scavenge water-soluble reactive species but do not prevent lipid peroxidation, and antioxidants that function specifically as inhibitors of lipid peroxidation without displaying activity against water-soluble reactive species.

The results of this work suggest that α-tocopherol is a potent inhibitor of lipid peroxidation in cultured fish cells, which supports the known activity of the vitamin E family of antioxidants in live fish. The observation that ascorbate, in the presence of haem iron, can cause lipid peroxidation in our cell-based model suggests that vitamin C should be used with caution in aquaculture feeds. It is possible that the benefits to the immune system derived from adequate vitamin C can be separated from its function as an antioxidant, and that in the case of fish capable of accumulating high levels of LC-PUFA in body tissues, the overdosing of vitamin C may promote lipid peroxidation under certain conditions. This is worthy of further investigation in live feeding trials, particularly in the case of marine finfish, which have a diet rich in LC-PUFA.

Appendix 1. Intellectual Property

All information arising from the project has to date been utilised for research purposes only and has not been implemented in a commercial setting. No intellectual property has been identified.

Appendix 2. Staff

Staff	Role	Location
Assoc. Prof. Kathryn Schuller	Principal Investigator	Flinders University
Dr. Peter Bain	Research Associate	Flinders University
Dr. Josephine Nocillado	Research Associate	Flinders University
Dr. Lin Koh	Research Assistant	Flinders University

Appendix 3. Chemicals and consumables

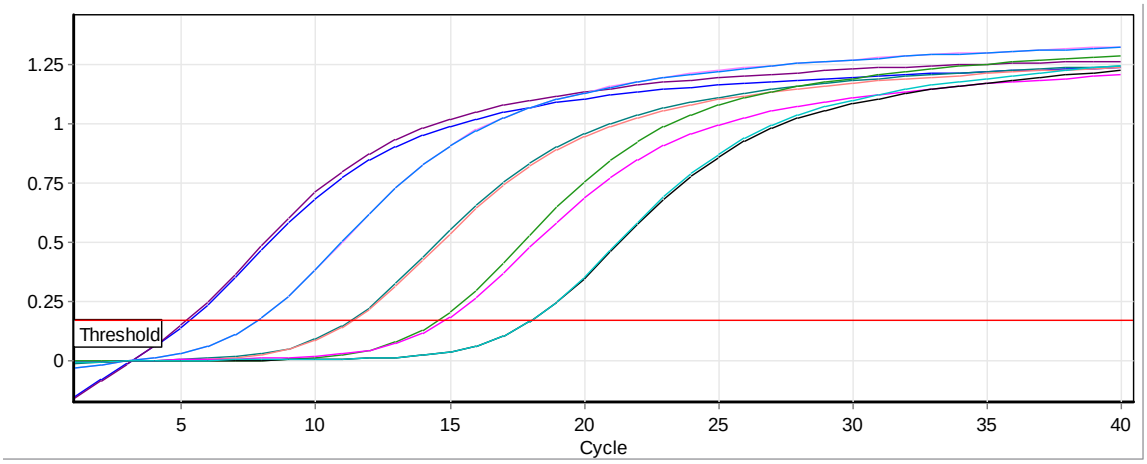
Chemical/consumable name	Abbreviation	Manufacturer / Supplier
2,2-diphenyl-1-picrylhydrazyl	DPPH	Sigma-Aldrich
2',7'-dichlorohihydrofluoescein diacetate	H_2DCFDA	Molecular Probes / Invitrogen (Life Technologies)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES	Amresco
4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY-C11$^{581/591}$	C_{11}-BODIPY$^{581/591}$	Molecular Probes (Invitrogen/Life Technologies)
6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid	Trolox	Sigma-Aldrich
10X buffer for Platinum Taq polymerase		Invitrogen (Life Technologies)
Acetic acid		Chem-Supply
Agarose		Amresco
Amphotericin B (FungiZone)		Invitrogen/Life Technologies
Butylated hydroxytoluene	BHT	Sigma-Aldrich
Calcium chloride	$CaCl_2$	Ajax-Finechem
Cell strainer/70 µm sieve		Beckton-Dickinson
Centrifuge tubes, 10 mL, sterile		Sarstedt
Chambered glass slides for cell culture (Lab-Tek II 8 chamber slides)		Nunc
Chloramphenicol		Sigma-Aldrich
Colchicine		
Collagenase VI	Collagenase	Sigma-Aldrich
Cryotubes		Nunc or Corning / In Vitro Technologies Aust.
Cumene hydroperoxide	Cumene-OOH	Sigma-Aldrich
Dimethylsufoxide, molecular biology grade	DMSO	Sigma-Aldrich
Ethanol, absolute, AR grade	EtOH	Chem-Supply / Southern Cross Scientific
Ethylenediaminetetra-acetic acid	EDTA	Sigma-Aldrich
Fish anaesthetic AQUI-S (clove oil-based)		AQUI-S
Flasks for cell culture, 75 cm^2, filter cap		Nunc / In Vitro Technologies Aust.
Foetal bovine serum	FBS	Hyclone (Thermo Scientific)
Formaldehyde		Sigma-Aldrich
Hemin		Sigma-Aldrich
Hydrochloric acid	HCl	Ajax
Liebovitz' L-15 medium	L-15	Gibco (Invitrogen/Life Technologies)
Magnesium chloride	$MgCl_2$	Chem-Supply
Magnesium sulphate, 50 mM	$MgSO_4$	Invitrogen (Life Technologies)

