Consequences of long-term feeding trout with plant-based diets on the regulation of energy and lipid metabolism : special focus on trans-generational effects and early stages

Viviana Lazzarotto

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par **Viviana LAZZAROTTO**

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Conséquences à long-terme d'une alimentation à base de matières premières végétales sur la régulation du métabolisme énergétique et lipidique chez la truite arc-en-ciel: focus particulier sur les effets trans-générationnels et les stades précoces.

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Devant la commission d'examen formée de :

Mme. IZQUIERDO, Marisol, Professeur, Université de Las Palmas de Gran Canaria (ES)	Rapporteur
M. ZAMBONINO, José Luis, Directeur de Recherche, Ifremer	Rapporteur
Mme. GRUFFAT, Dominique, Chargée de Recherche, HDR, INRA	Examinateur
Mme, BOLLIET, Valérie, Professeur, Université de Pau et des Pays de l'Adour	Président
Mme. MEDALE, Françoise, Directrice de recherche, INRA	Directeur de thèse

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Abbreviations	Full name
β-actin	beta-actin
Δ9	delta-9-desaturases
Δ6	delta-6-desaturases
Δ5	delta-5-desaturases
6PFKM	phosphofructokinase, muscle
18S	18S ribosomal RNA
AAs	amino acids
ACO	acyl-CoA oxidase
ACTA1	actin, alpha 1, skeletal muscle
ANOVA	analysis of variance
ARA	arachidonic acid
ATP	adenosine triphosphate
BPC	bean protein concentrate
C-diet	commercial like diet
COM-diet	COM-broodstock commercial diet
CKM	creatine kinase, muscle
COX5B	cytochrome c oxidase subunit Vb
COX7A2L	cytochrome c oxidase subunit VIIa polypeptide 2 like
CPT-II	carnitine palmitoyl transferase-II
cRNA	complementary RNA
CTSS	cathepsin S
CTSZ	cathepsin Z
CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1
DARS	aspartyl-tRNA synthetase
DHA	docosahexaenoic acid
DHCR7	7-dehydrocholesterol reductase
DNA	deoxyribonucleic acid
EAAs	essential amino acids
EASE	expression analysis systematic explorer
EFA	essential fatty acids
EF1α	eukaryotic translation elongation factor 1 alpha 1
Elovl2	elongation of very long chain fatty acids like-2
Elovl5	elongation of very long chain fatty acids like-5
EPA	eicosapentaenoic acid
EPRS	glutamyl-prolyl-tRNA synthetase
FA	fatty acids
FAAH	fatty acid amide hydrolase

FAME	fatty acyl methyl esters
FAS	fatty acid synthase
FC	fold change
FDR	false discovery rate
FI	feed intake
FM	fish meal
FO	fish oil
FUCA1	fucosidase, Alpha-L- 1 (tissue)
FUCA 2	fucosidase, Alpha-L- 2 (plasma)
G6PD	glucose-6-phosphate-dehydrogenase
GCK	glucokinase (hexokinase 4)
GE	gene expression
GO	gene ontology
GPD1	glycerol-3-phosphate dehydrogenase 1
GSI	gonadosomatic index
HK2	hexokinase-2
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1
IARS	isoleucyl-tRNA synthetase
LARS	leucyl-tRNA synthetase
LC-PUFAs	long chain-polyunsaturated fatty acids
LDHA	lactate dehydrogenase A
LPL	lipoprotein-lipase
MAN2B1	mannosidase, alpha, class 2B, member 1
M-diet	FM-FO based diet
MDH2	malate dehydrogenase 2, NAD (mitochondrial)
ME	malic enzyme
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acid
MYBPC1	myosin binding protein C, slow type
MYBPC2	myosin binding protein C, fast type
NAD$^+$	nicotinamide-adenine dinucleotide (oxidized form)
NADH	nicotinamide-adenine dinucleotide (reduced form)
PCBs	polychlorinated bisphenols
PCR	polymerase chain reaction
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PG	prostaglandins
PGK1	phosphoglycerate kinase 1
PYGL	phosphorylase, glycogen, liver

