4 THE USE OF FISH CELL LINES TO INVESTIGATE LCPUFA SYNTHESIS

4.1 Introduction

4.1.1 Culturing fish cells

Fish cells, like mammalian cells, can be maintained *in vitro* using primary cultures, which are temporary, or cell lines, which are permanent (Bols and Lee 1991). The morphology of primary cell cultures changes with increasing time in culture (Ghioni *et al.* 1997). A continuous cell line is obtained through the successful subculturing of a primary culture (Bols and Lee 1991). Cell lines can be derived from a number of fish organs and tissues such as gonad, liver, pancreas, kidney, heart, spleen, skeletal muscle and nervous tissue (Bols and Lee 1991). Fish cell lines are advantageous to use because they become fairly homogeneous during early passaging, which makes them reproducible, and they have the ability to be cryopreserved, which provides a convenient source of cells (Bols and Lee 1991). *In vitro* fish cells are more sensitive than whole organisms because multiple defence mechanisms are not present (Visoottiviseth and Chanwanna 2001). Thus, *in vitro* cells respond to treatments at much lower concentrations than may affect a whole organism.

The first cell line from a poikilothermic animal was the rainbow trout cell line (RTG-2) (Gravell and Malsberger 1965). The body temperature of a fish varies with the temperature of its surrounding medium. Therefore, fish cell cultures are eurythermic meaning they are able to adapt to growth and survival over a wide temperature range which is referred to as the cellular endurance zone (Fijan *et al.* 1983; Babich and Borenfreund 1991; Bols and Lee 1991). Poikilothermic fish cell lines are generally grown at higher temperatures than the fish encounters in their natural habitat (Tocher *et al.* 1988). For all fish species cultured to date, cells are anchorage dependent, attaching and growing as a monolayer on the flask surface (Babich and Borenfreund 1991). Growth at pH 7.4 is optimal for most cells to reach confluence within 5-7 days (Freshney 2000; Williams *et al.* 2003). Fish cell cultures are advantageous

because of their low cost, sensitivity, versatility and reproducibility (Visoottiviseth and Chanwanna 2001).

4.1.2 Choosing fish cell lines

The aquaculture industry and economic importance of many fish species have lead to the development of many continuous cultures of fish cells. The growth of the salmonid aquaculture industry which includes salmon, trout, chars and whitefish species along with the cyprinid aquaculture industry which includes carp, goldfish and danios has led to these two families being highly represented in cell lines (Fryer and Lannan 1994). Although many fish cell lines have been developed there still remain aquaculture species without *in vitro* models. In the American Tissue Culture Collection (ATCC) holdings there are only two species from the order Perciformes, white bass (*Morone chrysops*) (CRL-2773) and bluegill (*Lepomis macrochirus*) (CCL-91) (www.atcc.org). There are two finfish aquaculture species of commercial importance in South Australia, SBT and YTK, both from the order Perciformes, which currently do not have cell lines. These marine aquaculture industries would benefit greatly from the development of tuna and kingfish cell lines. SBT cell cultures are required because of the expense and logistical difficulties of working with SBT due to their large size, inability to be bred in captivity and high commercial value. Likewise, YTK cell cultures are required because of their commercial value and the inefficient use of whole fish in trials which could be conducted quicker and cheaper with a cell line model.

4.1.3 The use of fish cell lines

Fish cell lines are no longer only used in virology studies. Applications now include investigating cytogenetics, toxicology, carcinogenesis, immunology, temperature effects and cell physiology (Tocher *et al.* 1995a). Of particular interest to us is cellular metabolism and fish cell lines provide a way to investigate PUFA synthesis and metabolism. The homogenous cell population can be supplemented with PUFA under controlled conditions. The metabolism of the supplemented PUFA in cell lines from freshwater, anadromous and marine fish species may be different due to

differences in their natural diet and requirement for PUFA. It has been accepted that marine fish have a greater dietary requirement for LCPUFA than anadromous and freshwater fish (Ghioni *et al.* 1999; Tocher and Ghioni 1999). Therefore, the fatty acid metabolism in marine, anadromous and freshwater cell lines should be representative of the PUFA requirements and natural diet of the source organism.

Fish cell lines obtain their PUFA source from the mammalian fetal bovine serum (FBS) supplement which is added to the growth medium. The mammalian FBS is the serum of choice for fish cell culture due to the difficulties in obtaining fish serum and the risk of introducing viruses (Tocher *et al.* 1988). When investigating PUFA metabolism in cultured fish cell lines, we must keep in mind the altered fatty acid profile of cultured fish cells compared to the fish tissues. Fish cells in culture contain lower total n-3 PUFA and higher total n-6 PUFA than the cells they were derived from (Tocher *et al.* 1988; Tocher and Dick 1991). Conditions for culturing fish cells are bordering on being essential fatty acid (EFA) deficient which is demonstrated by the drastic fatty acid composition changes which occur (Ghioni *et al.* 1997).

PUFA metabolism studies with fish cell lines have reflected the findings from *in vivo* studies using the same fish species (Ghioni *et al.* 1997). Some fish cell lines have shown limited ability to convert C_{18} to C_{20} PUFA which was deemed to be due to limited $C_{18\text{-}20}$ elongase activity or Δ5desaturase activity (Ghioni *et al.* 1999; Tocher and Ghioni 1999). For example, the turbot cell line (TF) showed limited $C_{18\text{-}20}$ elongase activity and the gilthead sea bream cell line (SAF-1) showed limited Δ5desaturase activity compared to the Atlantic salmon cell line (AS) (Tocher and Dick 1990; Tocher and Mackinlay 1990; Ghioni *et al.* 1999; Tocher and Ghioni 1999; Ghioni *et al.* 2001). These deficiencies in the LCPUFA synthesis pathway are consistent with the known nutritional requirements of marine species for LCPUFA (Ghioni *et al.* 1999; Tocher and Ghioni 1999). Investigating the LCPUFA synthesis pathway using cell cultures is a less expensive and quicker alternative to fish feeding trials. However, there is no guarantee that the enzymes involved in the LCPUFA metabolic pathway will be expressed in cultured cells the same way they are expressed in live fish (Tocher 2003). In cultured mammalian cells, the desaturase activity tends to be lost before elongase activity (Maeda *et al.* 1978). Therefore, fish cell lines are useful for investigating the LCPUFA synthesis pathway, but it is

important to determine if the activity of the pathway is a reflection of the situation *in vivo*.

4.1.4 The use of the FHM and CHSE-214 cell lines to investigate LCPUFA synthesis

The northern fathead minnow (*Pimephales promelas*) epithelial cell line, designated FHM, was derived from the tissue posterior to the anus, excluding the caudal fin, of normal male and female adults (Gravell and Malsberger 1965). The FHM cell line was first successfully cultivated in 1962 (Gravell and Malsberger 1965). FHM cells were found to grow in Eagle's basal and minimum essential media with Hanks' or Earle's salts or Leibovitz L-15 medium over a temperature range of 14-34°C (Gravell and Malsberger 1965). The FHM cell line was developed for animal viral research (Gravell and Malsberger 1965; Solis and Mora 1970). In this study we will use the FHM cell line as a model for investigating LCPUFA synthesis in cell lines from freshwater fish species.

The Chinook salmon (*Oncorhynchus tshawytscha*) epithelial cell line, designated CHSE-214, was derived from pooled normal embryos (Lannan *et al.* 1984). The CHSE-214 cell line was first successfully cultivated in 1964 (Lannan *et al.* 1984). CHSE-214 cells were found to grow in Eagle's minimum essential medium (EMEM) with Earle's salts over a temperature range of 21-24°C (Lannan *et al.* 1984). The CHSE-214 cell line proved to be very useful for investigating salmonid viruses (Lannan *et al.* 1984). In this study we will use the CHSE-214 cell line as a model for investigating LCPUFA synthesis in cell lines from anadromous fish species.

Several studies have used fish cell lines as a model for whole organisms to investigate LCPUFA synthesis. The essential fatty acid deficient-epithelioma papulosum cyprini (EFAD-EPC) cell line from carp (*Cyrpinus carpio*) has been the most extensively studied because it can be grown in EFA deficient media to avoid complications caused by the FBS (Tocher *et al.* 1995a; Tocher *et al.* 1996; Tocher and Dick 2001). The EFAD-EPC cells displayed Δ6desaturase and Elovl5 activity when supplemented with ^{14}C labelled fatty acids but limited Δ5desaturase (Tocher *et*

al. 1995a; Tocher *et al.* 1996). Other cell lines from freshwater or anadromous species have been studied including topminnow (*Poeciliopsis lucida*) (PLHC-1), rainbow trout (RTG-2), Atlantic salmon (AS) and Chinook salmon (CHSE-214) (Tocher 1990; Tocher and Dick 1990; Tocher *et al.* 1989; Tocher *et al.* 1995). The CHSE-214 cells had the same LCPUFA synthesis enzymatic activity as the EFAD-EPC cells, while PLHC-1, RTG-2 and AS cells had higher Δ5desaturase activity together with Δ6desaturase and Elovl5 activity (Tocher *et al.* 1989; Tocher 1990; Tocher and Dick 1990; Tocher and Dick 1991; Tocher *et al.* 1995; Tocher *et al.* 1996). The cell lines examined from the marine species turbot (TF) and gilthead sea bream (SAF-1) have different Δ5desaturase activities. The TF and SAF-1 cells have Δ6desaturase activity, limited Elovl5 activity and the SAF-1 cells have lower Δ5desaturase activity compared to the TF cells (Tocher *et al.* 1989; Ghioni *et al.* 1999; Tocher & Ghioni 1999; Ghioni *et al.* 2001).

4.1.5 Aims

The work in the following chapter aims to compare the LCPUFA synthesis pathway in freshwater, anadromous and marine fish cell lines. The FHM and CHSE-214 cell lines will be used as model freshwater and anadromous species, respectively. Primary cell cultures will be developed from two commercially important marine aquaculture species in SA, SBT and YTK. We aim to supplement the fish cell lines with n-3 and n-6 PUFA to investigate if the LCPUFA synthesis pathway in the cell lines can be used as a model for fish *in vivo*.

4.2 Methods

4.2.1 Cell line culture materials

4.2.1.1 The fish cell lines

All cell lines used in this study were obtained from Dr Mark Crane at the Australian Animal Health Laboratory (AAHL) Fish Diseases Laboratory, Commonwealth Scientific and Industrial Research Organisation (CSIRO) Livestock Industries,

Geelong, Vic, Australia. The same source of cells was used throughout the study, with cells undergoing weekly passaging to maintain cell numbers.

The fathead minnow (*Pimephales promelas*) epithelial cell line (FHM) was received on 25/02/2004 and the cells were grown at 25°C.

The chinook salmon (*Oncorhynchus tshawytscha*) embryonic cell line (CHSE-214) (ATCC catalogue no. CRL 1681) was received on 26/08/2008 and the cells were grown at 22°C.

The bluegill (*Lepomis macrochirus*) fry caudal trunk cell line (BF-2) (ATCC catalogue no. CCL 91) was received on 26/08/2008 and the cells were grown at 22°C.

The barramundi (*Lates calcarifer*) kidney cell line (LCK) was received on 30/10/2008. The LCK cell line was initiated, propagated and cloned by Dr Nick Moody (AAHL, Fish Diseases Laboratory, CSIRO Livestock Industries, Geelong, Vic, Australia). It was a clonal cell line originating from a single cell and it was grown at 25°C.

The rainbow trout (*Oncorhynchus mykiss*) gonad cell line (RTG-2) (ATCC catalogue no. CCL 55) was received on 30/10/2008 and the cells were grown at 22°C.

The koi (*Cyprinus carpio*) fin cell line (KF-1) was received on 26/08/2008. The KF-1 cell line was supplied to the AAHL by Keith Way, from the Centre for Environment, Fisheries and Science (CEFAS), Weymouth, United Kingdom on 10/05/2005. The cells were grown at 25°C.

4.2.1.2 The media

All of the media and reagents used were obtained from Invitrogen™ Australia Pty. Ltd. (Mount Waverley, Vic, Aust.) unless otherwise stated.

The FHM and KF-1 cells were maintained in Gibco® Leibovitz's L-15 medium, which contained L-glutamine, galactose, sodium pyruvate and vitamins. Leibovitz's L-15 medium uses phosphates and free-base amino acids (L-arginine, L-histidine and L-cysteine) for its buffer system. Ten % (v/v) FBS and 100 IU penicillin/100 µg streptomycin per mL were added to the L-15 medium to make the complete growth medium.

The CHSE-214 and LCK cells were maintained in EMEM with Earle's salts and non-essential amino acids, which contained L-glutamine. EMEM contained 10 mM HEPES buffer pH 7.2-7.5, 10% (v/v) FBS and 100 IU penicillin/100 µg streptomycin per mL to make the complete growth medium.

The RTG-2 cells were maintained in the same medium used for the CHSE-214 and LCK cells, except the medium was buffered with 1.5g L^{-1} sodium bicarbonate (Sigma-Aldrich®, Australia), rather than HEPES.

The BF-2 cells were maintained in Hank's minimum essential medium (HMEM) which contained L-glutamine. HMEM contained 23.5 mM Tris/20 mM HCl buffer (Sigma-Aldrich®, Australia), 10% (v/v) FBS and 100 IU penicillin/100 µg streptomycin per mL to make the complete growth medium.

Phosphate buffered saline (PBSA) pH 7.3 without Ca^{2+}/Mg^{2+}, containing 8 g L^{-1} sodium chloride, 0.2 g L^{-1} potassium chloride, 1.15 g L^{-1} disodium hydrogen phosphate and 0.2 g L^{-1} potassium dihydrogen phosphate was used for washing the cells (Oxoid Australia Pty Ltd, Thebarton, SA, Aust.).

Trypsin-versene (T/V) solution, which is the same as trypsin-EDTA, was used to detach cells from the culture flasks. 10X T/V has a final concentration of 0.5 g of trypsin and 0.2 g EDTA•4Na L^{-1}. T/V (1X) was prepared by diluting 1 mL T/V (10X) solution in 9 mL sterile PBSA.

4.2.2 Cell line culture methods

4.2.2.1 Passaging cells

Passaging cell lines was done in a Class II Biohazard Safety Cabinet (BSC). The work was conducted in a Class II BSC because it is designed with inward air flow at a velocity of 75-100 linear feet per minute to protect personnel, contains a HEPA-filtered downward vertical laminar airflow for product protection, and a HEPA-filtered exhaust air for environmental protection.

The working surface was sterilised with 70% (v/v) ethanol. The old growth medium was decanted from the 25 cm^2 or 75 cm^2 cell culture flask and discarded into a discard bottle. The cell monolayer was rinsed with PBSA to remove all traces of growth medium. Two or 3 mL of T/V solution was added to a 25 cm^2 or 75 cm^2 flask, respectively, on the opposite side to the cell monolayer. The flask was gently rocked to detach the cell monolayer. Trypsin activity was stopped by the addition of growth medium at a volume three times the volume of T/V solution. The resuspended cells were transferred to a sterile 50 mL centrifuge tube and centrifuged for 5 min at 400 *g* in a Jouan CR312 centrifuge. The supernatant containing the T/V solution and growth medium was removed, leaving the pellet. The pellet was resuspended in a known volume of growth medium. A 75 cm^2 flask was seeded at approximately 2 x 10^6 cells in 20 mL growth medium. 100% confluent monolayers were obtained within 5 days when FHM and CHSE-214 cells were incubated at 25°C or 22°C, respectively.

4.2.2.2 Haemocytometer counting of cells

A haemocytometer microscope slide was used for determining the number of cells in a suspension under a microscope. The haemocytometer grid contains nine 1 mm^2 squares that are divided up into 4 x 4 medium squares measuring 0.2 mm^2 each. The middle square is further divided with each tiny square measuring 0.05 mm^2. The cell pellet was resuspended in growth medium and a cell count was performed prior to seeding culture flasks with cells. The haemocytometer and cover slip were cleaned before use and between samples with 70% (v/v) ethanol. The cover slip was placed

on the haemocytometer and 10 µl of the cell suspension was loaded under the cover slip. The haemocytometer was placed under a light microscope at 20X magnification. The number of cells in four quadrants of the haemocytometer was counted and the average calculated. If there were too many cells to accurately count, a dilution was performed.

The number of cells per mL from the haemocytometer count is determined by taking into account the area and dilution factors. Each square counted is 0.1 mm^3. 1 cm^3 is equal to 1 mL, therefore 0.1 mm^3 x 10^4 is equal to 1 cm^3. Therefore, the total number of cells in 1 mL of suspension equals to: Cells/mL = total count x 10^4 x dilution factor

4.2.2.3 Cell viability

A 10 µl aliquot of the cell suspension was added to 10 µl 0.4% (w/v) Trypan blue solution. Viable and non-viable cells were counted using a haemocytometer. Viable cells with an intact cell membrane appear golden in colour as they exclude Trypan blue. Non-viable cells cannot exclude Trypan blue and are stained blue. If there were too many cells to accurately count, the cells were diluted using Trypan blue solution at a ratio of 2:1. The percent viability was determined by dividing the number of viable cells by the total number of cells.

4.2.2.4 Cryopreservation of cell lines

4.2.2.4.1 Cell freezing

Cells were harvested from a 75 cm^2 flask using trypsin-versene solution, as described in section 4.2.2.1. The cell pellet was resuspended in 3 mL of FBS containing 7% (v/v) dimethyl sulfoxide (DMSO). Cryovials containing 1 mL of the cell suspension were stored overnight at -80°C, then transferred into a liquid N_2 storage tank for long term storage.