malonaldeyde bis(dimethyl acetal)		Sigma-Aldrich
Moloney Murine Leukaemia Virus reverse transcriptase	M-MuLV RT	New England Biolabs (NEB) / GeneSearch
Neutral red	NR	Gurr/BDH Chemical
Oligonucleotides (incl. primers)		GeneWorks Aust.
Penicillin-streptomycin stock solution (100X)	Pen-strep	Gibco (Invitrogen/Life Technologies)
Phosphate-buffered saline, sterile, prepared from tablets	PBS	Oxoid
Platinum Taq polymerase		Invitrogen (Life Technologies)
Platinum® SYBR® Green qPCR SuperMix UDG		Invitrogen (Life Technologies)
QIAquick gel extraction kit		Qiagen
QIAquick PCR purification kit		Qiagen
RNeasy kit		Qiagen
RNAlater		Ambion
RNase inhibitor		Promega
Thiobarbituric acid	TBA	Sigma-Aldrich
Trichloroacetic acid		Sigma-Aldrich
Tris(hydroxymethylaminomethane)	Tris or Tris-Cl	Sigma-Aldrich
Trypan blue		Gurr/BDH Chemical
Trypsin/EDTA stock solution (10X)	Trypsin-EDTA	Gibco (Invitrogen/Life Technologies)

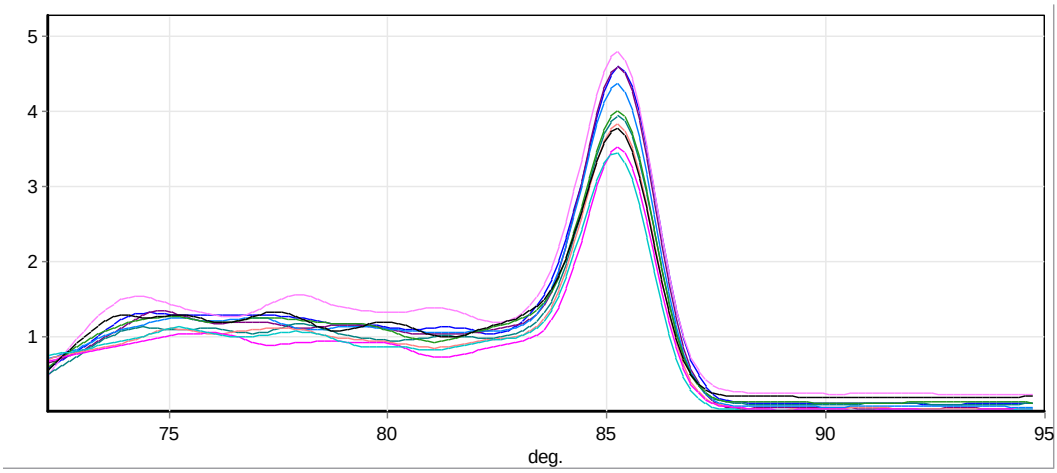
Appendix 4. Primer efficiency determination for qRT-PCR

A2.1 Beta-actin

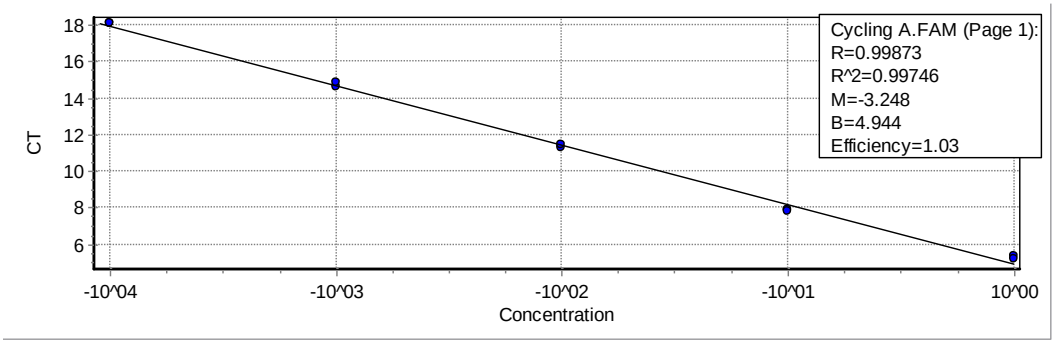
Amplification curve:



Melt curve (a single peak indicates a single specific product):