PYGM	phosphorylase, glycogen, muscle
POPs	persistent organic pollutants
RNA	ribonucleic acid
RT-qPCR	real time -quantitative polymerase chain reaction
SAT	saturated fatty acid
SD	standard deviation
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein
SPC	soy protein concentrate
TAG	triacylglycerol
V-diet	V-totally plant-based diet
VEG-diet	VEG-broodstock totally plant-based diet
VO/VOs	vegetable oil/vegetable oils

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Introduction (français)

Développement de l'aquaculture et son rôle dans le système alimentaire mondial

Au cours de 30 dernières années, la consommation mondiale de poisson et de crustacés s'est accrue, passant de 50 Millions de tonnes (Mt) en 1980 à 131 Mt en 2012 (FAO 2014). Cette augmentation ne résulte pas seulement de la croissance de la population mondiale mais aussi de la hausse de la consommation per capita; d'environ 10 kg/personne en 1970, elle a atteint près de 20 kg/personne en 2013 (FAO 2014), et représente actuellement en moyenne 17% de l'apport protéique. Cette augmentation de la consommation de poisson a été couverte par le développement spectaculaire de la production aquacole qui est passée de moins de 4 Mt en 1980 à environ 67 Mt aujourd'hui. En effet, du fait de leur limitation par des quotas, les captures de pêche sont restées stables depuis 40 ans autour de 90 Mt, dont 60-67 Mt utilisées pour l'alimentation humaine. Aujourd'hui, environ 50% du poisson consommé au niveau mondial provient de l'aquaculture.

Cette hausse continue de la production aquacole (+8.8% en volume moyen de production par an) a conduit à une forte augmentation de la demande en aliments piscicoles, dont la production a nécessité de plus en plus de matières premières qui les composent. Or, depuis le début du développement de l'aquaculture, les farines (FP) et les huiles de poisson (HP), fabriquées à partir des captures de pêche minotière (pêche spécifique d'espèces particulières comme l'anchois du Pérou, le merlan bleu, le chinchard destinées à la fabrication de farines et huiles) ont été les ingrédients majoritaires des aliments aquacoles. Mais, limitées par la disponibilité de la ressource, les captures issues de la pêche minotière ont été maintenues autour de 25 Mt/an au cours des quatre dernières décennies. La production de FP et HP ne pouvant pas couvrir la demande toujours croissante pour l'aquaculture, la recherche de ressources alimentaire alternatives est devenue une nécessité.

Vers une aquaculture durable

Dans ce contexte, les ingrédients végétaux ont reçu une attention particulière durant les dernières décennies, principalement en raison de leur disponibilité et de leur prix compétitif (Gatlin, Barrows et al. 2007, Hardy 2010). Grâce à d'intenses recherches, des progrès notables ont été réalisés concernant le remplacement de la FP et l'HP par des produits végétaux dans les aliments aquacoles (Naylor, Hardy et al. 2009). Cependant, l'utilisation de taux élevés

d’ingrédients végétaux en remplacement des matières premières d'origine marine a des inconvénients, qui sont principalement dus à des différences de teneur et de composition en protéines, acides aminés (AA), cholestérol et acides gras (AG) entre produits végétaux et ingrédients d'origine marine. Ces différences de composition induisent des effets métaboliques et physiologiques variables selon les espèces de poisson, leur stade de développement et les taux de substitution. En conséquence, les niveaux de remplacement ont été adaptés selon les espèces et le stade de développement des poissons. Il n'est pas encore possible de remplacer totalement à la fois la FP et l'HP dans les aliments des espèces d'eau tempérée élevées en Europe, telles que la truite, sans altérer la production. En particulier, les aliments pour les géniteurs et les jeunes stades de poisson contiennent encore des proportions élevées de FP et HP pour assurer de bonnes performances de reproduction et le développement des alevins.