4.2.2.4.2 Cell thawing

To revive cells which had been cryopreserved, the cryovial was thawed at room temperature. The thawed cell suspension was added to a 25 cm^2 flask, containing 10 mL of the appropriate growth medium.

4.2.3 Genomic DNA extraction from fish cell lines

Genomic DNA was extracted from a confluent 75 cm^2 flask of fish cells using the DNeasy Blood and Tissue Kit (Qiagen, Australia) following the spin column protocol of purification of total DNA from animal cells.

4.2.4 Identification of cell lines using cytochrome oxidase subunit I (cox1) gene barcoding

A 717 bp fragment of the cytochrome oxidase subunit I (*cox*1) gene was amplified from approximately 250 ng of genomic DNA. PCR amplification using the FishF1 - 5'-TCAACCAACCACAAAGACATTGGCAC-3' and FishR1 - 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3' primers (Ward *et al.* 2005) and Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, Australia) was performed with an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 68°C for 45 s, followed by a final extension at 68°C for 5 min. The gel purified PCR products were cloned into the pGEM®-T Easy Vector (Promega, WI, USA) and then sequenced.

4.2.5 Supplementation of FHM and CHSE-214 cells with fatty acids

4.2.5.1 Preparation of the media supplemented with fatty acids

Free fatty acids (FFA) were diluted in analytical grade ethanol to prepare 10 mg mL^{-1} stock solutions and stored at -20°C. Fatty acid supplemented media were prepared immediately prior to use. The FFA stock solution was diluted in L-15 containing 2%

(v/v) FBS to obtain final concentrations ranging from 1-20 μM. The FFA were bound to fatty acid free bovine serum albumin (FAF-BSA) (Best *et al.* 2006). FAF-BSA was dissolved in the pre-warmed medium before adding the FFA. The molar ratio of FFA to FAF-BSA was 4:1. The serum was reduced from 10% to 2% (v/v) in the media since FBS contained fatty acids, including LA, AA, EPA and DHA, which were supplemented as FFA.

4.2.5.2 Culturing cells in fatty acid supplemented media

Preliminary experiments were conducted to determine the conditions required for investigating the accumulation of fatty acids in the FHM and CHSE-214 cell phospholipids. The effect of the percentage of FBS, the concentration of supplemented FFA and the length of time required for incorporation and product formation were investigated. For the data presented in this chapter the FHM and CHSE-214 cells were cultured to approximately 80% confluence in L-15 containing 10% (v/v) FBS prior to the experiment. Cells were seeded into the 25 cm^2 flasks and cultured in 10 mL of L-15 containing 2% FBS with FFA bound to FAF-BSA for 24 h at 25°C or 22°C, respectively. The experiments were conducted with cells over a passage range of 13 passages.

4.2.5.3 Harvesting cells after fatty acid supplementation

The cells were harvested using T/V solution as described in section 4.2.2.1 following the 24 h incubation with the fatty acid supplemented medium. Approximately 10^6 cells were centrifuged as described in section 4.2.2.2 and the pellet was then resuspended in 1 mL of PBSA prior to storing at -80°C until fatty acid analysis.

4.2.6 Fatty acid analysis

4.2.6.1 Extraction of phospholipids from FHM and CHSE-214 cells

Phospholipids were extracted from samples using chloroform:methanol as outlined for total lipid extraction in section 2.2.8.1. The $C_{17:0}$ phospholipid internal standard (Sigma-Aldrich®) was added. After drying the lipid under nitrogen, it was

resuspended in 150 µl chloroform:methanol (9:1 by volume) and spotted onto 0.3 mm Silica Gel (60H) thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany). Phospholipids were separated by TLC using a mobile phase of petroleum spirits:glacial acetic acid (3:1 by volume). The phospholipid band remained where the lipid was spotted while the remaining lipid classes migrated towards the top of the plate. The phospholipid band was visualized under ultraviolet light and scraped into 5 mL glass vials containing 2 mL of 1% (v/v) sulphuric acid in methanol. The phospholipid fraction was methylated at 70°C for 3 h, shaking every 30 min. FAME were extracted into 250 µl of distilled H_2O and 500 µL of heptane. After mixing well and allowing the layers to separate, the top heptane layer was transferred to a gas chromatography vial containing anhydrous sodium sulphate. The samples were concentrated under nitrogen to a final volume of 100 µl, transferred into a gas chromatography insert and analysed by gas chromatography.

4.2.6.2 Extraction of total lipid from cell culture growth medium

Total lipid was extracted from cell culture growth medium basically according to the method in section 3.2.31.3. One mL of L-15 medium, L-15 + FBS or FBS was transferred to a glass extraction tube, the weight was recorded and 0.5 mL cold saline was added. The data on the L-15 medium and FBS can be seen in Table 4.1.

4.2.6.3 Analysis of FAME by gas chromatography

FAME were analysed by gas chromatography as outlined in section 2.2.8.4. A typical GC profile of FAME derived from FHM cell phospholipids can be seen in Figure 4.1.

Table 4.1 The fatty acid composition of L-15 medium, FBS and L-15 medium containing 10% (v/v) FBS. The total fatty acids were extracted from the FBS, L-15 medium or L-15 + 10% FBS and analysed by gas chromatography as described in section 4.2.6. The data are the mean ± SE (n=3). ND, not detected.

Fatty Acid	L-15	FBS	L-15 + 10% FBS	L-15	FBS	L-15 + 10% FBS
		% distribution			$\mu g\ mL^{-1}$	
16:0	52.1 ± 0.7	23.0 ± 0.2	29.7 ± 0.7	1.5 ± 0.1	43.8 ± 1.0	6.9 ± 0.4
18:0	47.9 ± 0.7	18.8 ± 0.1	21.2 ± 0.3	1.4 ± 0.1	35.7 ± 0.7	4.9 ± 0.1
Total Sats	100 ± 0.0	45.3 ± 0.3	53.0 ± 0.4	2.9 ± 0.1	86.1 ± 1.8	12.3 ± 0.5
16:1n-7	ND	1.3 ± 0.0	2.4 ± 0.2	ND	2.4 ± 0.1	0.6 ± 0.1
18:1n-9	ND	15.3 ± 0.0	16.4 ± 0.6	ND	29.1 ± 0.5	3.8 ± 0.3
18:1n-7	ND	5.0 ± 0.0	5.2 ± 0.3	ND	9.5 ± 0.2	1.2 ± 0.1
Total MUFA	ND	23.5 ± 0.2	24.1 ± 0.8	ND	44.7 ± 0.7	5.6 ± 0.4
Total n-9	ND	17.3 ± 0.2	16.4 ± 0.6	ND	32.8 ± 0.5	3.8 ± 0.3
Total n-7	ND	6.3 ± 0.0	7.7 ± 0.3	ND	11.9 ± 0.2	1.8 ± 0.1
18:2n-6	ND	7.7 ± 0.2	4.2 ± 0.1	ND	14.5 ± 0.2	1.0 ± 0.1
18:3n-6	ND	0.3 ± 0.0	ND	ND	0.6 ± 0.0	ND
20:2n-6	ND	0.2 ± 0.0	ND	ND	0.5 ± 0.0	ND
20:3n-6	ND	3.4 ± 0.0	3.6 ± 0.1	ND	6.5 ± 0.1	0.8 ± 0.1
20:4n-6	ND	9.9 ± 0.0	9.8 ± 0.4	ND	18.7 ± 0.4	2.3 ± 0.2
22:4n-6	ND	1.8 ± 0.0	1.3 ± 0.7	ND	3.4 ± 0.1	0.3 ± 0.2
22:5n-6	ND	ND	ND	ND	ND	ND
Total n-6	ND	23.3 ± 0.2	18.9 ± 0.2	ND	44.2 ± 0.5	4.4 ± 0.1
18:3n-3	ND	ND	ND	ND	ND	ND
18:4n-3	ND	ND	ND	ND	ND	ND
20:3n-3	ND	ND	ND	ND	ND	ND
20:5n-3	ND	0.3 ± 0.0	ND	ND	0.6 ± 0.1	ND
22:5n-3	ND	3.7 ± 0.1	2.4 ± 0.2	ND	7.0 ± 0.2	0.5 ± 0.0
22:6n-3	ND	4.0 ± 0.1	1.7 ± 0.9	ND	7.7 ± 0.2	0.4 ± 0.2
Total n-3	ND	8.1 ± 0.1	4.1 ± 1.0	ND	15.3 ± 0.4	0.9 ± 0.2

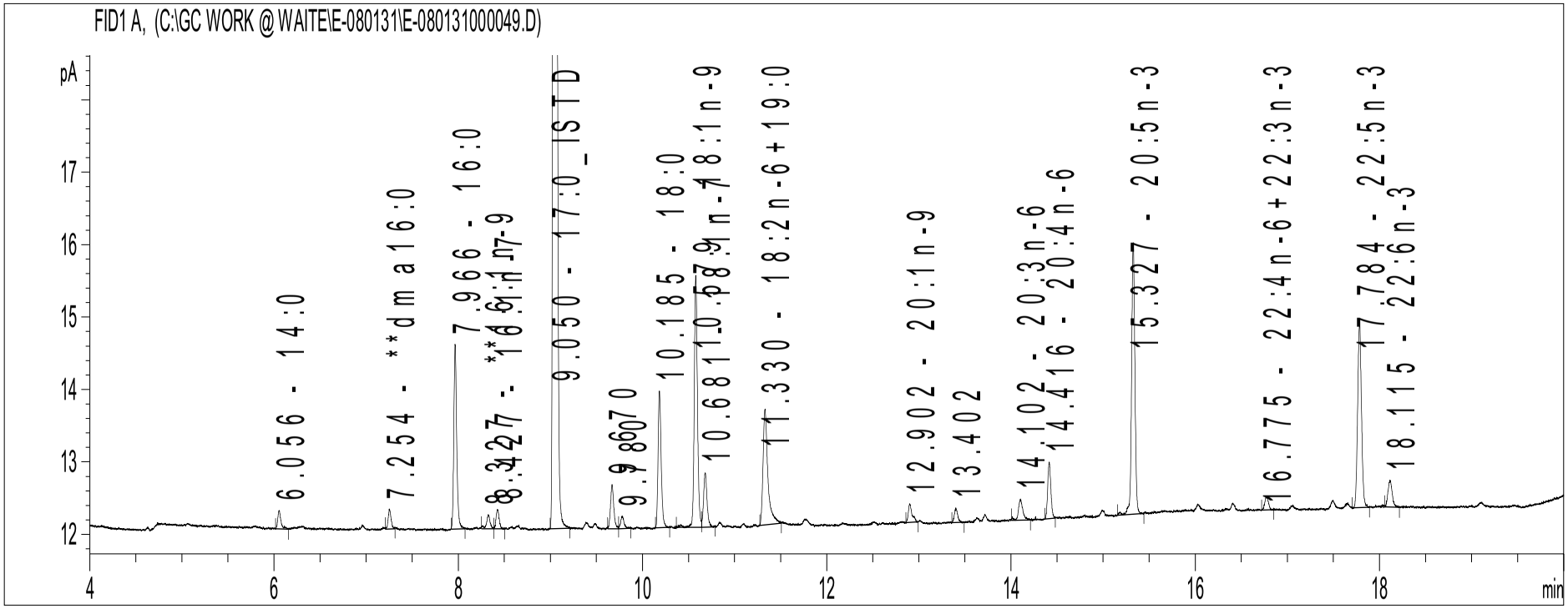

Figure 4.1 A typical gas chromatogram of fatty acid methyl esters derived from FHM cell phospholipids. The FHM cells were supplemented with 5 μM of 20:5n-3 for 24 h and then harvested for fatty acid analysis, as described in section 4.2.6.

4.2.7 qRT-PCR analysis of FHM cells

Total RNA was extracted from FHM cells covering the surface of a 25 cm^2 flask using the RiboPure^{TM} Kit (Ambion, TX, USA), according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA as outlined in section 3.2.9. qRT-PCR was carried out basically according to section 3.2.9. The housekeeping gene *β-actin* was suitable for use as an endogenous reference gene (Livak and Schmittgen 2001). The *β-actin* primers were designed based on an alignment of Chinook salmon (EF550597), rainbow trout (AF157514), SBT (EF452499.1) and fathead minnow (*Pimephales promelas*) (EU195887). *β-actin* was amplified using the β-actin F - 5'- GACCTGTATGCCAACACCGTGCT-3' and the β-actin R - 5'- ATCCAGACAGAGTATTTACGCTCAGGT-3' primers. The efficiencies of the FHM *β-actin*, *Δ6desaturase* and *elongase* primers were tested with a 5-step, 10-fold cDNA dilution series of the PCR product.

4.2.7.1 Calculation of gene transcript levels

Relative quantification was used to calculate the change in expression (fold change) of the target gene normalized to an endogenous reference gene and relative to the untreated control, according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The *C*t value represents the PCR cycle at which fluorescence above a base line signal can be first detected. The ΔCt ($Ct_{\text{gene of interest}} - Ct_{\beta\text{-actin}}$), $\Delta\Delta Ct$ ($\Delta Ct_{\text{Gene of interest treated}} - \Delta Ct_{\text{Gene of interest untreated}}$) and times-fold ($2^{-\Delta\Delta Ct}$) were calculated. The $\Delta\Delta Ct$ for the untreated control is zero, so by definition the fold change equals one (Livak and Schmittgen 2001). Replicate experiments (n=3) were performed for each gene.

4.2.8 Primary cell culture development

4.2.8.1 The media

Three media formulations were used in the primary cell culture development.

1. Leibovitz L-15 medium containing 20% (v/v) FBS and 100 IU penicillin/100 µg streptomycin per mL.
2. RPMI 1640 containing 20% (v/v) FBS and 100 IU penicillin/100 µg streptomycin per mL.
3. RPMI 1640 containing AmnioMax II complete medium and 100 IU penicillin/100 µg streptomycin per mL.

4.2.8.2 Sampling of the fish tissue

The following work was conducted by Lynette Williams (AAHL, Fish Diseases Laboratory, CSIRO Livestock Industries, Geelong, Vic, Australia) and Alexandra Korte and Melissa Gregory (Flinders University, SA, Australia).

4.2.8.2.1 Trial 1

Two YTK were harvested from the South Australian Research and Development Institute (SARDI) Aquatic Sciences facilities (West Beach, SA, Australia). Fish were killed in ice water and transported back to Flinders University for sampling. Sampling took place 2-2.5 h post-mortem. The surfaces of the fish were sterilized with 98% (v/v) ethanol before dissection in a Class II BSC. Tissue samples were taken from the heart, liver and white muscle of both fish. The samples were pooled and finely diced in Petri dishes, ensuring tissue was covered with growth medium number 3.

4.2.8.2.2 Trial 2

Two SBT (3-4 kg) were harvested from a commercial farm located near Port Lincoln, SA and tissue samples were taken from the heart, liver, red muscle and white muscle. The tissue samples were transported by air from Port Lincoln to Adelaide. Processing of the tissue took place at two time points, approximately 8 h and 14 h post-mortem. Tissue samples were taken from both fish. The samples were pooled and finely diced in Petri dishes, ensuring tissue was covered with growth medium number 3.

4.2.8.3 Methods of dissociation of the tissue

Various methods of dissociation were used in the preparation of tissue for primary cell culture development. For each of the dissociation methods the tissue samples were transferred to sterile PBSA and rinsed thoroughly. The fat was removed from the tissue. The membrane layer covering the tissue was separated from the tissue and they were both placed into separate dishes. Using a scalpel, the tissue samples were cut into approximately 1 mm^2 cubes. The finely cut tissue samples were placed into 50 mL tubes and washed three times or more with PBSA to remove any blood. Between washes, the samples were allowed to stand at room temperature.

4.2.8.3.1 Fine dissociation

Each of the finely dissected tissue types (approximately 1 mm^2 cubes) was split into three 25 cm^2 flasks, each containing one of the growth media (section 4.2.8.1), and the flasks were incubated at 25°C.

4.2.8.3.2 Mechanical dissociation

The blood-free, finely dissected tissue was placed into a Petri dish with a small volume of PBSA. A sterile syringe was used to mechanically break the tissue into smaller fragments until all pieces were the same size and evenly dispersed in the PBSA. Each of the mechanically dissociated tissue types was split into three 25 cm^2 flasks, each containing one of the growth media (section 4.2.8.1), and the flasks were incubated at 25°C.

4.2.8.3.3 Enzyme dissociation

The finely dissected tissue was treated with two methods of enzyme dissociation.

4.2.8.3.3.1 Method 1 - Cold pre-exposure

The finely cut tissue samples were incubated at 4°C for 24 h in 10 mL g^{-1} tissue of 0.25% (v/v) trypsin in RPMI 1640 medium. After 24 h, the dissociated tissue was filtered through a sterile sieve. The suspension was centrifuged at 500 *g* for 3 min. The supernatant containing the trypsin was removed and the pellet was resuspended in 10 mL of medium number 1 (section 4.2.8.1). Two mL of resuspended cells were used to seed 75 cm^2 flasks, while 1 mL of cells was used to seed 25 cm^2 flasks.