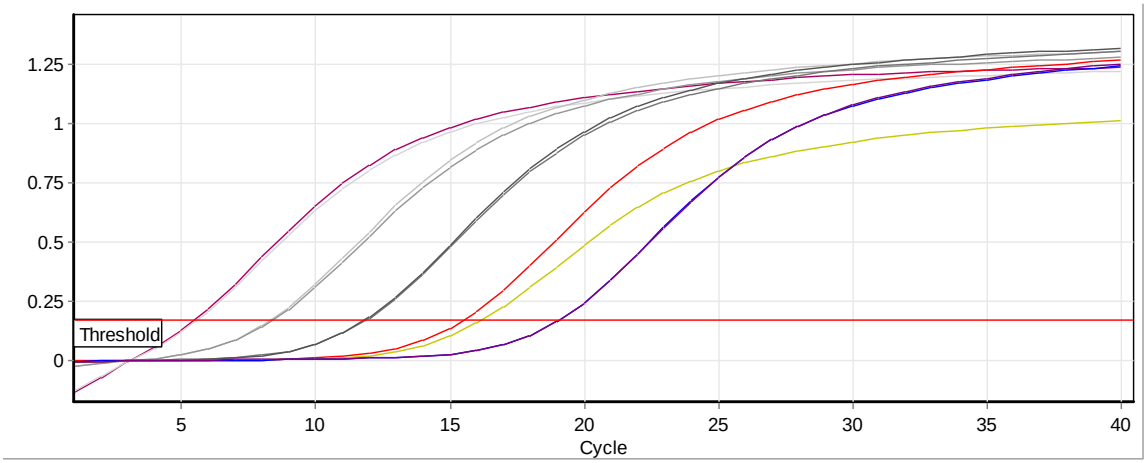


Standard curve and efficiency calculation: (efficiency of 1 = 100%):

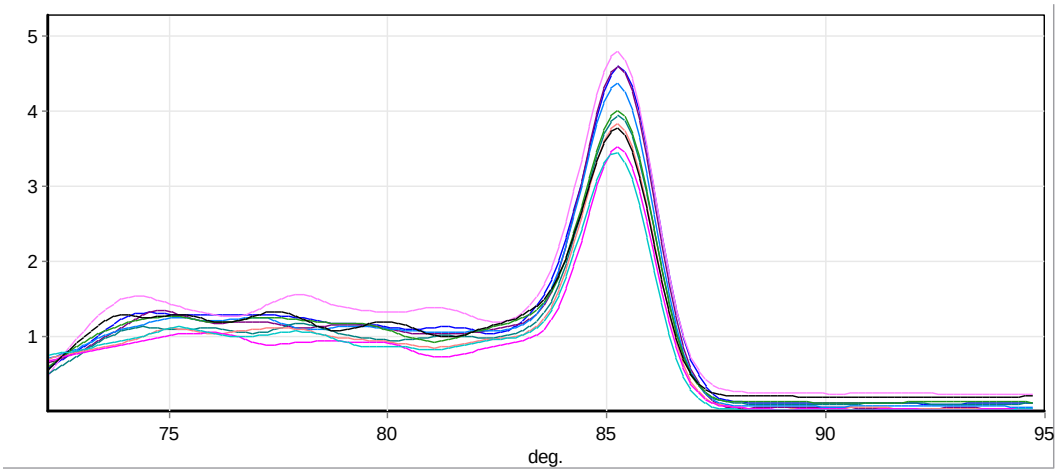


A2.2 Glutathione peroxidase 1

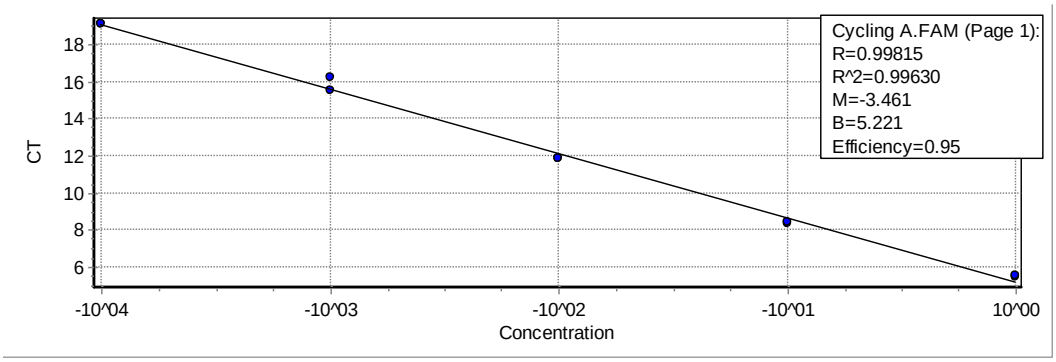
Amplification curve:



Melt curve (a single peak indicates a single specific product):

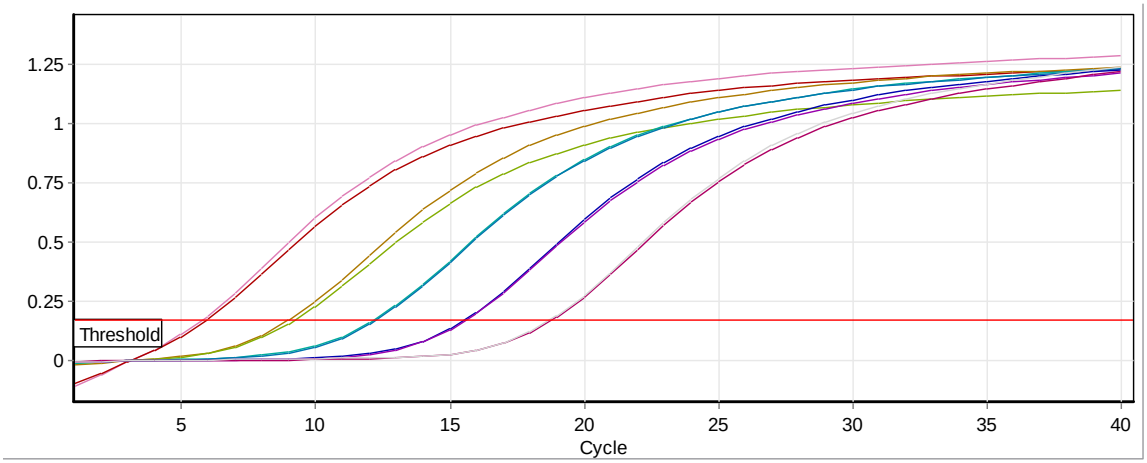


Standard curve and efficiency calculation: (efficiency of 1 = 100%):