Toutes les études prospectives indiquent que la production aquacole doit encore augmenter pour pouvoir répondre à la demande toujours croissante de consommation de produits aquatiques (Hardy 2010, Hixson 2014). Continuer à réduire davantage les quantités de FP et HP dans les aliments aquacoles est donc indispensable pour permettre le développement d’une aquaculture durable.

La présente thèse a pour objectif général d’analyser les effets potentiels d’un remplacement conjoint et total de la FP et HP par des ingrédients végétaux pendant tout le cycle d’élevage de la truite arc-en-ciel (Oncorhynchus mykiss). Une des originalités de la thèse est d'étudier notamment les effets résultant de l'alimentation des femelles reproductrices. En conséquence, cette introduction présente les particularités de la nutrition lipidique et protéique des poissons, et en particulier le rôle des lipides et des protéines durant la période de reproduction. Ensuite, un état des connaissances sur les effets du remplacement de la FP et/ou de l’HP par des produits végétaux sur le métabolisme et la physiologie des poissons est résumé, avant de présenter les hypothèses qui ont été à l'origine des travaux réalisés.

Les lipides: nutrition et métabolisme chez les poissons

Les lipides apportés par l’alimentation ont plusieurs rôles:

- la couverture des besoins en acides gras essentiels (AGE), c’est-à-dire non synthétisés par l’organisme (Corraze and Kaushik 1999).
- l'apport d'énergie digestible, d'autant plus important chez les poissons d'eau tempérée élevés en Europe que ces espèces digèrent mal les glucides complexes (Guillaume 2001)

- la contribution à l'absorption intestinale des vitamines liposolubles et des pigments caroténoïdes.

Les besoins en lipides et en acides gras

Établir les besoins spécifiques des poissons en terme de lipides alimentaires est compliqué en raison des différents facteurs qui peuvent les affecter: la nature chimique des lipides, leur rôle fonctionnel, la compétition avec les autres macronutriments (protéines et glucides) pour la production d'énergie ou encore la température du milieu (NRC 2011). En général, il est admis qu'un taux de lipides de 10-20% de la matière sèche de l'aliment (selon les espèces) suffit à garantir une utilisation efficace des protéines pour la croissance, sans entrainer de dépôt excessif de lipides dans les tissus du poisson (Cowey and Sargent 1979, Watanabe 1982, Sargent, Henderson et al. 1989, Corraze 2001).

La nature des AGE à apporter par l'alimentation pour couvrir les besoins des poissons dépend fortement de l'espèce et diffère entre espèces d'eau douce et espèces marines (NRC 2011). Cette différence est essentiellement due à la capacité des poissons à bio-convertir les acides gras à 18 atomes de carbone (18 C) en acides gras à plus longue-chaine (AGPI-LC), elle est donc liée à l'activité de deux classes d'enzymes spécifiques: les élongases et les desaturases.

La synthèse endogène et la bioconversion des acides gras

Chez les poissons; le foie est le site majeur de synthèse des acides gras, contrôlée par le complexe enzymatique acide gras synthase (Corraze 2001). Comme les autres organismes, les poissons ont la capacité de synthétiser de novo les acides gras saturés 16:0 (acide palmitique) et 18:0 (acide stéarique) en différentes proportions selon les espèces (Cook 1996, Sargent, Tocher et al. 2002). Ils sont aussi capables de convertir ces deux acides gras en acides gras mono-insaturés, tels que le 18:1 n-9, grâce à l'action de la Δ9-desaturase. Par contre, les poissons, comme les autres vertébrés, sont dépourvus des enzymes Δ12-desaturase et Δ15-desaturase, qui sont nécessaires pour convertir le 18:1 n-9 en 18:2 n-6 et le 18:2 n-6 en 18:3 n-3, respectivement (Tocher 2003). Aussi, les acides linoléique (18:2 n-6) et linolénique (18:3 n-3) sont des acides gras essentiels c'est-à-dire qu'ils doivent être apportés par l'aliment (Sargent, Tocher et al. 2002, Tocher 2003). Ces deux acides gras peuvent ensuite être allongés et désaturés en acides gras polyinsaturés à plus longue chaine (AGPI-LC): l'acide arachidonique (ARA) de la série n-6, et les acides eicosapentaénoïque (EPA) et docosahexaénoïque (DHA) de la série n-3.