4.2.8.3.3.2 Method 2 - Warm treatment

Flasks containing the finely cut tissue and 40 mL of 2.5% (v/v) trypsin were placed on a magnetic stirrer. The tissue samples were agitated at 100 rpm for 30 min at 37°C. Heart, white muscle and red muscle required a further 30 min of agitation to dissociate the tissues. The disaggregated cells were collected by centrifugation at 500 *g* for 3 min at 25°C. Each pellet was resuspended in 10 mL of medium number 1 (section 4.2.8.1) and incubated at 4°C for 24 h. After 24 h, the dissociated tissues were filtered through a sterile sieve. The resulting cell suspensions were centrifuged at 500 *g* for 3 min. The supernatant containing the trypsin was removed and the pellet was resuspended in 10 mL of medium number 1 (section 4.2.8.1). Two mL of resuspended cells were used to seed 75 cm^2 flasks, while 1 mL of cells was used to seed 25 cm^2 flasks.

4.2.8.4 Primary cell culture maintenance

Cultures were routinely (once per week) inspected for any signs of contamination including bacterial or fungal growth. Observations on cell attachment, migration from cell clusters and growth were recorded. Each week after the primary cultures were seeded the growth medium was replaced if necessary. Cells were passaged when flasks were approximately 70% confluent.

4.2.9 Bioinformatics analysis

Phylogenetic analysis was performed as outlined in section 3.2.2. The insect phylum was chosen to represent the basal phylum.

4.2.10 Statistical analysis

When required, one-way ANOVA was performed according to section 2.2.11.

4.3 Results

4.3.1 Identification of fish cell lines

The aim of these experiments was to confirm the species of origin of the various cell lines developed in our laboratory or obtained from the AAHL Fish Diseases Laboratory (CSIRO Livestock Industries, Geelong, Vic, Australia). The SBT gonad cell line (SBT-G), developed by Alexandra Korte in our laboratory and the Epithelioma papulosum cyprinid (EPC) cell line from carp, obtained from the AAHL Fish Diseases Laboratory, were routinely used in our laboratory. The mtDNA D-loop, *cox*1 and internal transcribed spacer of 18S rRNA (ITS1) sequences from the SBT-G and EPC cell lines showed that these were both FHM cell lines (This work was conducted by Josephine Nocillado). Furthermore, cell lines from the S*almonidae* Chinook salmon and rainbow trout, the *Cyprinidae* carp, the *Latidae* barramundi and the *Centrarchidae* bluegill were obtained from the AAHL Fish Diseases Laboratory. These fish represent species from fresh, anadromous and marine environments, each with different dietary requirements. Phylogenetic analysis confirmed that carp, barramundi and bluegill do not form any distinct clades with the Chinook salmon and rainbow trout clade (Figure 4.2). However, there also appears to be no clear relationship between the carp, barramundi and bluegill sequences (Figure 4.2). The *cox*1 gene identification of these cell lines was performed. The 717 bp *cox*1 products amplified from each cell line (Figure 4.3) were sequenced. Sequencing revealed the CHSE-214 cell line *cox*1 was 100% identical to chinook salmon *cox*1 (EF609421). The BF-2 cell line *cox*1 was also 100% identical to chinook salmon *cox*1 (EF609421), yet only 38% identical to bluegill *cox*1 (EU524740). The KF-1 cell line *cox*1 was 95% identical to the snakehead murrel (*Channa striata*) *cox*1 (EU342203), yet only 40% identical to carp *cox*1 (EU524556). The LCK cell line *cox*1 was 99% identical to bluegill *cox*1 (EU524740), yet only 38% identical to barramundi (EU189379). The RTG-2 cell line *cox*1 was 100% identical to rainbow trout *cox*1 (EF609420). Thus, the identity of the CHSE-214 and RTG-2 cells was confirmed whereas the BF-2, KF-1 and LCK cells were found to originate from a different species.

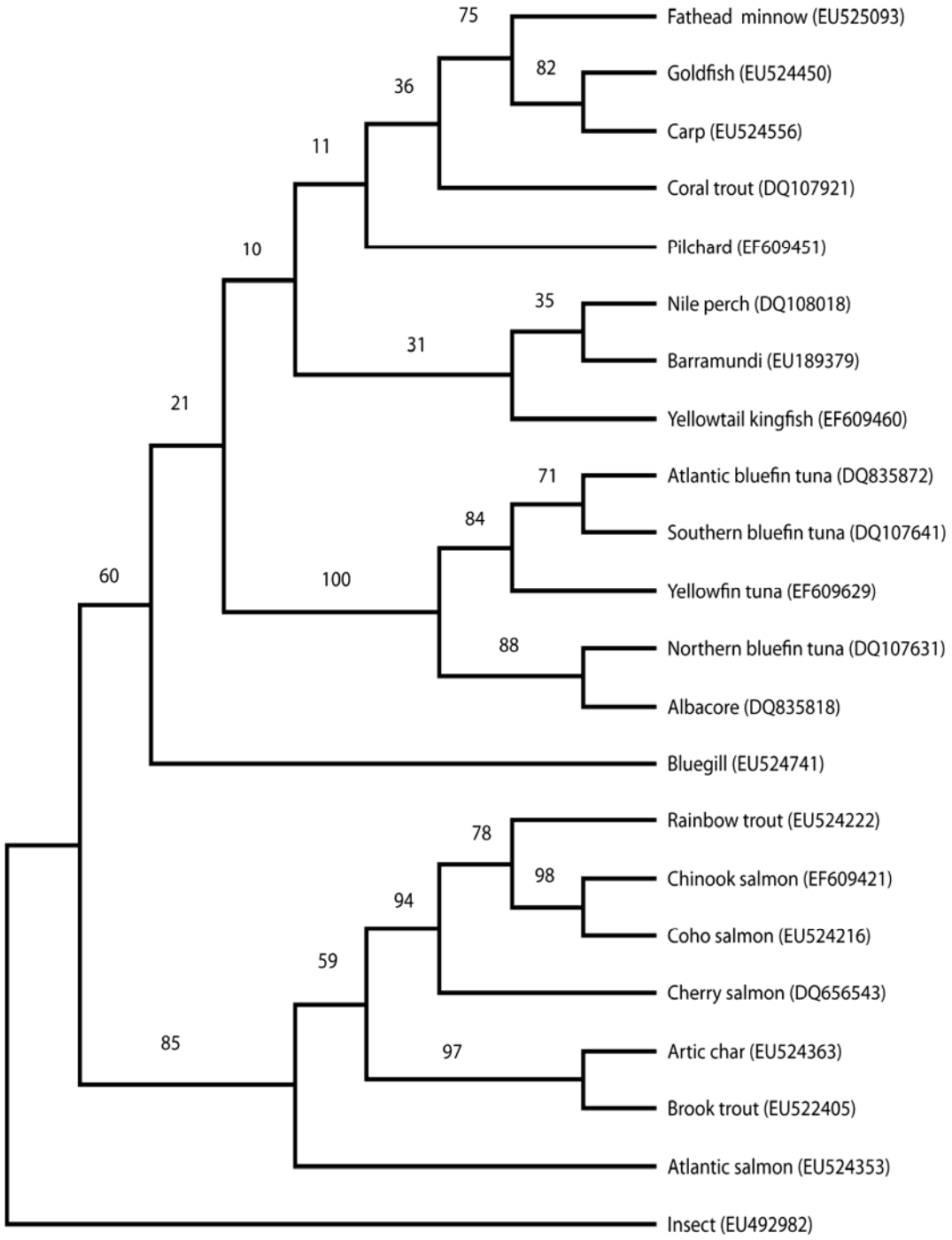

Figure 4.2 A phylogenetic tree comparing the *cox*1 nucleotide sequences from various fish species. The accession numbers are from the GenBank database. The tree was constructed using maximum parsimony analysis with ClustalX and PAUP* (Swofford 2003). The numbers on the branches represent the frequencies with which the tree topology presented was replicated after 1,000 bootstrap iterations. Insect was chosen as the out group.

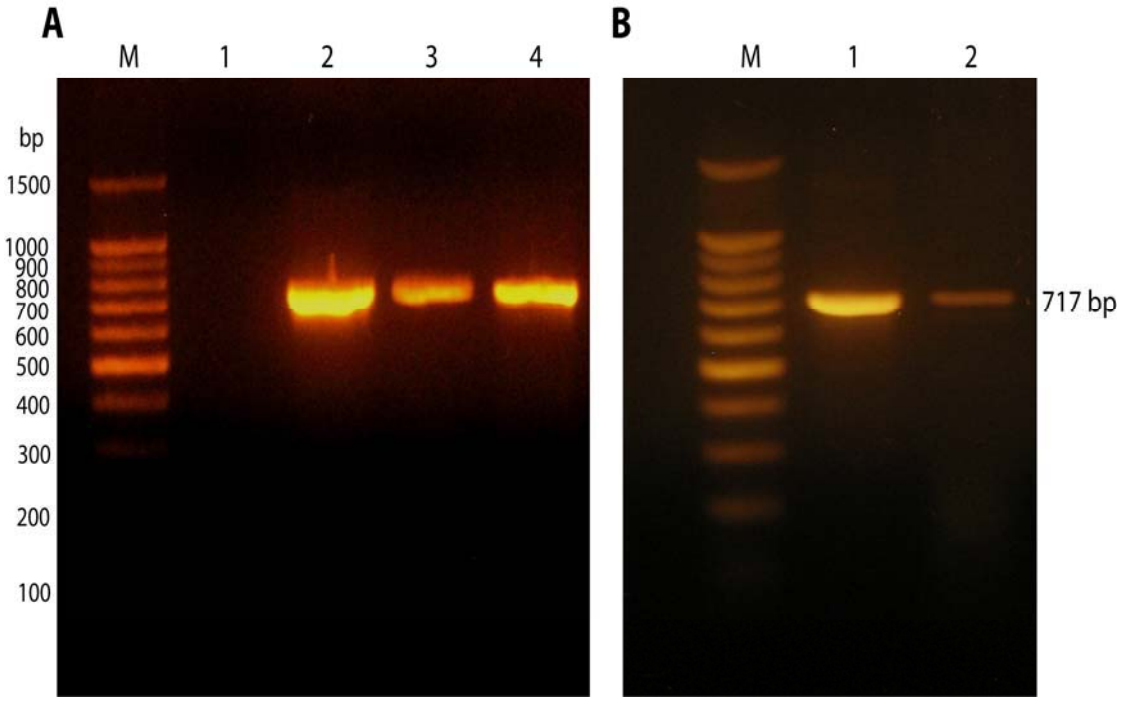

Figure 4.3 Visualisation of the *cox*1 gene amplification from the fish cell lines. The *cox*1 fragment can be seen at 717 bp. PCR was conducted as described in section 4.2.4.

Gel **A**

Lane 1 – negative control (no template)

Lane 2 - CHSE-214 cell line

Lane 3 - BF-2 cell line

Lane 4 - KF-1 cell line

Gel **B**

Lane 1 – LCK cell line

Lane 2 – RTG-2 cell line

4.3.2 Supplementing PUFA to cell lines

The FHM and CHSE-214 cell lines were chosen as model cell lines for species from freshwater and anadromous environments. The FHM and CHSE-214 cell lines were supplemented with individual PUFA to investigate the cell lines' ability to synthesize LCPUFA. The activity of the enzymes in the LCPUFA synthesis pathway was assessed by the synthesis of LCPUFA products.

4.3.2.1 The effect of storage on FHM cell phospholipids

We aimed to determine the most suitable way to store FHM cells prior to the extraction of phospholipids. The stability of the FHM cell phospholipids after different storage conditions was ranked in the order of fresh cells > cells stored at -70°C in 1 mL PSBA > cells stored at -70°C in DMSO (Figure 4.4). Although the phospholipids were most stable when the cells were fresh, this option was not always practical. The cells were stored at -70°C in 1 mL PSBA prior to extraction for all subsequent experiments.

4.3.2.2 The effect of FBS on FHM cell phospholipids

Cultured cells obtain their fatty acids from the FBS in the growth medium. Thus, the fatty acid composition of the FHM cells was predicted to reflect the fatty acid composition of the growth medium. The FHM cells grown in L-15 medium containing 10% (v/v) FBS showed uptake of specific fatty acids as seen by a decrease in the medium of the n-3 and n-6 PUFA, LA, AA, DPA and DHA after 24 h in culture (Figure 4.5). The most abundant n-6 PUFA in the medium was AA and the cells incorporate some AA, while a large amount remains in the medium after 24 h (Figure 4.5). After growing the FHM cells in L-15 medium containing 10% (v/v) FBS for 24 h there were no detectable levels of n-3 PUFA in the media. When less PUFA was supplied to the FHM cells by decreasing the FBS concentration in the medium from 10% to 2% (v/v), the amount of n-3 and n-6 PUFA in the cell phospholipids significantly decreased by 4-fold and 8.5-fold, respectively (Figure 4.6). The reduction in FBS concentration to 2% did not inhibit cell growth, whilst it lowered the amount of PUFA in the cell phospholipids, which allowed changes in the

cell phospholipid PUFA content to be seen when individual PUFA were supplemented to the cells. These data for the FHM cell phospholipids are summarised in Table 4.2, along with the profile of CHSE-214 cell phospholipids after growth in L-15 medium containing 2% FBS. Here it can be seen that the most abundant series of fatty acids in the FHM and CHSE-214 cell phospholipids were the monounsaturated fatty acids (MUFA), in particular 18:1n-9 (Table 4.2). The accumulation of DPA, DHA and AA in the cell phospholipids was most prominent when the FBS concentration was increased from 0-20% with a constant PUFA supplementation of 2 μM ALA (Figure 4.7).

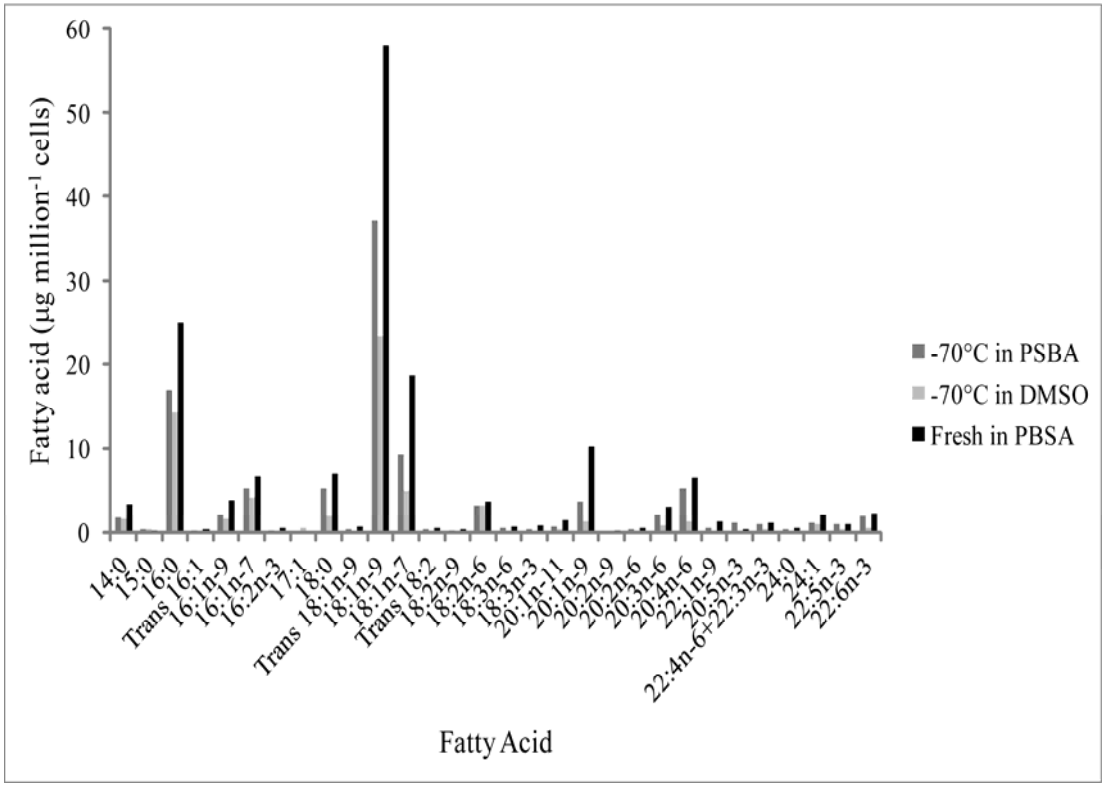

Figure 4.4 The effect of storage on the FHM cell phospholipids. The FHM cells were grown in L-15 medium containing 10% (v/v) FBS. The phospholipids were extracted for analysis using GC from the fresh cells in PBSA or after the cells were stored at -70°C in PSBA or DMSO, as outlined in section 4.2.6.1.

