INVESTIGATION OF THE LONG CHAIN POLYUNSATURATED FATTY ACID SYNTHESIS PATHWAY IN SOUTHERN BLUEFIN TUNA (*THUNNUS MACCOYII*) AND YELLOWTAIL KINGFISH (*SERIOLA LALANDI*)

BY

MELISSA K. GREGORY

A thesis submitted for the Degree of Doctor of Philosophy

The School of Biological Sciences

Flinders University

March 2010

TABLE OF CONTENTS

TABLE OF FIGURES

DATA TABLES

ABBREVIATIONS

AA	Arachidonic acid; 20:4n-6
AAHL	Australian Animal Health Laboratory
AESEC	Australasian Experimental Stockfeed Extrusion Centre
AGRF	Australian Genome Research Facility
ALA	α-linolenic acid; 18:3n-3
ANGIS	Australian National Genomic Information Service
ANOVA	Analysis of variance
AS	Atlantic salmon cell line
ATCC	American Tissue Culture Collection
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
bp	Base pair
BSC	Biohazard Safety Cabinet
cDNA	Complimentary DNA
CEFAS	Centre for Environment, Fisheries and Science
CHSE-214	Chinook salmon cell line
cm / mm	Centimetre / millimetre
*cox*1	Cytochrome oxidase subunit I
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DEPC	Diethyl pyrocarbonate
DHA	Docosahexaenoic acid; 22:6n-3
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DPA	Docosapentaenoic acid; 22:5n-3
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acid
EMEM	Eagle's minimum essential medium
EPA	Eicosapentaenoic acid; 20:5n-3
EPC	Epithelioma papulosum cyprinid cell line
EFAD-EPC	Essential fatty acid deficient-epithelioma papulosum cyprini
EQDM	Ethoxyquin dimer
ER	Endoplasmic reticulum
EU	Eurpoean Union
FAAL	Flinders Advanced Analytical Laboratory
FAF-BSA	Fatty acid free bovine serum albumin
FAME	Fatty acid methyl esters
FBS	Fetal bovine serum
FBW	Final body weight
FCR	Feed conversion ratio
FFA	Free fatty acid
FHM	Fathead minnow cell line
FLQ	Fillet lipid quality
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GSE	Grape seed extract
GSP	Gene specific primer
h	Hour

HMEM	Hank's minimum essential medium
HSD	Honestly significantly difference
HSI	Hepatosomatic index
IA	Index of atherogenicity
IBW	Initial body weight
IT	Index of thrombogenicity
ITS1	Internal transcribed spacer of 18S rRNA
IU	International unit
kb	Kilobase
kDa	Kilodalton
kg / g / mg / μg / ng	Kilogram / gram / milligram / microgram / nanogram
kL / L / mL / μl	Kilolitre / litre / millilitre / microlitre
LA	Linoleic acid; 18:2n-6
LB	Luria broth
LCPUFA	Long chain polyunsaturated fatty acids
LDL	Low-density lipoprotein
M / mM / μM	Molar / millimolar / micromolar
MDA	Malonaldehyde
min	Minute
MP	Maximum parsimony
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acids
NCBI	National Center for Biotechnology Information
ND	Not detected
nm	Nano metre
nmol	Nano mole
n-3	Omega-3
n-6	Omega-6
OD	Optical density
ORF	Open reading frame
PAUP*	Phylogenetic analysis using parsimony (*and other methods)
PBSA	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Propyl gallate
PLHC-1	Topminnow cell line
PUFA	Polyunsaturated fatty acids
qRT-PCR	Quantitative real time polymerase chain reaction
RACE-PCR	Rapid amplification of cDNA ends-polymerase chain reaction
RE	Restriction enzyme
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTG-2	Rainbow trout cell line
RT-PCR	Reverse transcriptase-polymerase chain reaction
SA	South Australia
SAF-1	Gilthead sea bream cell line
SAP	Shrimp alkaline phosphatase
SARDI	South Australian Research and Development Institute
Sats	Saturated fatty acids
SBT	Southern bluefin tuna
SBT-G	SBT gonad cell line