En général, la bioconversion en AGPI-LC est considérée plus efficace chez les poissons d'eau douce que chez les poissons marins. En particulier, chez la truite arc-en-ciel, cette voie permet une production efficace d'AGPI-LC à partir de l'acide linolénique (18:3 n-3) ou linoléique (18:2 n-6). La faible capacité de bioconversion observée chez les poissons marins pourrait s'expliquer par la relative abondance des AGPI-LC n-3, en particulier EPA et DHA, dans la chaine alimentaire du milieu marin qui aurait rendu dormantes les capacités de biosynthèse de ces AG (Sargent, Tocher et al. 2002).

Stockage et catabolisme des lipides

Chez les poissons, les lipides de réserve sont principalement des triglycérides (TAG), qui sont stockés dans différents tissus : tissu adipeux péri-viscéral, tissu adipeux sous-cutané, muscle et foie, en proportions variables selon les espèces. Chez les salmonidés ils sont stockés en priorité dans le tissu adipeux péri-viscéral ainsi que dans le muscle (Corraze and Kaushik 2009), tandis que chez les poissons marins comme le bar on les retrouve en majorité au niveau du foie et des viscères (Corraze and Kaushik 1999).

Les lipides stockés dans les tissus servent de réserves énergétiques, mobilisables en cas de besoin. Comme chez les mammifères, les AG sont libérés suite à l'hydrolyse des TAG. Ils sont ensuite oxydés grâce à la β-oxydation. Ce processus qui a lieu principalement dans la matrice mitochondriale (et dans une moindre mesure dans les peroxysomes) consiste en une succession de réactions au cours desquelles les AG sont découpés progressivement en acetyl-CoA. L'acetyl-CoA peut ensuite être métabolisé via le cycle de Krebs pour produire du NADH, qui participe à la fourniture d'énergie métabolique sous forme d'ATP via la phosphorylation oxydative.

Les réserves lipidiques tissulaires sont utilisées par le poisson comme source d'énergie à long terme, notamment lorsque les besoins énergétiques sont accrus, comme par exemple pendant la migration et pendant la reproduction. Durant ces périodes, les lipides sont mobilisés dans un premier temps à partir des sites principaux de stockage et dans un deuxième temps à partir des sites de stockage secondaires.

Lipides et acides gras dans la reproduction des poissons

La maturation sexuelle est un processus caractérisé par une forte demande en terme de nutriments et d'énergie. L'alimentation des géniteurs, et en particulier la composition en lipides et acides gras de l'aliment, est connue pour jouer un rôle clé dans l'efficacité de la

reproduction et la survie de la progéniture (Izquierdo, Fernandez-Palacios et al. 2001), les TAG représentant la principale source d'énergie métabolique pour la reproduction (Tocher 2003). L'EPA, le DHA, ainsi que l'ARA, stockés dans les ovules, jouent un rôle majeur dans la qualité des œufs et le développement des alevins.

Chez la truite, le tissu adipeux péri-viscéral est considéré comme la première source d'énergie pour la vitellogenèse. Après mobilisation des AG, le foie assure la synthèse des lipoprotéines (dont la vitellogénine) qui sont ensuite exportées. Ces lipoprotéines sont incorporées dans les ovules où elles constituent, avec les autres classes de lipides, les réserves énergétiques de l'œuf puis de l'alevin qui se développera ensuite.