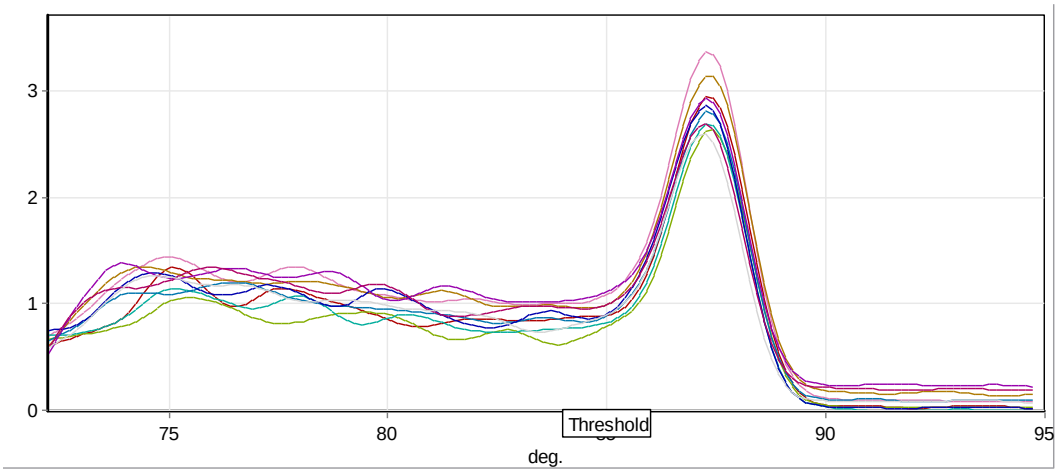


A2.3 Peroxiredoxin 1 (2-Cys peroxiredoxin)

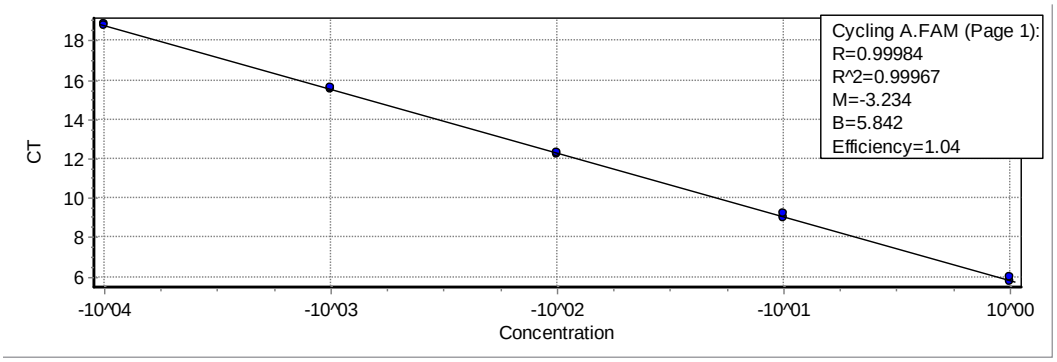
Amplification curve:



Melt curve (a single peak indicates a single specific product):



Standard curve and efficiency calculation: (efficiency of 1 = 100%):



Appendix 5. Pilot experiments investigating changes in gene expression in cultured cells by qRT-PCR in response to pro- and antioxidants

Cell type	Control	Treatment	Gene	Normalised to beta-actin	Not normalised
				(mRNA levels relative to control cultures)	
YTKF	10% FBS	1% FBS 6h	beta-actin	1.00	0.76
		serum dep 6h	beta-actin	1.00	0.95
		1% FBS 6h	GPx1	1.28	0.97
		serum dep 6h	GPx1	1.44	1.36
		1% FBS 6h	Prx1	1.28	0.97
		serum dep 6h	Prx1	1.32	1.25
EPC	Growth medium	10 µM Trolox 24h	beta-actin	1.00	0.85
		1 mM Trolox 24h	beta-actin	1.00	0.70
		10 µM Trolox 24h	GPx1	1.03	0.87
		1 mM Trolox 24h	GPx1	1.22	0.85
		10 µM Trolox 24h	Prx1	1.12	1.22
		1 mM Trolox 24h	Prx1	1.52	1.36
		10 mM H_2O_2 1h	beta-actin	1.00	0.72
		10 µM Trolox 24h + 10 mM H_2O_2 1h	beta-actin	1.00	0.76
		1 mM Trolox 24h + 10 mM H_2O_2 1h	beta-actin	1.00	0.57
		10 mM H_2O_2 1h	GPx1	0.92	0.66
		10 µM Trolox 24h + 10 mM H_2O_2 1h	GPx1	1.15	0.87
		1mM Trolox 24h + 10mM H2O2 1h	GPx1	1.12	0.64
		10mM H2O2 1h	Prx1	1.15	1.06
		10 µM Trolox 24h + 10 mM H_2O_2 1h	Prx1	1.28	1.25
		1 mM Trolox 24h + 10 mM H_2O_2 1h	Prx1	1.48	1.09
EPC	Vehicle control	10 µM paraquat 6h	beta-actin	1.00	1.22
		1 mM paraquat 6h	beta-actin	1.00	0.84
		10 µM paraquat 6h	GPx1	1.15	1.41
		1 mM paraquat 6h	GPx1	1.15	0.97