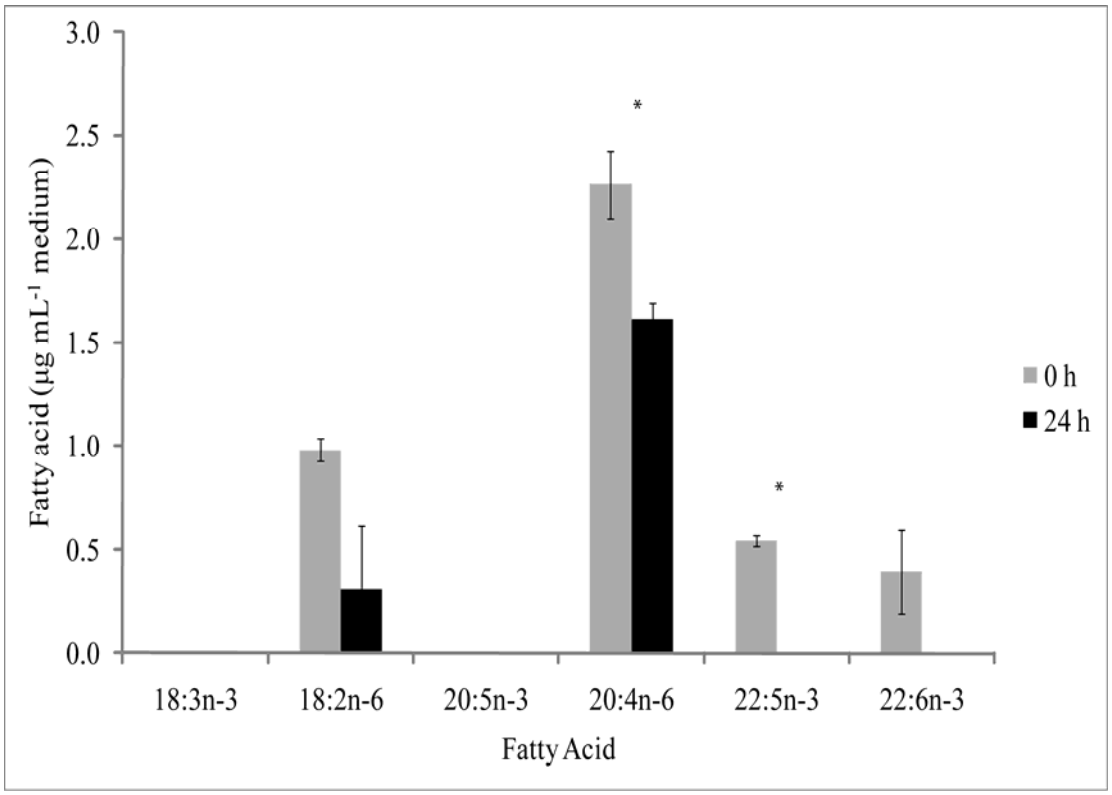

Figure 4.5 The n-3 and n-6 fatty acids found in L-15 medium containing 10% (v/v) FBS prior to incubation (0 h) and after incubation (24 h) with FHM cells. The fatty acids were extracted from the medium for analysis using GC as outlined in section 4.2.6.2. The data are the mean ± SE (n=3). An asterisk indicates a significant difference between the zero time and the 24 h samples.

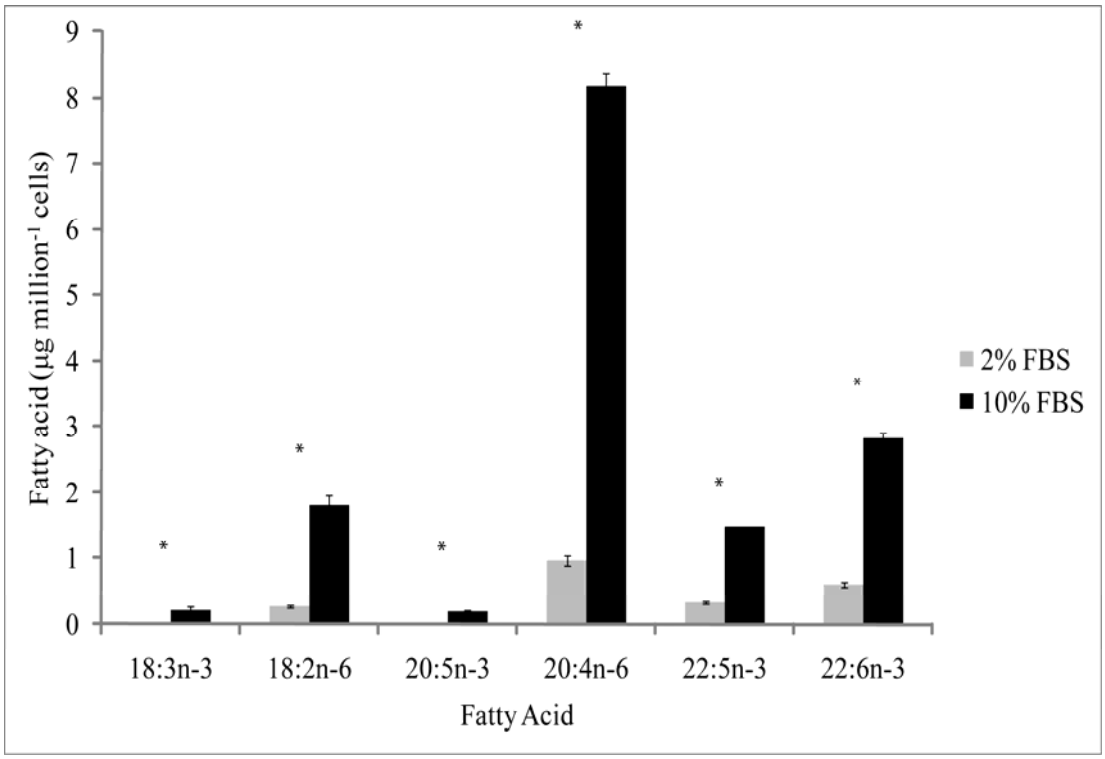

Figure 4.6 The n-3 and n-6 fatty acids found in the FHM cells after the cells were grown in L-15 medium containing 2% or 10% FBS. The phospholipids were extracted from the FHM cells after 24 h for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). An asterisk indicates a significant difference between the 2% and 10% (v/v) FBS samples.

Table 4.2 The fatty acid composition of FHM cells grown in L-15 medium containing 2% or 10% (v/v) FBS and CHSE-214 cells grown in L-15 medium containing 2% (v/v) FBS. The phospholipids were extracted from the cells after 24 h for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). ND, not detected.

Fatty Acid	FHM 10% (v/v) FBS	FHM 2% (v/v) FBS (μg million^{-1} cells)	CHSE-214 2% (v/v) FBS
16:0	17.7 ± 2.2	2.6 ± 0.2	1.1 ± 0.2
18:0	8.8 ± 0.3	2.3 ± 0.1	0.6 ± 0.1
Total Sats	31.7 ± 3.1	5.2 ± 0.3	2.3 ± 0.4
16:1n-7	2.3 ± 0.7	ND	0.3 ± 0.1
18:1n-9	24.3 ± 4.5	4.6 ± 0.3	2.0 ± 0.5
18:1n-7	8.0 ± 1.2	0.8 ± 0.1	0.1 ± 0.0
20:1n-9	2.8 ± 0.5	0.5 ± 0.1	ND
Total MUFA	42.7 ± 7.6	6.0 ± 0.5	2.6 ± 0.7
20:2n-9	2.9 ± 0.5	ND	ND
20:3n-9	1.6 ± 0.3	ND	ND
Total n-9	36.6 ± 6.3	5.2 ± 0.4	2.4 ± 0.6
Total n-7	10.2 ± 1.9	0.8 ± 0.1	0.4 ± 0.1
18:2n-6	1.8 ± 0.2	0.3 ± 0.0	0.2 ± 0.0
18:3n-6	0.4 ± 0.1	0.1 ± 0.1	ND
20:2n-6	0.4 ± 0.0	ND	ND
20:4n-6	8.2 ± 0.2	1.0 ± 0.1	0.2 ± 0.0
22:4n-6	1.3 ± 0.0	0.1 ± 0.0	ND
Total n-6	14.9 ± 0.5	1.8 ± 0.1	0.5 ± 0.1
18:3n-3	0.2 ± 0.1	ND	ND
20:5n-3	0.2 ± 0.0	ND	ND
22:5n-3	1.5 ± 0.0	0.3 ± 0.0	0.0 ± 0.0
22:6n-3	2.8 ± 0.1	0.6 ± 0.0	0.4 ± 0.1
Total n-3	4.8 ± 0.2	1.2 ± 0.0	0.5 ± 0.1
Total Fat (μg mL^{-1})	99.5 ± 12.3	14.1 ± 1.0	6.1 ± 1.0

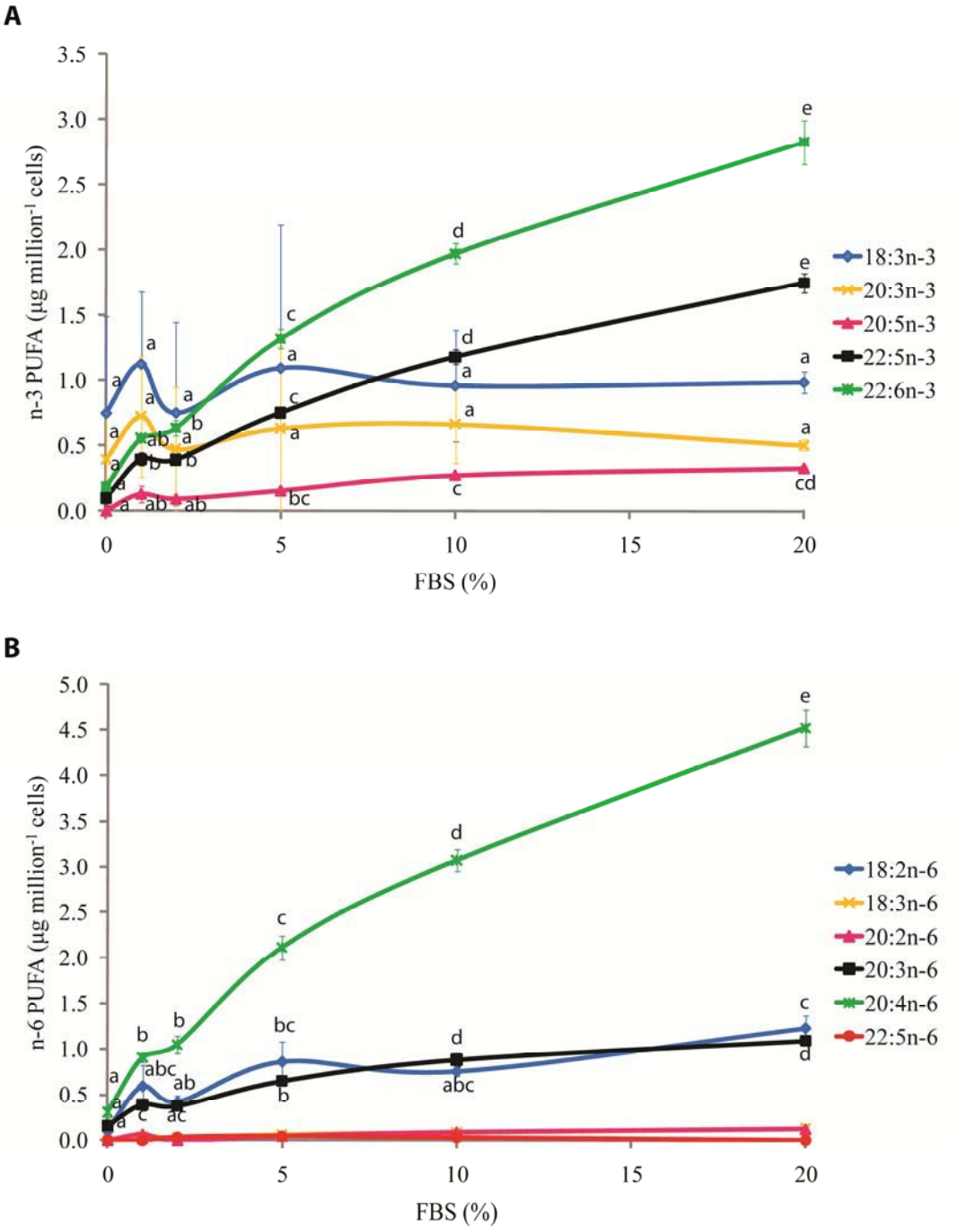

Figure 4.7 The effect of FBS in the L-15 medium on the n-3 PUFA (A**) and n-6 PUFA (**B**) in the FHM cell phospholipids.** The FHM cells were grown in L-15 medium containing various percentages of FBS and supplemented with 2 µM of ALA, as described in section 4.2.5. The phospholipids were extracted for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). Values with different symbols are significantly different from each other within each fatty acid group. There was no significant difference within each fatty acid group when there are no symbols.

4.3.2.3 Accumulation of n-3 fatty acids in FHM cells

The aim of this experiment was to investigate LCPUFA synthesis in the FHM cells following supplementation with individual n-3 fatty acids. The FHM cells were supplemented with the n-3 fatty acids ALA, EPA and DHA.

The accumulation of ALA in cell phospholipids was concentration dependent, increasing from 0 μg to 6.3 μg over the concentration range 1-20 μM (Figure 4.8A). Following supplementation with ALA we expected to see an accumulation of one or more of the products in the LCPUFA synthesis pathway, EPA, DPA and DHA. Contrary to our expectations, the level of EPA, DPA and DHA in cell phospholipids remained relatively unchanged across the range of ALA concentrations supplied to the cells (Figure 4.8). However, the accumulation of 20:3n-3 was dependent on the concentration of ALA supplied to the cells (Figure 4.8). The level of 20:3n-3 in cell phospholipids increased 2.8-fold over the ALA concentration range 1-10 μM and then began to plateau at ALA concentrations greater than 10 μM (Figure 4.8A). This suggests that the elongation of ALA to 20:3n-3 occurred through an alternate LCPUFA synthesis pathway.

The accumulation of EPA in cell phospholipids produced a bell-shaped curve as the concentration of EPA increased. The accumulation was concentration dependent, increasing from 0.1 μg to 3.8 μg over the concentration range 1-10 μM, followed by a 1.4 μg decrease over the concentration range 10-20 μM (Figure 4.9A). Interestingly, the accumulation of EPA after supplementation with 5 μM or 20 μM was not significantly different (Figure 4.9). The accumulation of DPA, presumably through elongation of EPA, followed the same trend described for EPA accumulation. The accumulation of DHA requires DPA to be further elongated by Elovl2, desaturated by Δ6desaturase and undergo β-oxidation. The level of DHA in the cell phospholipids was unchanged over an EPA supplementation range of 1-5 μM indicating that these enzymes were not active (Figure 4.9B).

DHA accumulation from supplementation with ALA or EPA was not seen. To assess if DHA accumulation after supplementation with ALA and EPA was due to a limitation in DHA incorporation into the cell phospholipids, preformed DHA was

supplemented to the medium. DHA was readily incorporated into the cell phospholipids, increasing from 0.6 μg to 3.7 μg over the concentration range 1-10 μM (Figure 4.10). The accumulation of DHA in the cell phospholipids was linear when cells were supplied up to 2 μM DHA, followed by a plateau at concentrations greater than 2 μM (Figure 4.10). While DHA accumulation was linear (< 2 μM), the level of DPA and EPA did not significantly change. When DHA accumulation in the cell phospholipids began to plateau, the level of EPA significantly increased suggesting retroconversion of DHA to EPA (Figure 4.10).

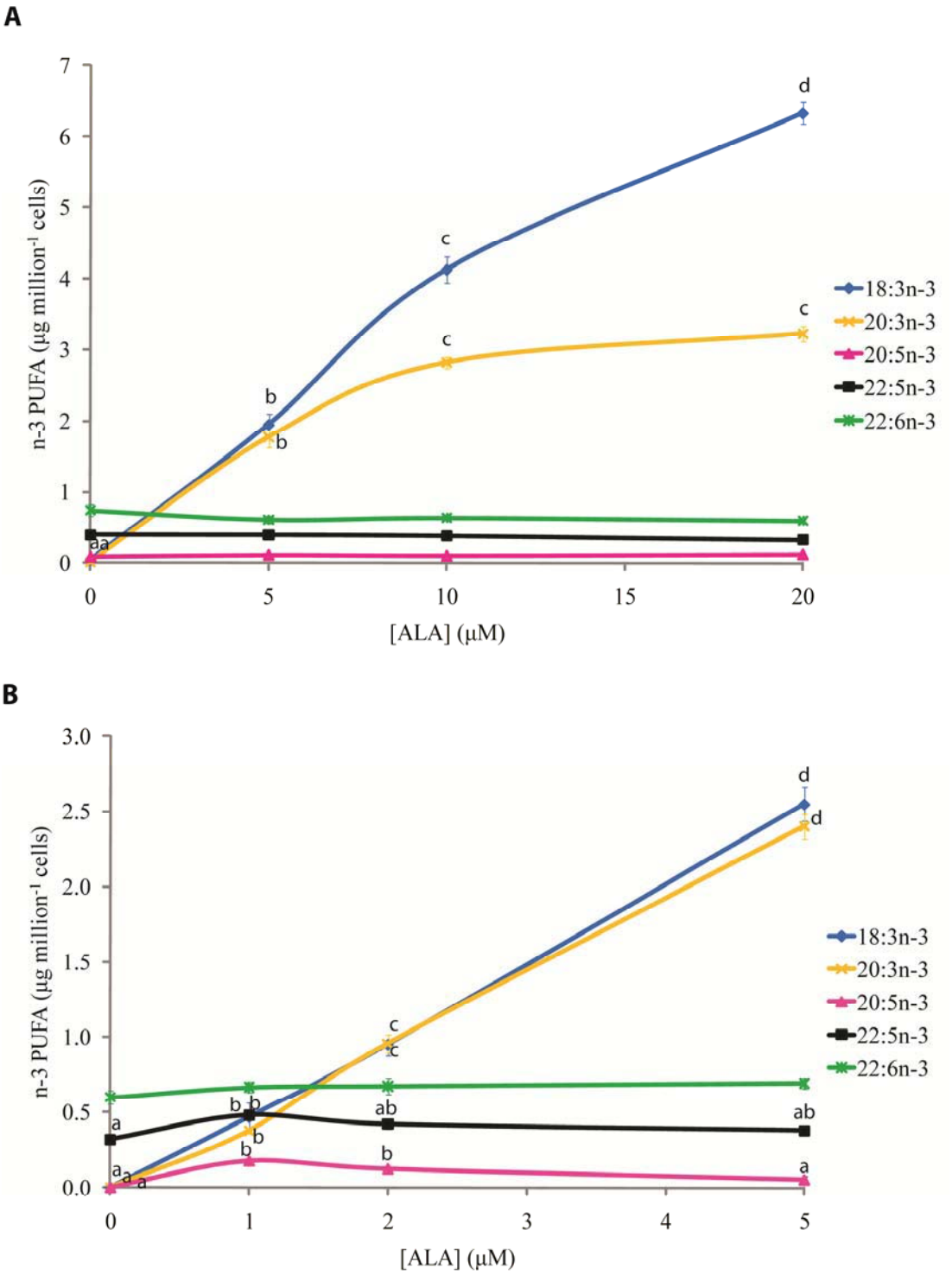

Figure 4.8 The n-3 PUFA in FHM cell phospholipids after cell supplemention with 5-20 µM (A**) or 1-5 µM (**B**) of ALA.** The FHM cells were grown as described in section 4.2.5. The phospholipids were extracted for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). Values with different symbols are significantly different from each other within each fatty acid group. There was no significant difference within each fatty acid group when there are no symbols.