Les protéines: nutrition et métabolisme chez les poissons

Les besoins

Chez les poissons, comme chez les autres organismes, les acides aminés, qu'ils proviennent de l'absorption intestinale des produits de digestion des protéines alimentaires, de la synthèse de novo et des inter-conversions, ou l'hydrolyse des protéines corporelles, peuvent être utilisées pour:

- la synthèse des protéines corporelles de structure et des autres composants azotés ;
- servir de précurseurs pour d'autres composants (le glucose notamment mais aussi des acides gras) en fournissant les carbones pour le métabolisme intermédiaire ;
- l'oxydation pour la production d'énergie.

A la différence des autres animaux, les poissons utilisent une part importante des protéines alimentaires pour la fourniture d'énergie. Les protéines apportées par l'alimentation doivent donc couvrir à la fois les besoins azotés pour la croissance, en particulier les besoins en acides aminés indispensables que le poisson n'est pas capable de synthétiser et une partie des besoins énergétiques des poissons. De ce fait, la teneur en protéines des aliments piscicoles est 2 à 3 fois plus élevée que celle des autres animaux d'élevage (de l'ordre de 38 à 45 % de la matière sèche de la ration pour les salmonidés et 42 à 55% pour les poissons marins). Le niveau alimentaire optimum en protéines dépend de la proportion des autres macronutriments (glucides et lipides) et de leur digestibilité. En conséquence, l'apport alimentaire est le plus souvent raisonné en terme de rapport protéines/énergie digestible.

Les AAI pour les poissons sont bien identifiés et sont les mêmes que pour les autres animaux : arginine, histidine, isoleucine, leucine, lysine méthionine, phénylalanine, thréonine,

tryptophane et valine. Des valeurs seuils ont été déterminées chez les principales espèces d'élevage piscicole (NRC 2011); elles sont proches des valeurs connues pour les mammifères et les oiseaux.

Protéines et acide aminés dans la reproduction des poissons

Tout comme les lipides, les protéines provenant de l'alimentation peuvent aussi affecter les performances de reproduction chez le poisson, maturation des gonades, production des œufs, développement des stades précoces, du fait de leur implication dans la synthèse des protéines structurales et de composés tels que les enzymes et les hormones (Izquierdo, Fernandez-Palacios et al. 2001). Une corrélation positive a été montrée entre l'apport protéique alimentaire, la taille des géniteurs et la production des descendants (De Silva and Anderson 1995). Cependant, le rôle des protéines alimentaires dans la reproduction et le développement de la progéniture est moins bien connu que celui des lipides. En outre, les effets du remplacement de la FP par des sources protéiques végétales sur la reproduction ont été peu étudiés.

Les huiles végétales et les effets du remplacement

Caractéristiques des huiles végétales

La principale différence entre les HP et les huiles végétales (HV) concerne leur composition en AG. Alors que les HP sont caractérisées par une forte teneur en AGPI-LC n-3, notamment en EPA et DHA, les HV en sont, sans exception, totalement dépourvues. Les HV contiennent des proportions variables d'acide α-linolénique (18:3 n-3), linoléique (18:2 n-6) et oléique (18:1 n-9), mais aussi d'acides gras saturés. Les autres différences sont liées au fait que les HV contiennent des phytostérols, et pas de cholestérol et ont une teneur plus faible en vitamine A et D que les HP.

Effets du remplacement

D'après Turchini, Torstensen et al. (2009), les lipides jouent un rôle mineur dans l'attractivité de l'aliment. Chez les poissons, la perception sensorielle s'appuie majoritairement sur des composés azotés, des nucléotides et nucléosides. Cependant, dans la pratique de production, les HP utilisées sont rarement pures et peuvent en conséquence contenir certaines de ces molécules attractantes. Ce phénomène pourrait en partie expliquer que le remplacement des HP par des HV soit parfois associé à une réduction de la prise alimentaire (Glencross 2009).