		10 µM paraquat 6h	Prx1	0.87	1.06
		1 mM paraquat 6h	Prx1	0.82	0.69
EPC	Medium only	100 µM ascorbate	beta-actin	1.00	1.17
			GAPDH	0.83	0.97
			GPx1	0.75	0.87
			Prx1	0.93	1.09
YTK1hep	Vehicle control	10 µM t-BHQ 6h	beta-actin	1.00	1.08
		100 µM t-BHQ 6h	beta-actin	1.00	1.17
		10 µM t-BHQ 6h	GPx1	1.11	1.20
		100 µM t-BHQ 6h	GPx1	1.05	1.23
		10 µM t-BHQ 6h	Prx1	1.12	1.21
		100 µM t-BHQ 6h	Prx1	0.79	0.92
		10 µM t-BHQ 6h	Gpx4	1.30	1.41
		100 µM t-BHQ 6h	Gpx4	1.11	1.30
EPC	Vehicle control	100 µM Trolox	beta-actin	1.00	0.89
		100 µM Trolox	GPx1	1.12	1.00
		100 µM Trolox	Prx1	1.03	0.92
EPC	10% FBS	serum deprivation 6h	beta-actin	1.00	0.67
		1 mM paraquat 6h	beta-actin	1.00	0.87
		serum deprivation 6h	GPx1	2.88	1.93
		1 mM paraquat 6h	GPx1	2.80	2.43
		serum deprivation 6h	Prx1	1.58	1.06
		1 mM paraquat 6h	Prx1	1.12	0.97

References

ABRAMOFF, M. D., MAGELHAES, P. J. & RAM, S. J. 2004. Image Processing with ImageJ. *Biophotonics International,* 11**,** 36-42.

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. & LIPMAN, D. J. 1990. Basic local alignment search tool. *J Mol Biol,* 215**,** 403-10.

ANDOH, T., CHOCK, P. B. & CHIUEH, C. C. 2002. The roles of thioredoxin in protection against oxidative stress-induced apoptosis in SH-SY5Y cells. *J Biol Chem,* 277**,** 9655-60.

ATABAY, C., CAGNOLI, C. M., KHARLAMOV, E., IKONOMOVIC, M. D. & MANEV, H. 1996. Removal of serum from primary cultures of cerebellar granule neurons induces oxidative stress and DNA fragmentation: protection with antioxidants and glutamate receptor antagonists. *J Neurosci Res,* 43**,** 465-75.

BABICH, H. & BORENFREUND, E. 1990. Applications of the neutral red cytotoxicity assay to in vitro toxicology. *Altern. Lab. Anim.,* 18**,** 129-144.

BIRD, R. P. & DRAPER, H. H. 1984. Comparative studies on different methods of malonaldehyde determination. *In:* LESTER, P. (ed.) *Methods in Enzymology.* Academic Press.

BOESS, F., KAMBER, M., ROMER, S., GASSER, R., MULLER, D., ALBERTINI, S. & SUTER, L. 2003. Gene expression in two hepatic cell lines, cultured primary hepatocytes, and liver slices compared to the in vivo liver gene expression in rats: possible implications for toxicogenomics use of in vitro systems. *Toxicol Sci,* 73**,** 386-402.

BORENFREUND, E., BABICH, H. & MARTIN-ALGUACIL, N. 1988. Comparisons of two in vitro cytotoxicity assays. The neutral red (NR) and tetrazolium MTT test. *Toxicol. In Vitro,* 2**,** 1-6.

BRANSDEN, M. P., CARTER, C. G. & NOWAK, B. F. 2001. Alternative methods for nutrition research on the southern bluefin tuna, Thunnus maccoyii (Castelnau): evaluation of Atlantic salmon, Salmo salar L., to screen experimental feeds. *Aquaculture Research,* 32**,** 174-181.

BRIGELIUS-FLOHE, R. & BANNING, A. 2006. Part of the series: from dietary antioxidants to regulators in cellular signaling and gene regulation. Sulforaphane and selenium, partners in adaptive response and prevention of cancer. *Free Radic Res,* 40**,** 775-87.

BUCHANAN, J. G. & THOMAS, P. M. 2008. Improving the color shelf life of farmed southern bluefin tuna (Thunnus maccoyii) flesh with dietary supplements of vitamins E and C and selenium. *Journal of Aquatic Food Product Technology,* 17**,** 285-302.

CAPES-DAVIS, A., THEODOSOPOULOS, G., ATKIN, I., DREXLER, H. G., KOHARA, A., MACLEOD, R. A., MASTERS, J. R., NAKAMURA, Y., REID, Y. A., REDDEL, R. R. & FRESHNEY, R. I. 2010. Check your cultures! A list of cross-contaminated or misidentified cell lines. *International Journal of Cancer,* 127**,** 1-8.