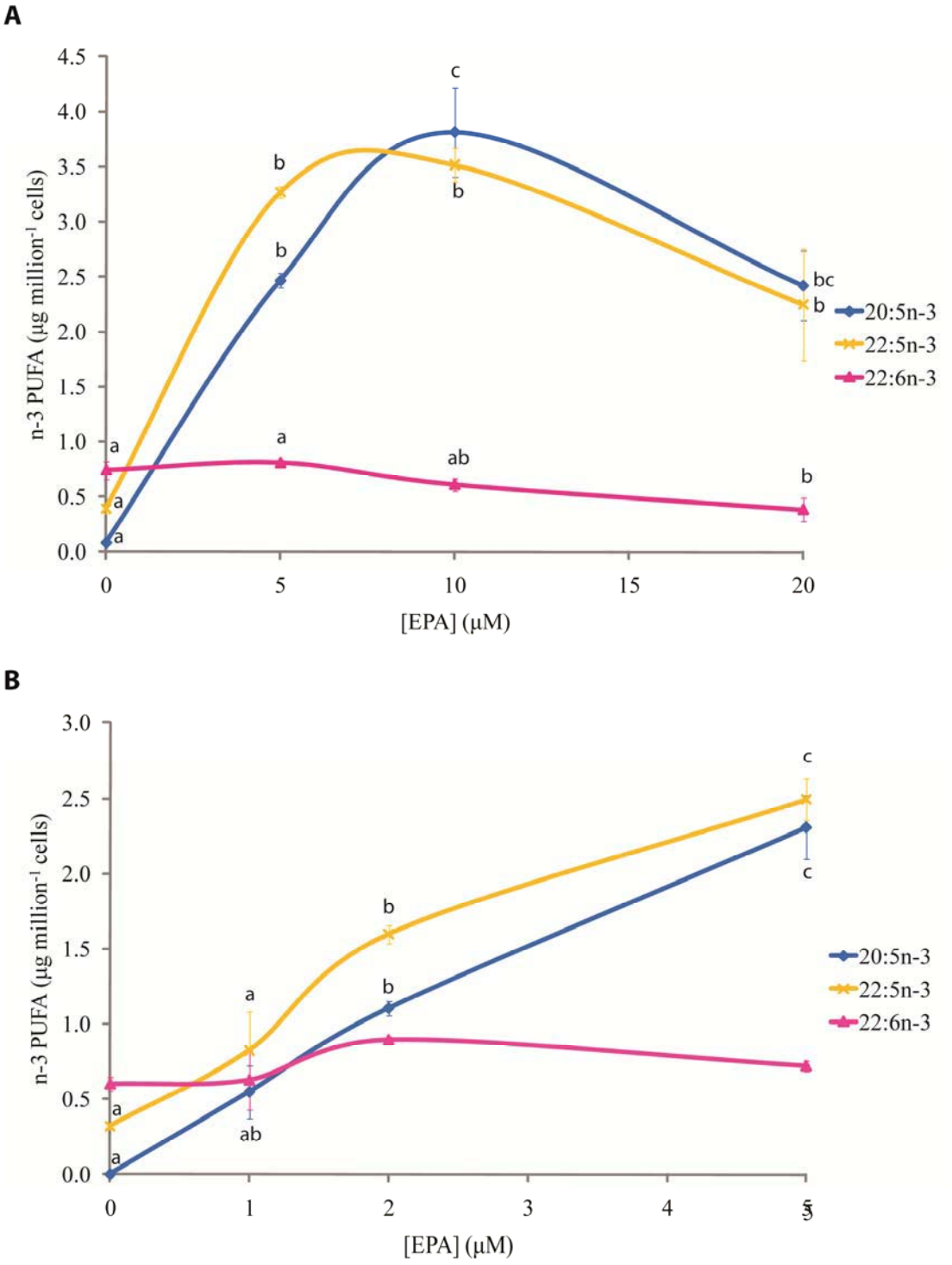

Figure 4.9 The n-3 PUFA in FHM cell phospholipids after cell supplemention with 5-20 µM (A) **or 1-5 µM** (B) **of EPA.** The FHM cells were grown as described in section 4.2.5. The phospholipids were extracted for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). Values with different symbols are significantly different from each other within each fatty acid group. There was no significant difference within each fatty acid group when there are no symbols.

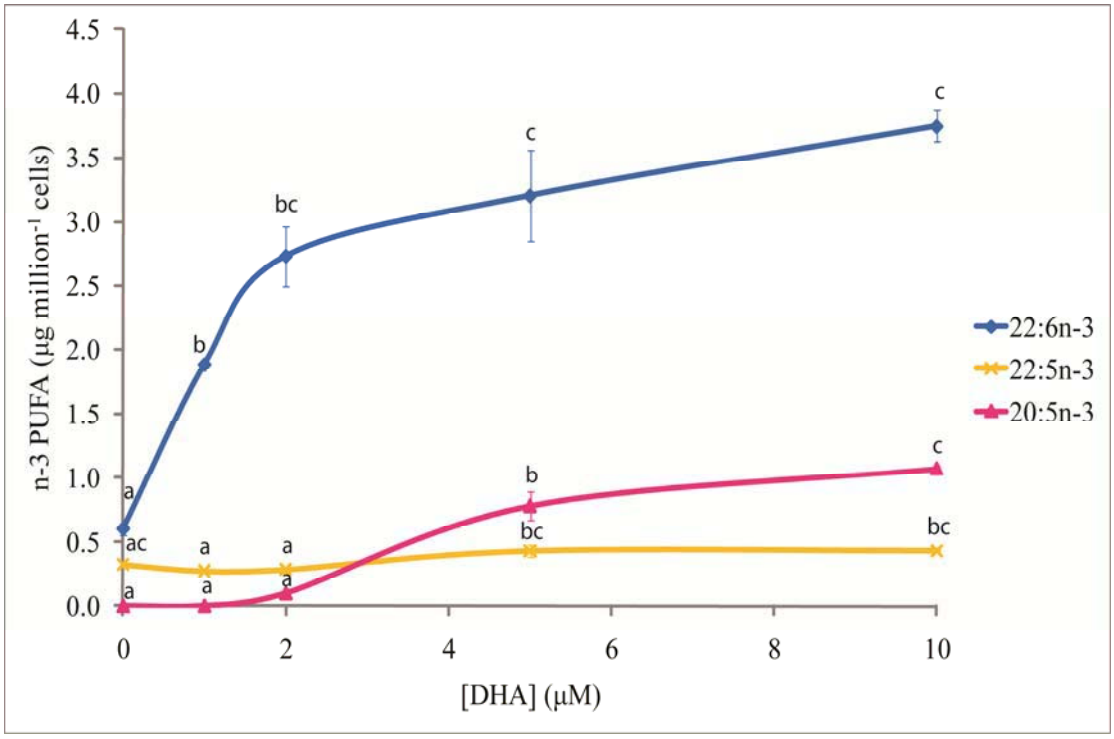

Figure 4.10 The n-3 PUFA in FHM cell phospholipids after cell supplemention with 1-10 µM of DHA. The FHM cells were grown as described in section 4.2.5. The phospholipids were extracted for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). Values with different symbols are significantly different from each other within each fatty acid group.

4.3.2.4 Accumulation of n-6 fatty acids in FHM cells

The aim of this experiment was to investigate LCPUFA synthesis in the FHM cells following supplementation with individual n-6 fatty acids. The FHM cells were supplemented with the n-6 fatty acids LA and AA.

The accumulation of LA in cell phospholipids was concentration dependent, increasing from 1.5 μg to 9.5 μg over the concentration range 1-20 μM (Figure 4.11A). The Δ6desaturation product of LA, 18:3n-6, did not accumulate in significant amounts. Although 18:3n-6 did not accumulate in the cells, we could detect 20:3n-6 after supplementation with 1-5 μM of LA (Figure 4.11). There was no Δ5desaturase activity seen as AA levels remained constant, despite increasing the concentration of supplemented LA. Using the LA supplementation range of 1-5 μM, we revealed that 20:2n-6 also significantly increased. Presumably, the elongation of LA to 20:2n-6 used an alternate LCPUFA synthesis pathway.

We have previously shown that AA was not accumulated after LA supplementation. To assess if AA accumulation after supplementation with LA was due to a limitation in AA incorporation into the cell phospholipids, preformed AA was supplemented to the medium. Initially it appeared that AA was not incorporated into the cell phospholipids over the concentration range 1-20 μM (Figure 4.12A). Also there was no accumulation of any products of AA. The amount of AA was lower in the cells supplemented with AA than control cells which were not receiving any PUFA supplementation. As performed with the other supplemented PUFA, the AA supplementation range of 1-5 μM was investigated. The amount of not only AA, but also the elongation product 22:4n-6, in the cell phospholipids were significantly increased by 2.6 μg and 1.8 μg, respectively (Figure 4.12B). The amounts of each of these PUFA in the cell phospholipid were dramatically higher in the 1-5 μM treatment than the 1-20 μM treatment.

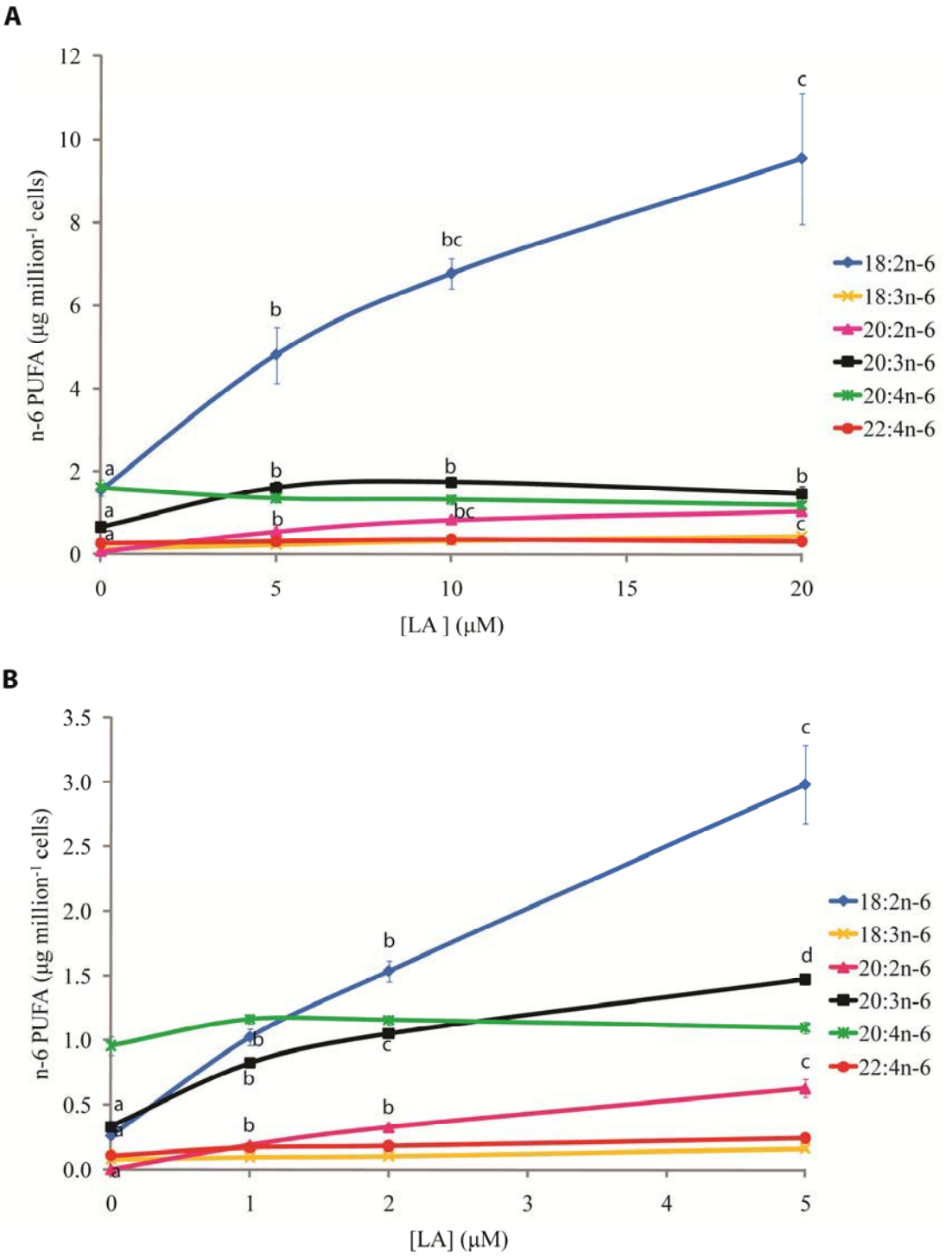

Figure 4.11 The n-6 PUFA in FHM cell phospholipids after cell supplemention with 5-20 µM (A) or 1-5 µM (B) of LA. The FHM cells were grown as described in section 4.2.5. The phospholipids were extracted for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). Values with different symbols are significantly different from each other within each fatty acid group. There was no significant difference within each fatty acid group when there are no symbols.

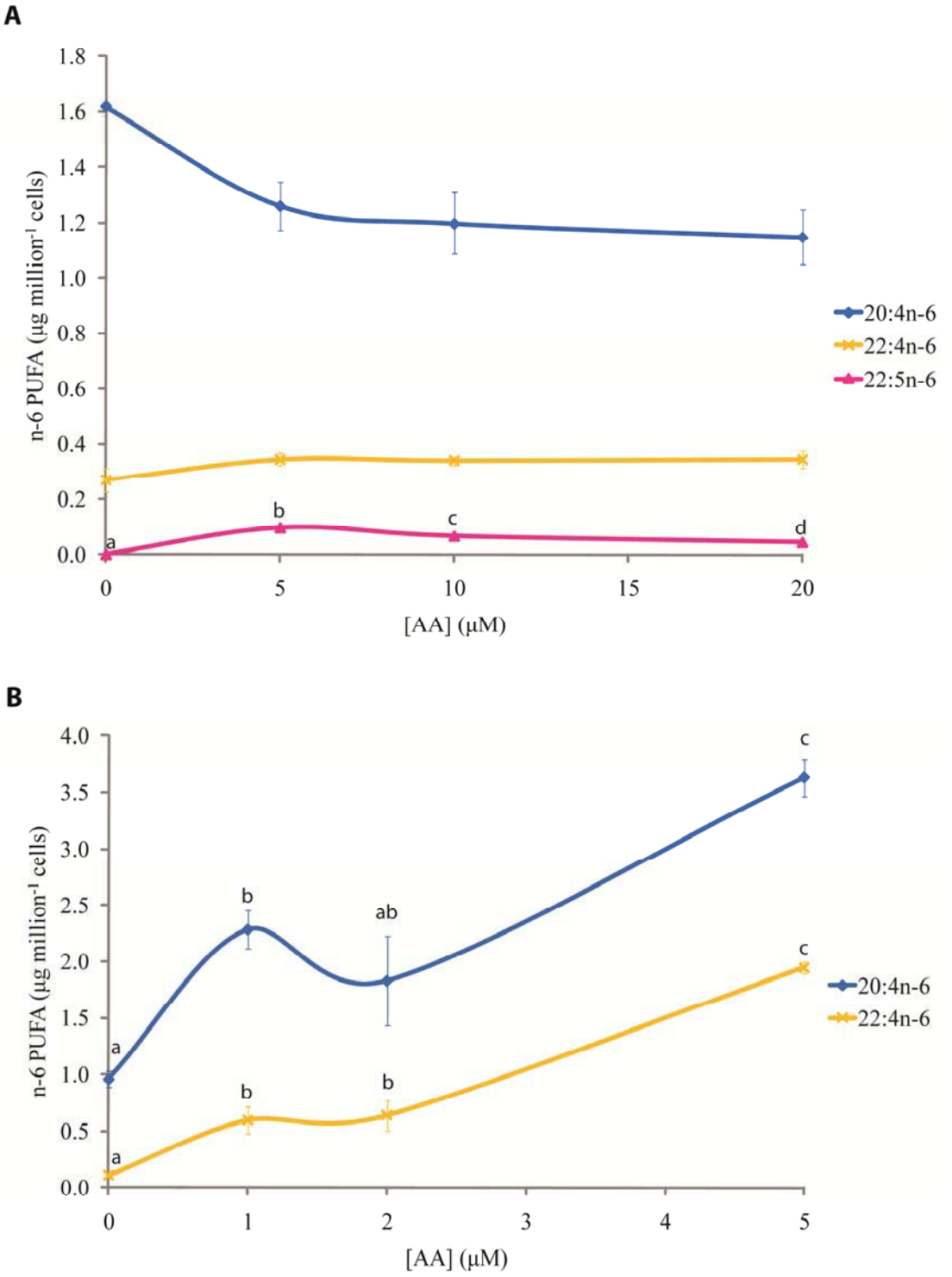

Figure 4.12 The n-6 PUFA in FHM cell phospholipids after cell supplemention with 5-20 µM (A) or 1-5 µM (B) of AA. The FHM cells were grown as described in section 4.2.5. The phospholipids were extracted for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). Values with different symbols are significantly different from each other within each fatty acid group. There was no significant difference within each fatty acid group when there are no symbols.