SC-U	Synthetic minimal defined medium for yeast without uracil
SD	Standard deviation
SDA	Stearidonic acid; 18:4n-3
SDM	Site directed mutagenesis
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
SGR	Specific growth rate
T/V	Trypsin-versene
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBHQ	Tert-butylhydroquinone
TBR	Tree bisection reconnection
TCA	Trichloroacetic acid
TF	Turbot cell line
TL	Total fish length
TLC	Thin layer chromatography
U	Units
UTR	Untranslated region
v/v	Volume/volume
WG	Weight gain
w/v	Weight/volume
w/w	Weight/weight
YEp	Yeast episomal plasmid
YPD	Yeast extract peptone dextrose
YTK	Yellowtail kingfish

ABSTRACT

The essential polyunsaturated fatty acids (PUFA) for humans, 18:3n-3 (α-linolenic acid, ALA) and 18:2n-6 (linoleic acid, LA), must be obtained through the diet because they cannot be synthesized. Humans consume a diet rich in n-6 fatty acids and are not able to convert the essential dietary n-3 PUFA ALA into n-3 long chain polyunsaturated fatty acids (LCPUFA) like 20:5n-3 (eicosapentaenoic acid, EPA), 22:5n-3 (docosapentaenoic acid, DPA) and 22:6n-3 (docosahexaenoic acid, DHA). In contrast to humans, fish are considered to have a functional LCPUFA synthesis pathway which can convert ALA into the LCPUFA derivatives EPA, DPA and DHA.

The vertebrate LCPUFA synthesis pathway requires three elongation and three desaturation steps to convert ALA to DHA. The fatty acyl Δ6desaturase and fatty acyl elongase, Elovl5, are both considered to be used twice. This thesis aimed to examine the LCPUFA synthesis pathway, in particular Elovl5 and Δ6desaturase, in freshwater, anadromous and marine fish species.

Three fish models were used to examine the accumulation of individual PUFA and their subsequent LCPUFA products. Yellowtail kingfish (YTK; *Seriola lalandi*) were used as an *in vivo* marine fish species model and were fed a diet containing a synthetic antioxidant, ethoxyquin, and/or a natural antioxidant, grape seed extract, for 8 weeks. The YTK fillet was found to bioaccumulate 2.5-fold more DHA than the level supplied in the diet. However, natural fish variation resulted in substantial variation in the proportion of DHA in the fillet. Interestingly, there was a significant decrease in the proportion of DHA in the fillet after storage at 4°C for 4 days, regardless of ethoxyquin or grape seed extract antioxidant protection.

Southern bluefin tuna (SBT; *Thunnus maccoyii*) are a large and economically valuable marine aquaculture species in South Australia. Whole SBT are essentially unobtainable for research as their supply is limited due to a strict wild-catch quota system and the inability to routinely breed them in captivity. To elucidate the enzymatic regulation of the SBT LCPUFA synthesis pathway, the *Saccharomyces*

cerevisiae expression system was used to characterise the *Elovl5* and *Δ6desaturase* genes from SBT liver tissue. The SBT *Elovl5* and *Δ6desaturase* cDNAs encoded predicted proteins which had the main structural characteristic features of microsomal fatty acyl elongases and desaturases, respectively, from mammals and other fish. The Elovl5 enzyme was very efficient at elongating C_{18} and C_{20} PUFA substrates, with higher activity towards the n-3 substrates than the n-6 substrates. The Δ6desaturase enzyme activity appeared to be low because desaturation products were not detected when the cultures were supplemented with various n-3 and n-6 PUFA. However, Δ6desaturase protein expression in the *S. cerevisiae* system was also low, thus making it difficult to determine the substrate specificity of the Δ6desaturase. This thesis went further to show that at least one fatty acyl elongase gene is expressed in a range of SBT tissues, while expression of *Δ6desaturase* appears to be limited.