CAVANAUGH, P. F. 1990. A semi-automated neutral red based chemosensitivity assay for drug screening. *Invest. New Drugs,* 8**,** 347-354.

CUMMINGS, S. W., ANSARI, G. A. S., GUENGERICH, F. P., CROUCH, L. S. & PROUGH, R. A. 1985. Metabolism of 3-tert-Butyl-4-hydroxyanisole by Microsomal Fractions and Isolated Rat Hepatocytes. *Cancer Research,* 45**,** 5617-5624.

DRUMMEN, G. P., MAKKINJE, M., VERKLEIJ, A. J., OP DEN KAMP, J. A. & POST, J. A. 2004. Attenuation of lipid peroxidation by antioxidants in rat-1 fibroblasts: comparison of the lipid peroxidation reporter molecules cis-parinaric acid and C11-BODIPY(581/591) in a biological setting. *Biochim Biophys Acta,* 1636**,** 136-50.

DRUMMEN, G. P. C., VAN LIEBERGEN, L. C. M., OP DEN KAMP, J. A. F. & POST, J. A. 2002. C11-BODIPY581/591, an oxidation-sensitive fluorescent lipid peroxidation probe: (micro)spectroscopic characterization and validation of methodology. *Free Radical Biology and Medicine,* 33**,** 473-490.

FIJAN, N., SULIMANOVIC, D., BEARZOTTI, M., MUZINIC, D., ZWILLENBERG, L. O., CHILMONCZYK, S., VAUTHEROT, J. F. & DE KINKELIN, P. 1983. Some properties of the Epithelioma papulosum cyprini (EPC) cell line from carp cyprinus carpio. *Annales de l'Institut Pasteur. Virologie,* 134**,** 207-220.

FLOYD, R. A. & LEWIS, C. A. 1983. Hydroxyl free radical formation from hydrogen peroxide by ferrous iron-nucleotide complexes. *Biochemistry,* 22**,** 2645-2649.

FRESHNEY, R. I. 2000. *Culture of Animal Cells. A Manual of Basic Techniques (4th ed.),* New York, Wiley-Liss Inc.

GALLI, G. & FRATELLI, M. 1993. Activation of apoptosis by serum deprivation in a teratocarcinoma cell line: inhibition by L-acetylcarnitine. *Exp Cell Res,* 204**,** 54-60.

HARRIS, A. J., DIAL, S. L. & CASCIANO, D. A. 2004. Comparison of basal gene expression profiles and effects of hepatocarcinogens on gene expression in cultured primary human hepatocytes and HepG2 cells. *Mutat Res,* 549**,** 79-99.

HEIKKILA, R. E. & MANZINO, L. 1987. Ascorbic Acid, Redox Cycling, Lipid Peroxidation, and the Binding of Dopamine Receptor Antagonistsa. *Annals of the New York Academy of Sciences,* 498**,** 63-76.

HIROSE, M., HAGIWARA, A., INOUE, K., ITO, N., KANEKO, H., SAITO, K., MATSUNAGA, H., ISOBE, N., YOSHITAKE, A. & MIYAMOTO, J. 1988. Metabolism of 2- and 3-tert-butyl-4-hydroxyanisole in the rat (III): Metabolites in the urine and feces. *Toxicology,* 53**,** 33-43.

ISCHIROPOULOS, H., GOW, A., THOM, S. R., KOOY, N. W., ROYALL, J. A. & CROW, J. P. 1999. Detection of reactive nitrogen species using 2,7-dichlorodihydrfluorescein and dihydrorhodamine 123. *In:* LESTER, P. (ed.) *Methods in Enzymology.* Academic Press.

ITOH, N., CAO, J., CHEN, Z.-H., YOSHIDA, Y. & NIKI, E. 2007. Advantages and limitation of BODIPY as a probe for the evaluation of lipid peroxidation and its inhibition by antioxidants in plasma. *Bioorganic & Medicinal Chemistry Letters,* 17**,** 2059-2063.

JIALAL, I. & GRUNDY, S. 1993. Effect of combined supplementation with alpha-tocopherol, ascorbate, and beta carotene on low-density lipoprotein oxidation. *Circulation,* 88**,** 2780-2786.

KARLSSON, M., KURZ, T., BRUNK, U. T., NILSSON, S. E. & FRENNESSON, C. I. 2010. What does the commonly used DCF test for oxidative stress really show? *Biochemical Journal,* 428**,** 183-190.

KENSLER, T. W., WAKABAYASHI, N. & BISWAL, S. 2007. Cell Survival Responses to Environmental Stresses Via the Keap1-Nrf2-ARE Pathway. *Annual Review of Pharmacology and Toxicology,* 47**,** 89-116.

KIM, H.-J., BARAJAS, B., WANG, M. & NEL, A. E. 2008. Nrf2 activation by sulforaphane restores the age-related decrease of TH1 immunity: Role of dendritic cells. *Journal of Allergy and Clinical Immunology,* 121**,** 1255-1261.e7.