4.3.2.5 Accumulation of n-3 fatty acids in CHSE-214 cells

The aim of this experiment was to investigate LCPUFA synthesis in the CHSE-214 cells following supplementation with individual n-3 fatty acids. The CHSE-214 cells were supplemented with the n-3 fatty acids ALA, EPA, DPA and DHA.

The accumulation of ALA in cell phospholipids increased from 0-0.2 μg over the concentration range 0-5 μM (Figure 4.13). Following supplementation with ALA we expected to see an accumulation of one or more of the products in the accepted LCPUFA synthesis pathway. However, the level of n-3 PUFA products remained unchanged across the range of ALA concentrations supplied to the cells (Figure 4.13).

The accumulation of EPA in cell phospholipids was concentration dependent, increasing from 0-0.5 μg over the concentration range 0-5 μM (Figure 4.14). Supplementation with 5 μM of EPA increased DPA and DHA accumulation significantly (Figure 4.14). Apparently, the LCPUFA synthesis enzymes Elovl5, Elovl2 and Δ6desaturase converted the 5 μM of supplemented EPA through to 24:6n-3, followed by β-oxidation to DHA.

We have previously shown that EPA supplementation resulted in DPA accumulation followed by accumulation of the subsequent end-product of the LCPUFA synthesis pathway, DHA. When the CHSE-214 cells were supplemented with DPA the level of DPA in the cell phospholipids increased from 0-0.5 μg following supplementation with 1 μM DPA, followed by a plateau between 1-5 μM (Figure 4.15). The accumulation of DHA followed a similar trend to DPA (Figure 4.15). The biggest increase in DHA accumulation occurred after 1 μM DPA supplementation, followed by a plateau (Figure 4.15).

DHA accumulation from supplementation with 5 μM of EPA or 1 μM of DPA was seen. DHA was shown to be readily incorporated in the cell phospholipids, increasing from 0-0.8 μg over the concentration range 0-5 μM (Figure 4.16). DHA accumulation was linear when supplemented with 1 μM (Figure 4.16). The significant rise in DPA after 1 μM DHA supplementation may have been due to

retroconversion of DHA to DPA (Figure 4.16). There was no significant retroconversion of DHA to EPA (Figure 4.16).

The accumulation of PUFA in the CHSE-214 cell phospholipids was lower than the accumulation in the FHM cell phospholipids. For example, over the EPA supplementation range 1-5 μM, the CHSE-214 cells accumulated 0.5 μg of EPA compared to the FHM cells which accumulated 2.2 μg of EPA.

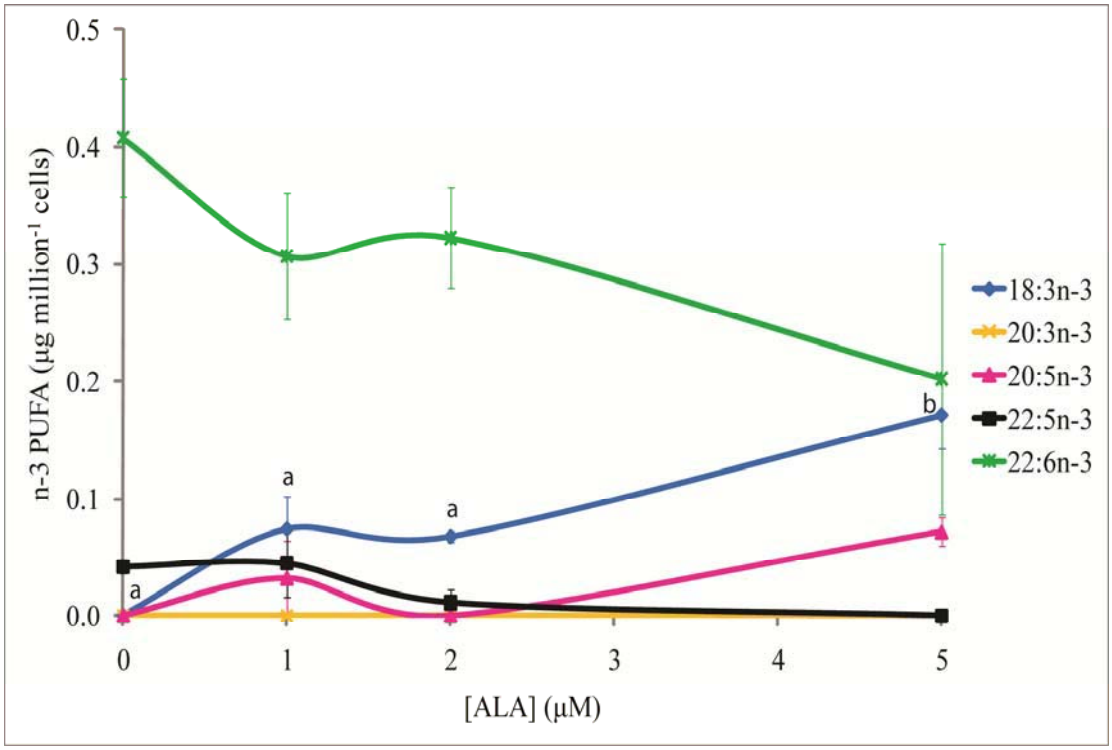

Figure 4.13 The n-3 PUFA in CHSE-214 cell phospholipids after cell supplemention with 1-5 µM of ALA. The CHSE-214 cells were grown as described in section 4.2.5. The phospholipids were extracted for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). Values with different symbols are significantly different from each other within each fatty acid group. There was no significant difference within each fatty acid group when there are no symbols.

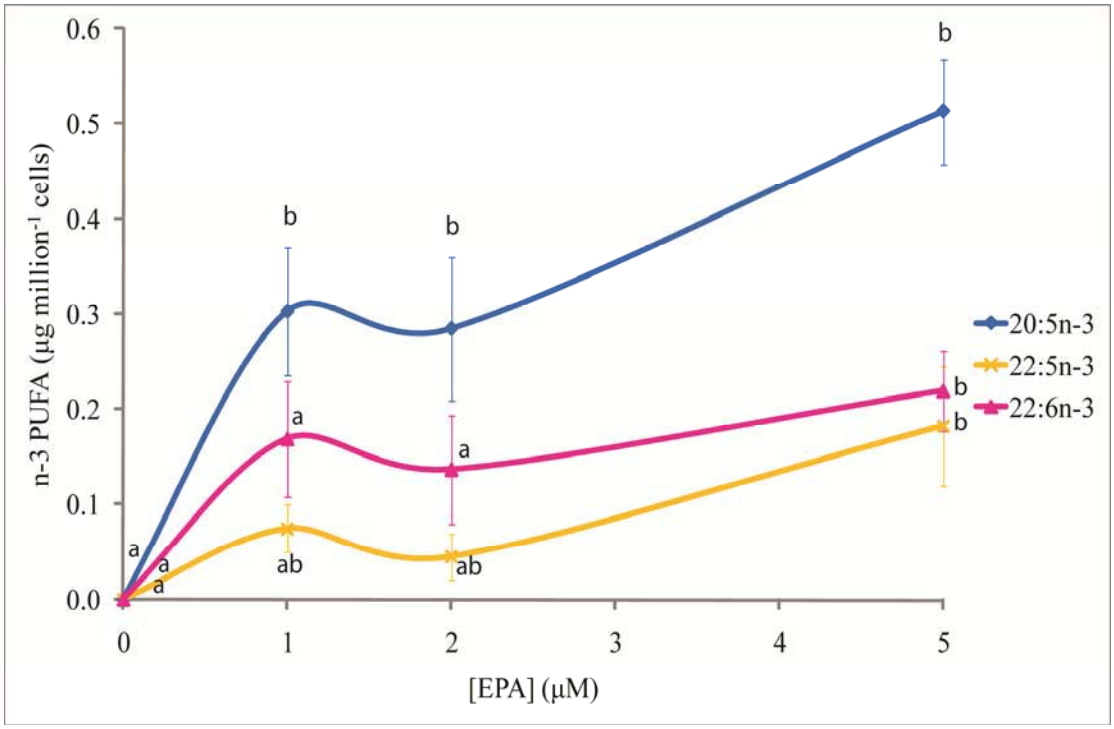

Figure 4.14 The n-3 PUFA in CHSE-214 cell phospholipids after cell supplemention with 1-5 µM of EPA. The CHSE-214 cells were grown as described in section 4.2.5. The phospholipids were extracted for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). Values with different symbols are significantly different from each other within each fatty acid group.

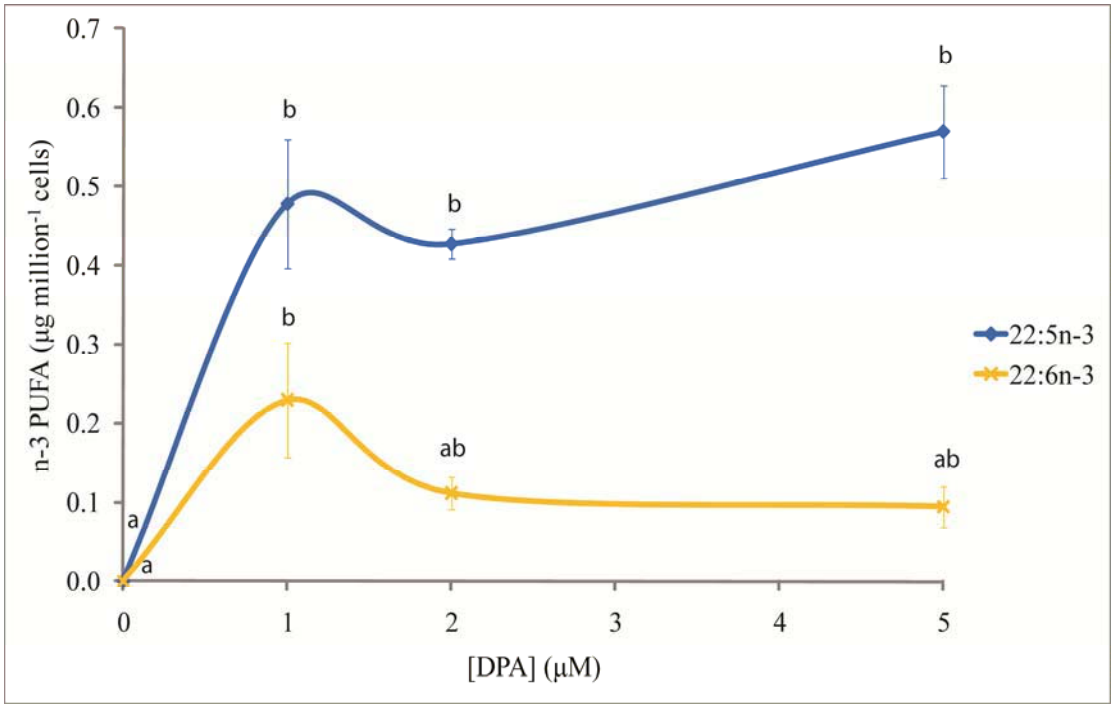

Figure 4.15 The n-3 PUFA in CHSE-214 cell phospholipids after cell supplemention with 1-5 µM of DPA. The CHSE-214 cells were grown as described in section 4.2.5. The phospholipids were extracted for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). Values with different symbols are significantly different from each other within each fatty acid group.

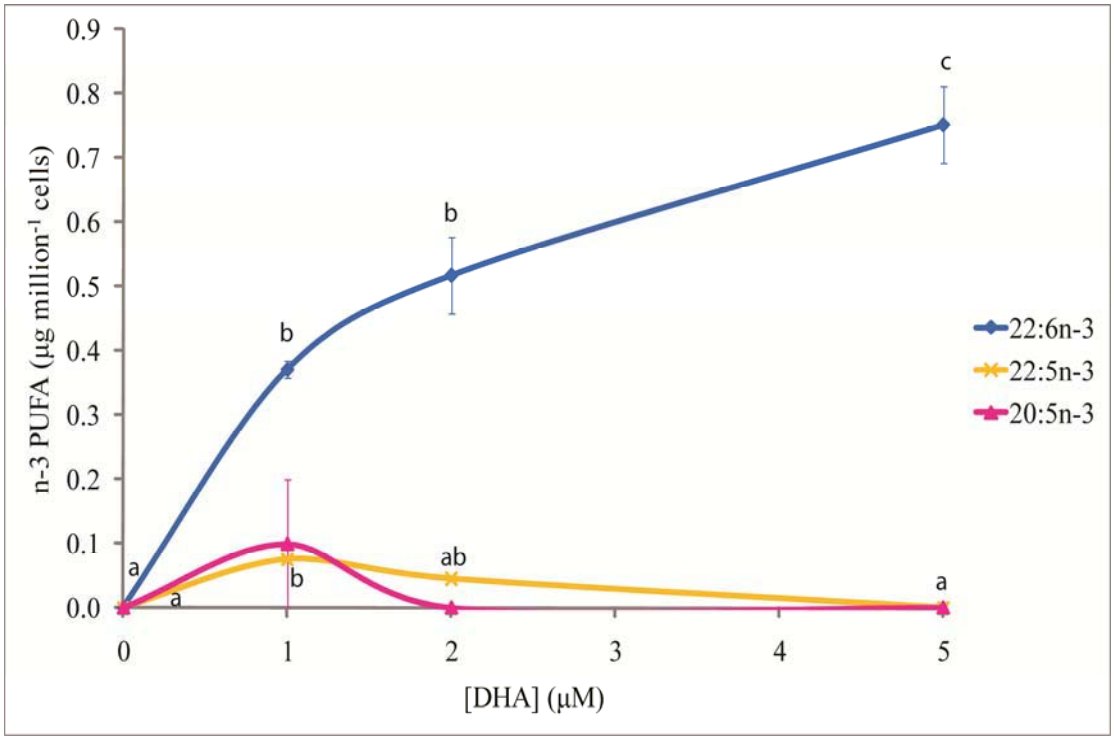

Figure 4.16 The n-3 PUFA in CHSE-214 cell phospholipids after cell supplemention with 1-5 µM of DHA. The CHSE-214 cells were grown as described in section 4.2.5. The phospholipids were extracted for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). Values with different symbols are significantly different from each other within each fatty acid group. There was no significant difference within each fatty acid group when there are no symbols.

4.3.2.6 Accumulation of n-6 fatty acids in CHSE-214 cells

The aim of this experiment was to investigate LCPUFA synthesis in the CHSE-214 cells following supplementation with individual n-6 fatty acids. The CHSE-214 cells were supplemented with the n-6 fatty acids LA and AA.

The accumulation of LA in cell phospholipids significantly increased over the concentration range 0-5 μM (Figure 4.17). The Δ6desaturase product of LA, 18:3n-6, and subsequent elongation product, 20:3n-6, were found to increase with supplementation of 2-5 μM of LA (Figure 4.17). However, 20:3n-6 was not further desaturated to AA and a reduction in the amount of AA in the cell phospholipids was observed (Figure 4.17).

AA was not accumulated in the cell phospholipids after LA supplementation. However, when preformed AA was supplemented to the medium, the accumulation of AA in the cell phospholipids was concentration dependent, increasing from 0-1.2 μg over the concentration range 0-5 μM (Figure 4.18). The level of 22:4n-6 remained unchanged in the cell phospholipids when the cells were supplemented with 1-2 μM of AA, but increased significantly with 5 μM of AA supplementation (Figure 4.18).