De nombreuses études ont montré qu'en dessous d'un seuil critique de substitution et lorsque les besoins en AGE sont couverts, l'incorporation d'huiles végétales dans l'aliment n'a pas d'impact négatif sur la croissance des poissons. En particulier, chez les salmonidés, l'HP peut être totalement remplacée par des HV (ou par un mélange d'HV), sans affecter la croissance et l'efficacité alimentaire à partir du moment où les besoins en AGE sont couverts, par exemple par les lipides résiduels contenus dans la farine de poisson (Turchini, Ng et al. 2010). L'utilisation des HV modifie peu la teneur globale en lipides musculaires des poissons quelle que soit l'espèce (Bell, McGhee et al. 2003, Richard, Kaushik et al. 2006). En revanche, le profil en acides gras du muscle est profondément modifié. La chair des poissons nourris avec des HV contient moins d'EPA et de DHA, et davantage d'AG caractéristiques des huiles végétales incorporées dans l'aliment (18:1 n-9, 18:2 n-6, 18:3 n-3).

Le changement de la composition en AG de l'aliment quand l'HP est remplacée par des HV, est connu pour affecter aussi les lipides plasmatiques et la lipogenèse, et ces modifications varient selon les HV utilisées.

Chez la plupart des espèces d'eau douce, dont la truite arc-en-ciel fait partie, une alimentation avec un régime riche en C18:3 n-3 caractéristique de certaines HV (comme les huiles de lin, de soja ou de colza) et pauvre voire dépourvu d'AGPI-LC n-3, induit une augmentation des niveaux d'activité des élongases et desaturases, permettant la production d'AGPI-LC n-3 : EPA et DHA (Tocher 2003). Cependant, cette bioconversion n'est pas suffisante pour compenser totalement la baisse d'apport de ces AGPI-LC par l'alimentation.

Du fait du rôle clé de ces deux AGPI-LC pour la qualité des œufs et la survie de la progéniture (Bell, Henderson et al. 1986, Izquierdo, Fernandez-Palacios et al. 2001), leur absence totale de l'aliment lorsque l'HP est totalement remplacée par des HV est connue pour affecter de manière négative les performances de reproduction (Izquierdo, Fernandez-Palacios et al. 2001). Les AGPI affectent aussi les performances de reproduction, via la régulation de la production des eicosanoïdes, et en particulier des prostaglandines impliquées dans différents aspects de la reproduction chez les poissons (Moore 1985).

Les protéines végétales et les effets du remplacement

Caractéristiques de la farine de poisson et des sources protéiques végétales

Plusieurs caractéristiques des FP rendent difficile leur remplacement par des farines végétales (FV) dans les aliments piscicoles. Les FP sont riches en protéines (65 à 72% de la matière sèche), contiennent tous les acides animées indispensables en quantités adéquates pour

couvrir les besoins des différentes espèces de poissons et apportent aussi des AGPI-LC n-3 et des minéraux. Les FV ont généralement des teneurs en protéines modérées; pour atteindre des concentrations protéiques aussi élevées que celles des FP, il faut recourir à des concentrés protéiques tels que les glutens. De plus, les sources protéiques végétales ont souvent des teneurs en certains AAI, comme la lysine et la méthionine, trop faibles pour couvrir les besoins des poissons. Les produits végétaux peuvent aussi contenir des facteurs antinutritionnels dont la concentration doit être limitée pour ne pas perturber la prise alimentaire et le métabolisme des poissons. Enfin, certaines FV ont des taux élevés de glucides, soit sous forme d'amidon, faiblement digestible par les salmonidés, soit sous forme de glucides pariétaux, indigestibles par les poissons.

Effets du remplacement

Les effets du remplacement de FP par FV varient principalement en fonction des sources de protéines végétales utilisées, du taux de substitution et de l'espèce. Chez les salmonidés, aucun effet négatif sur la croissance n'a généralement été observé jusqu'à un taux de remplacement de 80% lorsque le mélange de sources végétales (et éventuellement l'apport d'acides aminés de synthèse) permet de couvrir les besoins en protéines et en AAI (Gomes, Rema et al. 1995, Médale, Boujard et al. 1998, Glencross, Evans et al. 2004, Thiessen, Maenz et al. 2004, Pierce, Palti et al. 2008). Par contre, au-delà de ce taux, la croissance est généralement ralentie (De Francesco, Parisi et al. 2004, Barrows, Gaylord et al. 2007). La principale origine de cette réduction du taux de croissance est la baisse de la prise alimentaire, mais l'efficacité de transformation de l'aliment peut aussi être affectée.