KJAER, M. A., TODORCEVIC, M., TORSTENSEN, B. E., VEGUSDAL, A. & RUYTER, B. 2008. Dietary n-3 HUFA affects mitochondrial fatty acid beta-oxidation capacity and susceptibility to oxidative stress in Atlantic salmon. *Lipids,* 43**,** 813-827.

KOBAYASHI, M., LI, L., IWAMOTO, N., NAKAJIMA-TAKAGI, Y., KANEKO, H., NAKAYAMA, Y., EGUCHI, M., WADA, Y., KUMAGAI, Y. & YAMAMOTO, M. 2009. The Antioxidant Defense System Keap1-Nrf2 Comprises a Multiple Sensing Mechanism for Responding to a Wide Range of Chemical Compounds. *Mol. Cell. Biol.,* 29**,** 493-502.

LAVILLE, N., AIT-AISSA, S., GOMEZ, E., CASELLAS, C. & PORCHER, J. M. 2004. Effects of human pharmaceuticals on cytotoxicity, EROD activity and ROS production in fish hepatocytes. *Toxicology,* 196**,** 41-55.

LEBEL, C. P., ISCHIROPOULOS, H. & BONDY, S. C. 1992. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chemical Research in Toxicology,* 5**,** 227-231.

LEE, M., HYUN, D.-H., HALLIWELL, B. & JENNER, P. 2001. Effect of overexpression of wild-type and mutant Cu/Zn-superoxide dismutases on oxidative stress and cell death induced by hydrogen peroxide, 4-hydroxynonenal or serum deprivation: potentiation of injury by ALS-related mutant superoxide dismutases and protection by Bcl-2. *Journal of Neurochemistry,* 78**,** 209-220.

LEE, S.-Y., SONG, J.-Y., KWON, E.-S. & ROE, J.-H. 2008. Gpx1 is a stationary phase-specific thioredoxin peroxidase in fission yeast. *Biochemical and Biophysical Research Communications,* 367**,** 67-71.

MAHER, J. & YAMAMOTO, M. 2010. The rise of antioxidant signaling--The evolution and hormetic actions of Nrf2. *Toxicology and Applied Pharmacology,* 244**,** 4-15.

MARKOVIC, O. & MARKOVIC, N. 1998. Cell cross-contamination in cell cultures: The silent and neglected danger. *In Vitro Cellular & Developmental Biology - Animal,* 34**,** 1-8.

MIKKELSEN, L., HANSEN, H. S., GRUNNET, N. & DICH, J. 1993. Inhibition of fatty acid synthesis in rat hepatocytes by exogenous polyunsaturated fatty acids is caused by lipid peroxidation. *Biochim Biophys Acta,* 1166**,** 99-104.

MIKKELSEN, L., HANSEN, H. S., GRUNNET, N. & DICH, J. 1994. Cytoprotective effect of tocopherols in hepatocytes cultured with polyunsaturated fatty acids. *Lipids,* 29**,** 369-72.

NAGUIB, Y. M. A. 1998. A Fluorometric Method for Measurement of Peroxyl Radical Scavenging Activities of Lipophilic Antioxidants. *Analytical Biochemistry,* 265**,** 290-298.

NAGUIB, Y. M. A. 2000. Antioxidant Activities of Astaxanthin and Related Carotenoids. *Journal of Agricultural and Food Chemistry,* 48**,** 1150-1154.

NAKAJIMA, Y., INOKUCHI, Y., SHIMAZAWA, M., OTSUBO, K., ISHIBASHI, T. & HARA, H. 2008. Astaxanthin, a dietary carotenoid, protects retinal cells against oxidative stress in-vitro and in mice in-vivo. *J Pharm Pharmacol,* 60**,** 1365-74.

NEMES, Z., DIETZ, R. & LUTH, J. B. 1979. The pharmacological relevance of vital staining with neutral red. *Experientia,* 35**,** 1475-1476.

PALOZZA, P. & KRINSKY, N. I. 1992. Astaxanthin and canthaxanthin are potent antioxidants in a membrane model. *Archives of Biochemistry and Biophysics,* 297**,** 291-295.

PAP, E. H. W., DRUMMEN, G. P. C., WINTER, V. J., KOOIJ, T. W. A., RIJKEN, P., WIRTZ, K. W. A., OP DEN KAMP, J. A. F., HAGE, W. J. & POST, J. A. 1999. Ratio-fluorescence microscopy of lipid oxidation in living cells using C11-BODIPY581/591. *FEBS Letters,* 453**,** 278-282.

PFAFFL, M. W., HORGAN, G. W. & DEMPFLE, L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res,* 30**,** e36.

PORKKALA-SARATAHO, E., SALONEN, J. T., NYYSSONEN, K., KAIKKONEN, J., SALONEN, R., RISTONMAA, U., DICZFALUSY, U., BRIGELIUS-FLOHE, R., LOFT, S. & POULSEN, H. E. 2000. Long-Term Effects of Vitamin E, Vitamin C, and Combined Supplementation on Urinary 7-Hydro-8-Oxo-2'-Deoxyguanosine, Serum Cholesterol Oxidation Products, and Oxidation Resistance of Lipids in Nondepleted Men. *Arterioscler Thromb Vasc Biol,* 20**,** 2087-2093.