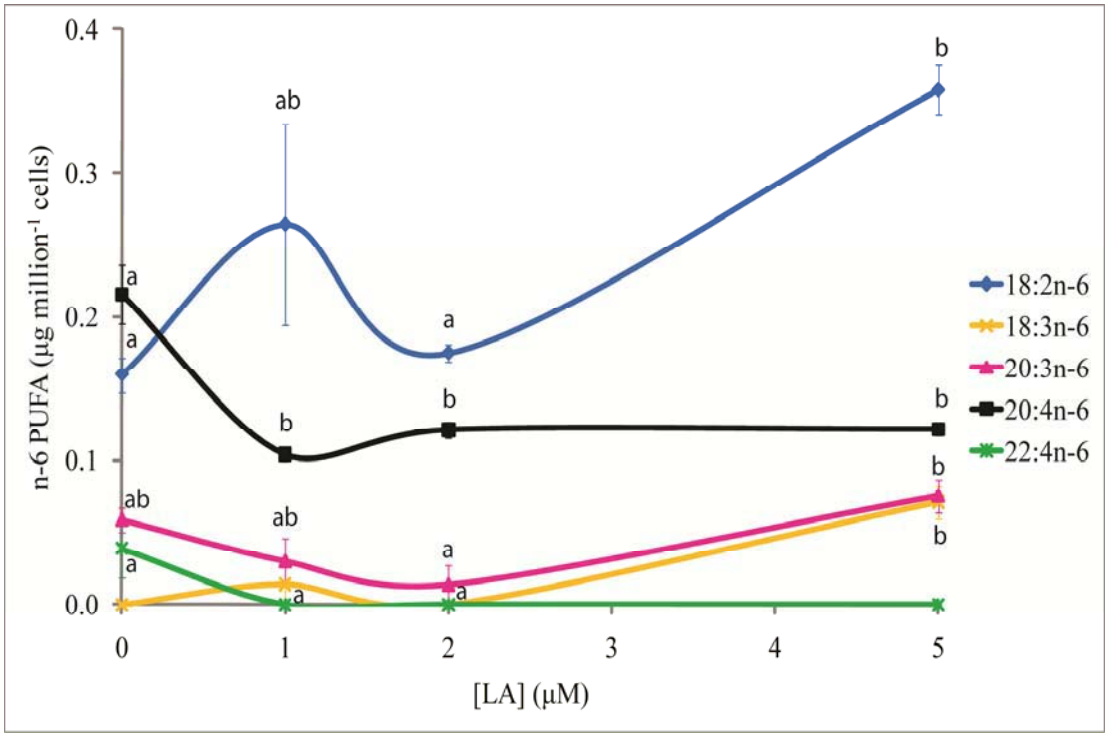

Figure 4.17 The n-6 PUFA in CHSE-214 cell phospholipids after cell supplemention with 1-5 µM of LA. The CHSE-214 cells were grown as described in section 4.2.5. The phospholipids were extracted for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). Values with different symbols are significantly different from each other within each fatty acid group. There was no significant difference within each fatty acid group when there are no symbols.

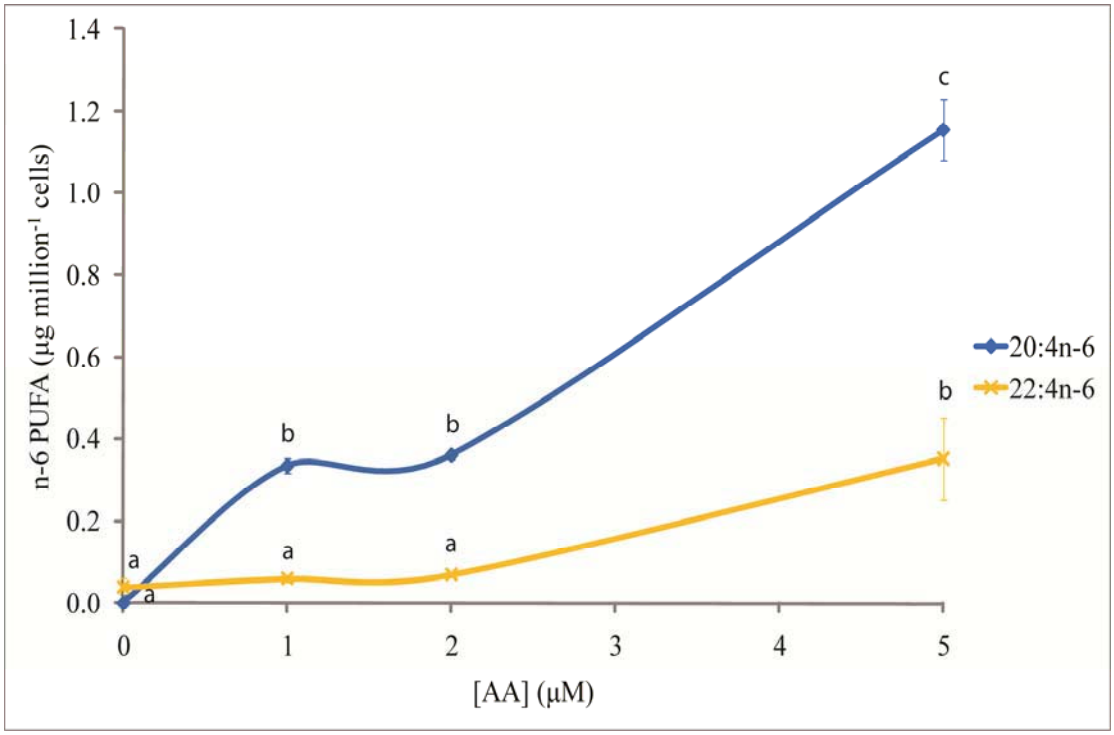

Figure 4.18 The n-6 PUFA in CHSE-214 cell phospholipids after cell supplemention with 1-5 µM of AA. The CHSE-214 cells were grown as described in section 4.2.5. The phospholipids were extracted for analysis using GC as outlined in section 4.2.6.1. The data are the mean ± SE (n=3). Values with different symbols are significantly different from each other within each fatty acid group.

4.3.3 qRT-PCR analysis of FHM cells

The change in expression of the Δ6desaturase and elongase genes in the FHM cells was investigated in response to supplementing the cell line with n-3 PUFA. Fish Δ6desaturase and elongase enzymes tend to have higher activity with the n-3 PUFA substrates rather than the n-6 PUFA substrates (Hastings *et al.* 2001; Agaba *et al.* 2005; Hastings *et al.* 2005; Morais *et al.* 2009). We hypothesized that the expression of the fatty acyl Δ6desaturase and elongase would be increased when cell cultures were supplemented with the PUFA substrates ALA and SDA, compared to the LCPUFA EPA, DPA and DHA.

4.3.3.1 Amplification efficiency

The amplification efficiency for the *Δ6desaturase, Elovl5* and *β-actin* primer pairs was assessed by measuring the C_T value with various cDNA template dilutions. A standard curve was produced and the reaction efficiencies for *Δ6desaturase, Elovl5* and *β-actin* were 0.84, 0.97 and 1, respectively. Thus, the reaction efficiency using the *Δ6desaturase* primer pair was somewhat lower than for the other two primer pairs but it was deemed to be satisfactory.

4.3.3.2 Amplification of Δ6desaturase, Elovl5 and β-actin PCR products

The *Δ6desaturase*, *Elovl5* and *β-actin* PCR products were analysed by gel electrophoresis to test for the presence of any contaminating genomic DNA or primer-dimer formation. The melt curve analysis at the end of the qRT-PCR cycling also checked for these contaminants. Agarose gel electrophoresis and melt curves of *Δ6desaturase*, *Elovl5* and *β-actin* PCR products, which were amplified from the cDNA standards, were analysed. A typical melt curve obtained after amplification of *Δ6desaturase* can be seen in Appendix D.3. The findings showed the primers were specific, there was no contaminating genomic DNA as the expected product size was seen and there was no visible primer-dimer formation.

4.3.3.3 Δ6desaturase and Elovl5 mRNA abundance

A typical qRT-PCR trace showing the amplification of *Δ6desaturase*, *Elovl5* and *β-actin* can be seen in Figure 4.19. Amplification of *Δ6desaturase* typically began at approximately 8 qRT-PCR cycles compared to *β-actin* at approximately 14 cycles and *Elovl5* at approximately 25 cycles (Figure 4.19). In FHM cells, the *Elovl5* was being expressed at lower levels compared to *β-actin* and *Δ6desaturase*. The C_T value of *Δ6desaturase* or *Elovl5* was compared to the *β-actin* C_T value to normalise the expression of *Δ6desaturase* or *Elovl5* between samples (ΔC_T).

In the presence of 2 μM of the n-3 PUFA, ALA, SDA, EPA, DPA or DHA, the expression of *Δ6desaturase* and *Elovl5* in the FHM cells was up regulated (Figure 4.20). The expression of *Δ6desaturase* and *Elovl5* was generally increased 2-fold in the presence of n-3 PUFA (Figure 4.20).

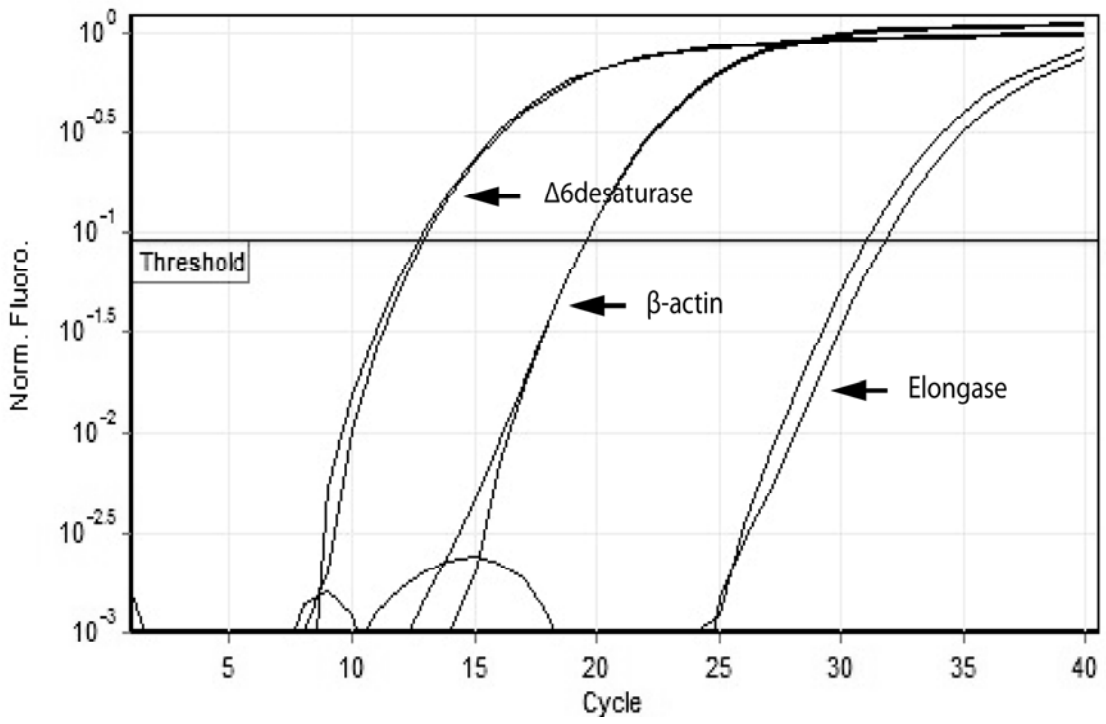

Figure 4.19 A typical qRT-PCR amplification trace for *Δ6desaturase*, *Elovl5* and *β-actin* abundance in FHM cells. The fluorescence measured was plotted against the qRT-PCR cycle number. The horizontal line represents the threshold cycle (C_T).

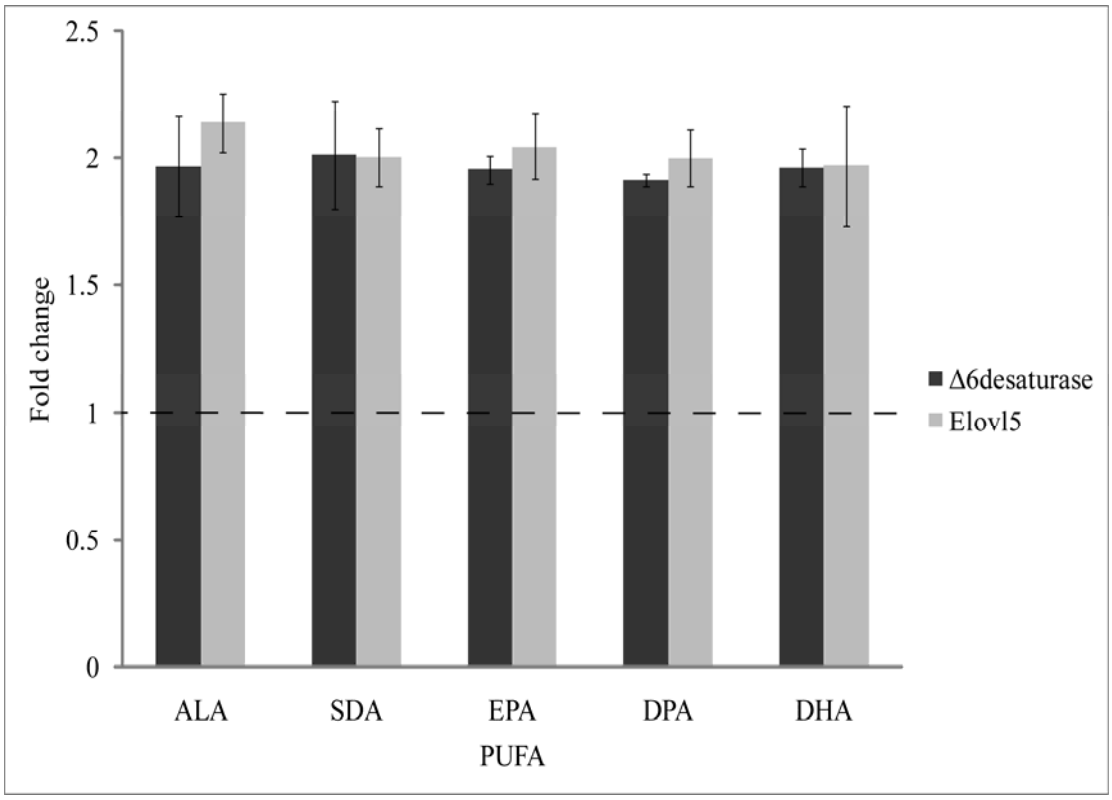

Figure 4.20 Relative expression of the FHM cell line Δ6desaturase and elongase genes after the cell line was supplemented with various PUFA. The fold-change in gene expression was determined using qRT-PCR as described in section 4.2.7. The relative expression was calculated after normalization against β-actin. The data are the mean ± SD (n=3).

4.3.4 Primary cell line development from two marine fish species

The work outlined in this chapter aimed to investigate the ability of cell lines from freshwater, anadromous and marine fish species to synthesize LCPUFA. YTK and SBT are two marine species which are important in the Australian aquaculture industry. YTK or SBT cell lines are currently not available, although they would be very useful in studying LCPUFA synthesis in these commercially valuable species. We aimed to develop primary cell cultures from YTK and SBT to enable us to compare the LCPUFA synthesis pathway in these marine species with the pathway from freshwater and anadromous species.

4.3.4.1 YTK primary cell culture development

Various YTK tissue types and methods of dissociating the tissue were used to develop the YTK primary cell cultures. After 2 days of culturing, pieces of liver tissue could be seen attached to the flasks from the cold enzyme dissociated and mechanically dissociated treatments, while individual liver cells were attached to the flasks from the warm enzyme dissociated treatment. Pieces of muscle tissue were attached to flasks from the warm enzyme dissociated treatment and muscle cells were attached to flasks from the cold enzyme dissociated treatment. Red blood cells could be seen in the heart cell cultures. After 7 days of culturing the YTK primary cells, liver cells were attached to flasks from all treatments, muscle cells were attached to flasks from the cold enzyme dissociated treatment and heart cells were attached to flasks from the fine dissociation treatment. The morphology of the YTK heart and liver primary cells can be seen in Figure 4.21. The most successful flasks containing the YTK liver primary cells were from the cold enzyme dissociated and mechanically dissociated treatments in L-15 medium and underwent two passages. There were no successful YTK primary cell cultures which became cell lines.

4.3.4.2 SBT primary cell culture development

Various SBT tissue types and methods of dissociating the tissue were used to develop the SBT primary cell cultures. After 2 days of culturing the SBT primary cells, most flasks contained a large amount of tissue debris, but few attached cells or

tissues. Heart cells were attached to flasks from the warm enzyme dissociated and fine dissociation treatments, as well as heart membrane cells. After 7 days of culturing the SBT primary cells, red muscle membrane cells, red muscle cells from the warm enzyme dissociated treatment, white muscle cells from the fine dissociation and heart cells from the warm enzyme dissociated, cold enzyme dissociated and fine dissociation treatments were attached. The morphology of the SBT heart and red muscle membrane primary cells can be seen in Figure 4.21. The most successful flasks containing SBT heart primary cells were from the warm enzyme dissociated treatment in L-15 medium and underwent three passages. There were no successful SBT primary cell cultures which became cell lines.