The FHM (fathead minnow; *Pimephales promelas*) and CHSE-214 (Chinook salmon; *Oncorhynchus tshawytscha*) epithelial cell lines were used as *in vitro* systems to examine the LCPUFA synthesis capabilities of freshwater and anadromous fish species, respectively. The fish cell lines were supplemented with n-3 and n-6 PUFA to investigate if the LCPUFA synthesis pathway in the cell lines could be used as a model for fish *in vivo*. This thesis confirmed that the CHSE-214 cells had functional Δ6desaturase, Elovl5 and Elovl2 enzymes, consistent with previous data. In contrast, the FHM cell line displayed the ability to elongate PUFA substrates but did not efficiently desaturate them. The low Δ6desaturase activity in the FHM cells lead to the investigation of the expression of *Δ6desaturase* and *Elovl5* genes in the FHM cells following n-3 PUFA supplementation. Approximately the same level of up-regulation was seen, regardless of the n-3 PUFA.

This thesis highlights the different LCPUFA synthesis pathway capabilities in freshwater, anadromous and marine fish species. These findings will help define dietary approaches to maintaining or enhancing the synthesis of LCPUFA in aquaculture fish species.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Melissa K. Gregory

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr Kathy Schuller, for her day to day support and guidance. A special thanks for all of your time and effort during my thesis preparation. I would like to thank my co-supervisors, Prof Bob Gibson and Prof Andy Ball. Bob, thankyou for your refreshing ideas and introducing me to the world of fatty acid research. Andy, thankyou for your comments, advice and honest opinion with my paper.

Thanks to Drew Sutton, Valene See and Peter Bain in the Schuller lab. Drew, thankyou for sharing an office and lab with me for 3.5 years and always listening, whether it was to my general chatter, gossiping, ranting or whinging! And for all of the science questions of course!

A huge thankyou must go to the people who made coming to Uni each day fun because I knew I was going to see some of my best friends. Chevaun, Patrick, Lexi, Drew, Mel P, Bart, Ana, Simon, Mel S and Emma - there are so many reasons to thank you! Firstly, for all of the fun times at work, in the corridor, the tearoom and in everyones offices, as well as all the great times we've had going away together or heading out for dinner and drinks. Secondly, for always being happy to help and offer science advice.

A special thanks to Chevaun for responding to my endless emails with thesis questions and for the friendship we've developed through science which will last forever! A very special thankyou to my boyfriend Bart for all of his support, both at work and home. Your ideas and enthusiasm helped me get through the hard patches and your patience with me while I've been writing up has been incredible. The days writing were made easier knowing we always had something fun planned together.

Mum and Dad, thankyou for providing me with the opportunity to be 'a professional student' and giving me the endless support which made it possible for me to go through this whole process. To my other family and friends, thankyou for always showing an interest in my PhD and asking when it's done. Well, it's done!

PUBLISHED WORK

Part of the work in this thesis has been published.

Publications:

Gregory, M.K., See, V.H.L., Gibson, R.A., Schuller, K.A. (2010) Cloning and functional characterisation of a fatty acyl elongase from southern bluefin tuna (*Thunnus maccoyii*), *Comparative Biochemistry and Physiology, Part B,* 155, 178-185.

Abstracts:

Gregory, M.K., See, V.H.L., Gibson, R.A., Schuller, K.A. Cloning and functional characterization of a fatty acyl elongase from southern bluefin tuna (*Thunnus maccoyii*), oral presentation at World Congress on Oils and Fats & 28th ISF Congress, Sydney, NSW, Australia, September 2009.

Schuller, K.A., **Gregory, M.K.**, Gibson, R.A., Cloning and characterization of a southern bluefin tuna (*Thunnus maccoyii*) fatty acyl elongase cDNA, oral presentation at Aquaculture Europe, Trondheim, Norway, August 2009.

Gregory, M.K., Gibson, R.A., Schuller, K.A. Omega-3 fatty acid conversion in the southern bluefin tuna gonad cell line, poster at International Society for the Study of Fatty Acids and Lipids (ISSFAL), Kansas City, Missouri, USA, May 2008.

Gregory, M.K., Buchanan, J., Gibson, R.A., Schuller, K.A. Yellowtail kingfish diet trial using grape seed extract as a natural antioxidant supplement, poster at the Aquafin CRC conference, Barossa Valley, SA, Australia, May 2007.