PUANGKAEW, J., KIRON, V., SATOH, S. & WATANABE, T. 2005. Antioxidant defense of rainbow trout (Oncorhynchus mykiss) in relation to dietary n-3 highly unsaturated fatty acids and vitamin E contents. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology,* 140**,** 187-196.

REILLY, C. A. & AUST, S. D. 1999. Measurement of Lipid Peroxidation. *Current Protocols in Toxicology.* John Wiley & Sons, Inc.

REPETTO, G., DEL PESO, A. & ZURITA, J. L. 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat. Protocols,* 3**,** 1125-1131.

ROSJO, C., BERG, T., MANUM, K., GJOEN, T., MAGNUSSON, S. & THOMASSEN, M. S. 1994. EFFECTS OF TEMPERATURE AND DIETARY N-3 AND N-6 FATTY-ACIDS ON ENDOCYTIC PROCESSES IN ISOLATED RAINBOW-TROUT (ONCORHYNCHUS-MYKISS, WALBAUM) HEPATOCYTES. *Fish Physiology and Biochemistry,* 13**,** 119-132.

ROYALL, J. A. & ISCHIROPOULOS, H. 1993. Evaluation of 2',7'-Dichlorofluorescin and Dihydrorhodamine 123 as Fluorescent Probes for Intracellular H2O2 in Cultured Endothelial Cells. *Archives of Biochemistry and Biophysics,* 302**,** 348-355.

ROZEN, S. & SKALETSKY, H. J. 2000. Primer3 on the WWW for general users and for biologist programmers. *In:* KRAWETZ, S. & MISENER, S. (eds.) *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Totowa, NJ: Humana Press.

SAMBROOK, J. 2001. *Molecular cloning : a laboratory manual / Joseph Sambrook, David W. Russell,* Cold Spring Harbor, N.Y. :, Cold Spring Harbor Laboratory Press.

SCHILDERMAN, P. A. E. L., RHIJNSBURGER, E., ZWINGMANN, I. & KLEINJANS, J. C. S. 1995. Induction of oxidative DNA damage and enhancement of cell proliferation in human lymphocytes in vitro by butylated hydroxyanisole. *Carcinogenesis,* 16**,** 507-512.

SCHNEIDER, J. E., BROWNING, M. M. & FLOYD, R. A. 1988. Ascorbate/iron mediation of hydroxyl free radical damage to PBR322 plasmid DNA. *Free Radical Biology and Medicine,* 5**,** 287-295.

SCHOLZ, S. & SEGNER, H. 1999. Induction of CYP1A in primary cultures of rainbow trout (Oncorhynchus mykiss) liver cells: concentration-response relationships of four model substances. *Ecotoxicol Environ Saf,* 43**,** 252-60.

SEGNER, H. & CRAVEDI, J. P. 2001. Metabolic activity in primary cultures of fish hepatocytes. *Altern Lab Anim,* 29**,** 251-7.

STÉPHAN, G., GUILLAUME, J. & LAMOUR, F. 1995. Lipid peroxidation in turbot (Scophthalmus maximus) tissue: effect of dietary vitamin E and dietary n - 6 or n - 3 polyunsaturated fatty acids. *Aquaculture,* 130**,** 251-268.

THOMPSON, J. L., SEE, V. H. L., THOMAS, P. M. & SCHULLER, K. A. 2010. Cloning and characterization of two glutathione peroxidase cDNAs from southern bluefin tuna (Thunnus maccoyii). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 156**,** 287-297.

TOCHER, D. R., MOURENTE, G., VAN DER EECKEN, A., EVJEMO, J. O., DIAZ, E., BELL, J. G., GEURDEN, I., LAVENS, P. & OLSEN, Y. 2002. Effects of dietary vitamin E on antioxidant defence mechanisms of juvenile turbot (Scophthalmus maximus L.), halibut (Hippoglossus hippoglossus L.) and sea bream (Sparus aurata L.). *Aquaculture Nutrition,* 8**,** 195-207.

TOPPO, S., FLOHÉ, L., URSINI, F., VANIN, S. & MAIORINO, M. 2009. Catalytic mechanisms and specificities of glutathione peroxidases: Variations of a basic scheme. *Biochimica et Biophysica Acta (BBA) - General Subjects,* 1790**,** 1486-1500.

TUSCHL, G., LAUER, B. & MUELLER, S. O. 2008. Primary hepatocytes as a model to analyze species-specific toxicity and drug metabolism. *Expert Opinion on Drug Metabolism & Toxicology,* 4**,** 855-870.

WALLACE, K. B. 1989. Glutathione-dependent metabolism in fish and rodents. *Environmental Toxicology and Chemistry,* 8**,** 1049-1055.

WARD, R. D., ZEMLAK, T. S., INNES, B. H., LAST, P. R. & HEBERT, P. D. N. 2005. DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 360**,** 1847-1857.

WINCKLER, J. 1974. Vital staining of lysosomes and other cell organelles of the rat with neutral Red. *Prog. Histochem. Cytochem.,* 6**,** 1-89.

YOSHINO, K., IIMURA, E., SAIJO, K., IWASE, S., FUKAMI, K., OHNO, T., OBATA, Y. & NAKAMURA, Y. 2006. Essential role for gene profiling analysis in the authentication of human cell lines. *Human Cell,* 19**,** 43-48.