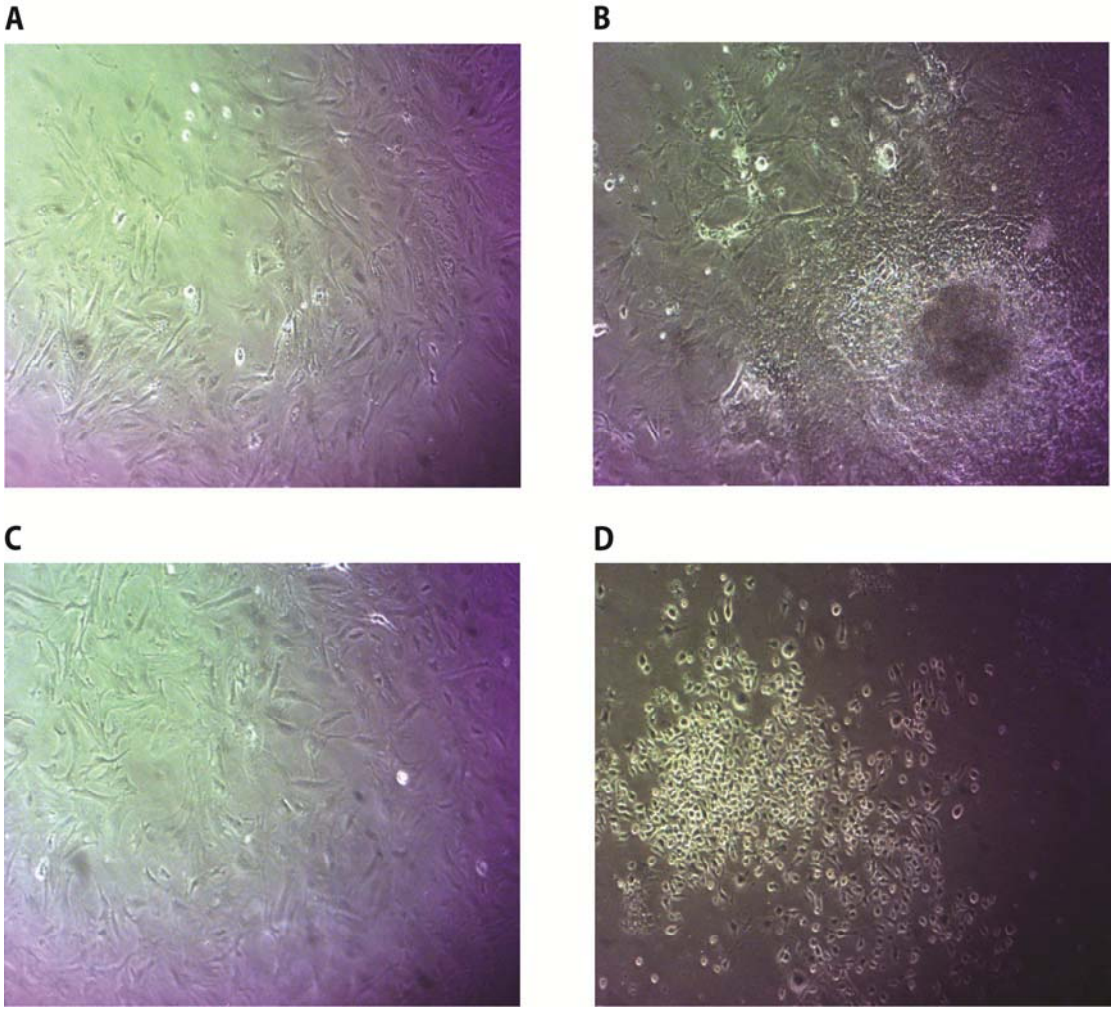

Figure 4.21 The development of the YTK and SBT primary cell cultures from various tissue types.

A. The YTK heart primary cell cultures after 7 days. The cells were obtained by fine dissociation and grown in medium number 2, as described in section 4.2.8.

B. The YTK liver primary cell cultures after 7 days. The cells were obtained by mechanical dissection and grown in medium number 1, as described in section 4.2.8.

C. The SBT heart primary cell cultures after 7 days. The cells were obtained by the warm enzyme dissociation treatment and grown in medium number 1, as described in section 4.2.8.

D. The SBT red muscle membrane primary cell cultures after 7 days. The cells were obtained by the warm enzyme dissociation treatment and grown in medium number 3, as described in section 4.2.8.

4.4 Discussion

The FHM and CHSE-214 cell lines were chosen as model cell lines for fish species from freshwater and anadromous environments, respectively. Supplementing various n-3 and n-6 PUFA to the growth medium of FHM and CHSE-214 cells at a concentration greater than 20 μM inhibited growth, possibly due to the production of lipid peroxides (Tocher and Dick 1991). The FHM and CHSE-214 cells proliferated with supplementations at concentrations below 20 μM FFA, possibly due to the PUFA providing essential nutrients (Tocher and Dick 1991). The growth of FHM and CHSE-214 cells was unimpaired when the growth medium contained 2% FBS, a reduced level compared with the normal maintenance level. This is in agreement with the unimpaired growth of other cell lines in media containing 2% FBS, as well as 2% FBS not significantly interfering with the PUFA supplementation experiments (Tocher *et al.* 1989).

The FHM cell line displayed the ability to elongate fatty acid substrates but did not efficiently desaturate fatty acids. Upon supplementation with ALA, LA, EPA and AA the elongation products 20:3n-3, 20:2n-6, DPA and 22:4n-6, respectively, accumulated in the cell phospholipids. The activity of Elovl5 to chain elongate EPA to DPA and AA to 22:4n-6 is considered to be through the accepted pathway. However, the elongation products 20:3n-3 and 20:2n-6 are not considered intermediates of the accepted metabolic pathway for the synthesis of LCPUFA from C_{18} PUFA (Figure 1.1). Upon supplementation with 18:2n-6, the immediate Δ6desaturase product 18:3n-6 did not significantly increase, but the subsequent elongation product 20:3n-6 was increased. We propose that it was unlikely that 20:3n-6 was obtained from 18:2n-6 by Δ6desaturation followed by elongation. The accumulation of 20:2n-6 and 20:3n-6 from 18:2n-6 supplementation suggests an alternative pathway involving Elovl5 followed by Δ8desaturase (18:2n-6 → 20:2n-6 → 20:3n-6) (Rivers *et al.* 1975) rather than the traditional Δ6desaturase followed by Elovl5 (18:2n-6 → 18:3n-6 → 20:3n-6). It is proposed that low Δ6desaturase activity in the FHM cells resulted in ALA and LA being shunted through an alternate pathway rather than proceeding through the accepted metabolic pathway.

The low Δ6desaturase activity in the FHM cells lead to the investigation of the expression of *Δ6desaturase* and *Elovl5* in the FHM cells following n-3 PUFA supplementation. The expression of the desaturase and elongase genes in whole fish is influenced by dietary levels of fatty acids, environmental factors and hormones (Zheng *et al.* 2004a; Zheng *et al.* 2005a). When the expression of these genes are influenced by dietary PUFA they may be regulated by an abundance of either the direct substrates, obtained through the diet, or removal of the products throughout the LCPUFA pathway (Zheng *et al.* 2004a; Mourente *et al.* 2005). In this study, the expression of both *Δ6desaturase* and *Elovl5* was 2-fold up regulated after the FHM cells were supplemented with n-3 PUFA. Approximately the same level of up regulation was seen, regardless of the n-3 PUFA. Although, the findings from the expression data of *Elovl5* should be considered with care, since the detection of these transcripts did not occur until the end of the qRT-PCR cycling. The gene expression data suggest that the *Δ6desaturase* and *Elovl5* are being up regulated in the FHM cells following PUFA supplementation, but the *Δ6desaturase* activity remains low. Our findings suggest that although there was an increase in the *Δ6desaturase* transcript level after PUFA supplementation, Δ6desaturase protein levels may remain unchanged in the FHM cells. The FHM cell line originates from a freshwater species which was expected to have more capacity to desaturate and elongate PUFA than marine cell lines. In general, salmonoids and freshwater fish have been found to have higher Δ6desaturase and elongase gene expression and enzyme activities compared to marine fish (González-Rovira *et al.* 2009).

The CHSE-214 cell line appeared to incorporate lower amounts of PUFA substrates than the FHM cell line and subsequently synthesized lower amounts of LCPUFA pathway products. However, the CHSE-214 cell line displayed the ability to desaturate and elongate fatty acids. The CHSE-214 cells' Δ6desaturase showed activity towards LA but not ALA. However, the accumulation of DHA in the cell phospholipids suggested that the Δ6desaturase showed activity towards the n-3 series after EPA or DPA supplementation. Most established cell lines lack the ability to produce DHA although the reason for this apparent enzymatic deficiency is unknown (Ghioni *et al.* 2001). The CHSE-214 cells are unique compared to the anadromous AS cells. Both cells have an active Δ6desaturase but when the AS cells were

supplemented with 25 μM of EPA an accumulation of EPA was seen but DHA levels were unaltered (Ghioni *et al.* 1999).

Elovl5 activity was exhibited with n-3 and n-6 PUFA substrates. Supplementation with LA resulted in the Δ6desaturase product 18:3n-6 being further elongated to 20:3n-6 and supplementation with EPA and AA resulted in elongation to DPA and 22:4n-6, respectively. The accumulation of DHA in the cell phospholipids indicated that an Elovl2 was active in CHSE-214 cells because Elovl2, Δ6desaturase and β-oxidation are required for conversion of DPA to DHA.

Retroconversion of DHA takes place by the saturation of the Δ4 double bond by 2,4-enoyl-CoA reductase and partial β-oxidation to shorten the chain length (Tocher and Dick 2001). The FHM cells appeared to retroconvert the supplemented DHA more than the CHSE-214 cells. The FHM cell phospholipids had a significant increase in the accumulation of EPA after 0-10 μM DHA supplementation. In contrast, the CHSE-214 cell phospholipids accumulated DPA after 1 μM DHA supplementation, while no significant increase in EPA accumulation was seen. The retroconversion of DHA has also been reported in EPC, EFAD-EPC and AS cells (Tocher and Dick 1990; Tocher and Dick 2001), while retroconversion in RTG-2 and TF cells was not significant (Tocher *et al.* 1989; Tocher 1990). The level of DHA in the CHSE-214 cell phospholipids may have been required for cellular function, as the accumulation of DHA was lower in CHSE-214 cell phospholipids than FHM cell phospholipids, and therefore retroconversion did not occur.

The most abundant series of fatty acids in the CHSE-214 and FHM cell phospholipids was the MUFA, in particular 18:1n-9. EFA deficient animals will use n-9 PUFA like 18:1n-9 to synthesize 20:3n-9, which accumulates in the tissue instead of AA (Tocher *et al.* 1995). The culturing of cells in lipid free medium causes an increase in MUFA compared to the original tissue (Tocher *et al.* 1995; Tocher *et al.* 1996; Ghioni *et al.* 1997). The fatty acid profile of fathead minnow was not available, but the fatty acid profile of the freshwater species carp (*Cyprinus carpio*), although not a direct comparison, demonstrates this finding. The flesh of carp contains 17.6% MUFA (Nichols *et al.* 1998) compared to 43% found in the FHM cell line cultured in L-15 containing 10% FBS. Conversely, the flesh of carp contains

41.8% n-3 PUFA (Nichols *et al.* 1998) compared to 4.8% found in the FHM cell line cultured in L-15 containing 10% FBS. Ghioni *et al.* (1997) showed that the total n-3 PUFA in rainbow trout skin cells being used for primary cell cultures decreased from 25% to 16% in 14 days. After 4 months in culture, the n-3/n-6 ratio decreased by 3.6-fold (Ghioni *et al.* 1997).

This study has confirmed that CHSE-214 cells have a functional Δ6desaturase, Elovl5 and Elovl2, consistent with previous data (Tocher *et al.* 1995a). These enzymes appear to be selective towards particular substrates. However, this apparent selectivity towards PUFA substrates may actually be enzymatic down regulation caused by the abundance of supplemented PUFA. The AS cell line has a fully functional LCPUFA synthesis pathway but supplementation with 50 μM of EPA or DHA significantly reduced the LCPUFA synthetic activity of the pathway (Ghioni *et al.* 1999; Zheng *et al.* 2009a). The AS cells may be a reliable model of the LCPUFA synthesis pathway for Atlantic salmon because dietary EPA and DHA also effect the transcription of Δ6desaturase in whole Atlantic salmon (Zheng *et al.* 2009a).

We have compared the LCPUFA synthesis pathway in a freshwater and an anadromous fish cell line. The FHM and CHSE-214 cell lines were chosen as model freshwater and anadromous species after characterization of the cell lines. The sequence of the mitochondrial *cox*1 gene can be used as a molecular barcode to differentiate animal species (Herbert *et al.* 2003; Hanner and Gregory 2007). The *cox*1 gene has a rate of molecular evolution three times greater than 12S or 16S ribosomal DNA, which allow discrimination of groups within a species (Herbert *et al.* 2003). Previously, 207 species of Australian fish have been identified using a 665 bp fragment of the *cox*1 gene (Ward *et al.* 2005). We chose to confirm the species of origin of the cell lines developed or acquired by our laboratory using *cox*1 barcoding. The NCBI database contained *cox*1 sequences of each of the species from which the cell lines originated. Two cell lines were routinely used in our laboratory prior to *cox*1 identification. The EPC cell line was obtained from colleagues and used since 2004, while the SBT-G cell line was developed in our laboratory by Alexandra Korte in 2006 and was characterised by chromosomal analysis. However, *cox*1 identification revealed that both of these cell lines were of FHM origin. It was unlikely that our original source of EPC cells were contaminated by FHM in our

facilities as these cells have never been used by any group in The School of Biological Sciences, Flinders University. The AAHL Fish Diseases Laboratory (CSIRO Livestock Industries, Geelong, Vic, Australia), have recently used *cox*1 identification of their EPC cells to reveal they were in fact FHM cells. The practice of obtaining cell lines from colleagues is common. A study conducted showed that 69% of scientists obtained cell lines from another researcher or another source and nearly half had not identified the cell line once obtained (Hughes *et al.* 2007). The 'fault' of our laboratory was that we did both of the above. We speculate that the SBT-G cell line was cross-contaminated by the FHM cells, which at that point were thought to be EPC cells, through the use of shared equipment such as safety cabinets, incubators and automated pipettes. Cross-contamination is a common occurrence with approximately one third of cell lines containing cells from other species or unrelated cells from the same species (Hughes *et al.* 2007). It is estimated that 15-20% of publications have used misidentified and cross-contaminated cell lines (Nardone *et al.* 2007). Although cross-contamination and misidentification has been recognised since the 1950s, these problems are common today for many cell lines, including one of the most commonly used cell lines, HeLa (Hughes *et al.* 2007; Nardone *et al.* 2007). We propose that the FHM cells are robust in culture, very much like HeLa cells, and were able to overgrow the SBT-G cells (Hughes *et al.* 2007; Nardone *et al.* 2007).

In addition to the FHM cell line, five other fish cell lines were obtained and characterized. The bluegill and koi cell lines were from species of freshwater origin, the Chinook salmon and rainbow trout cell lines were from species of anadromous origin and the barramundi cell line was from a catadromous species. Disappointingly, only two out of the five cell lines obtained were found to be correctly identified. Therefore, in our laboratory we experienced a cell line misidentification rate of 71%. The AAHL Fish Diseases Laboratory (CSIRO Livestock Industries, Geelong, Vic, Australia), where the cell lines were obtained, acquired cell lines from other researchers and the ATCC. Moreover, the reputable cell bank, ATCC, have recently characterized their EPC cell line as FHM cells through *cox*1 testing (www.atcc.org). The importance of characterizing cell lines is clearly not well understood and is continuously being overlooked. The impacts of working with misidentified cell lines

are the enormous waste of research funding and the ultimate publication of misleading results (Hughes *et al.* 2007).

The development of primary cell cultures from two commercially important marine aquaculture species in SA, SBT and YTK, was attempted. The optimal conditions for culturing fish cells have not been well studied (Ghioni *et al.* 1997). Establishing fish cultures and maintaining continuous growth of cell lines is done in media and supplements which are optimized for mammalian cell lines (Ghioni *et al.* 1997). The Leibovitz L-15 and RPMI 1640 media supplemented with 20% FBS or AmnioMax II were used for establishing primary SBT and YTK cultures, although conditions were not optimal for maintaining the fish cells in culture. However, it is common practice for primary fish cell cultures to be established under these conditions. The most successful SBT and YTK primary cultures of heart and liver cells, respectively, were most stable in the L-15 medium, as previously seen with rainbow trout skin cells (Ghioni *et al.* 1997). Although the SBT heart and YTK liver primary cultures underwent three and two passages, respectively, they did not become continuous cultures. These cells progressively lost their potential to differentiate as they were cultured (Freshney 2000). In the end, the LCPUFA synthesis pathway in these marine fish cell cultures could not be studied through PUFA supplementations.

We have found that cell lines from freshwater and anadromous fish species can not clearly be differentiated by their ability to synthesis LCPUFA through desaturase and elongase activity. While FHM, PLHC-1 and EFAD-EPC cells originated from freshwater species which should have active LCPUFA synthesis pathways due to their natural diets, the cell lines do not all display a fully functional LCPUFA synthesis pathway (Tocher *et* al 1995; Tocher *et al.* 1996). Interestingly, the three cell lines which originated from anadromous species, CHSE-214, AS and RTG-2, do not all have the same active LCPUFA synthesis enzymes (Tocher *et al.* 1989; Tocher *et al*. 1995a). The Δ6desaturase and elongase activities seen in the CHSE-214 cells used in this study confirm the findings of Tocher *et al.* (1995a). However, the lack of Δ5desaturase activity was also seen in the freshwater EFAD-EPC cells (Tocher *et al.* 1996) and the marine SAF-1 cells (Tocher and Ghioni 1999), thus making it difficult to use species habitat and diet as a means to classify LCPUFA synthesis ability in fish cell lines. Evolutionarily fathead minnow are most closely related to common

carp, both being from the order Cypriniformes. However, the absence of not only Δ5desaturase activity but also Δ6desaturase activity in the FHM cell line highlights the need for caution when using cell lines for predicting the LCPUFA synthesis situation *in vivo.* When cell lines are maintained in culture the loss or down regulation of desaturase and elongase activities appears be common. An early study showed that three out of six mammalian cell lines examined did not have Δ6desaturase activity (Maeda *et al.* 1978).