Le remplacement de la FP par des sources végétales a généralement peu d'effet sur la composition en lipides du muscle (Alvarez, Lopez-Bote et al. 1999) même si une faible variation des niveaux d'AGPI-LC a parfois été observée (Médale and Kaushik 2009). Les quantités d'AGPI-LC présents dans la FP étant plus faibles que dans les HP, le remplacement de la FP a cependant un impact moins fort sur le profil en AG. La conséquence principale du remplacement de FP par des FV sur le métabolisme lipidique est la diminution du taux de cholestérol plasmatique chez la truite arc-en-ciel (Kaushik, Cravedi et al. 1995) malgré une stimulation de l'expression des gènes impliqués dans la biosynthèse du cholestérol (Tacchi, Secombes et al. 2012). Plusieurs études ont mis en évidence, notamment chez le bar, un effet du remplacement de la FP sur la lipogenèse hépatique via la diminution de l'activité de la glucose-6-phosphate déshydrogénase, de l'enzyme malique et de l'acide gras synthétase

(Dias, Alvarez et al. 2005). Une étude réalisée chez le saumon a montré que l'expression de certains gènes impliqués dans la bioconversion des AGPI-LC n-3 (i.e. Δ6- et Δ5-desaturase) était stimulée dans l'intestin (Tacchi, Secombes et al. 2012).

Les protéines alimentaires peuvent potentiellement influencer les performances de reproduction des poissons, en termes de fécondité, fertilisation et développement des alevins (Izquierdo, Fernandez-Palacios et al. 2001). Cependant, les connaissances des effets des protéines végétales sur les performances de reproduction des poissons sont limitées car très peu d'études ont été réalisées sur ce sujet. Un des effets majeurs rapporté dans la littérature scientifique est la réduction du taux de croissance des géniteurs, qui peut affecter la taille des œufs (les géniteurs plus petits produisant des œufs de plus faible diamètre), et en conséquence la taille des alevins. Un autre effet du remplacement de FP par des FV concerne la maturation des gonades mais davantage d'études sont nécessaires pour apporter des précisions.

Effets d'un remplacement conjoint de la farine et l'huile de poisson par des sources végétales

La plupart des études réalisées en remplaçant de façon conjointe FP et FV par des substituts végétaux ont montré un cumul des effets négatifs observés lors du remplacement soit de FP soit de FV. Un des effets récurrents est la diminution de la prise alimentaire lorsque les taux de substitution sont élevés (>70-80%), ce qui entraine une baisse du taux de croissance, comme cela a été montré chez le saumon (Torstensen, Espe et al. 2008). Les effets du remplacement conjoint de la FP et l'HP par des ingrédients végétaux sur la réponse métabolique des poissons ont été peu étudiés, et les études ont été focalisées principalement au niveau de l'intestin et du foie. En général, les métabolismes des protéines et des lipides (surtout l'élongation des AG et du cholestérol) sont les plus affectés.

Peu d'études ont été consacrées aux effets du remplacement conjoint de la FP et l'HP par des végétaux sur la reproduction. Cependant, lorsque les deux ingrédients marins sont totalement remplacés en même temps, l'apport alimentaire en AGPI-LC n-3 est nul. Etant donné le rôle clé que ces AG jouent dans la reproduction, et la faible efficacité de la bioconversion du précurseur alimentaire C18:3 n-3, on peut émettre l'hypothèse que la production des œufs et le développement des descendants peuvent être affectés.

Objectifs et questions principales

La plupart des études conduites jusqu'à présent se sont focalisées sur les effets du remplacement de la FP et /HP par des végétaux pendant une partie du cycle de vie du poisson, et en particulier pendant la phase de croissance. Les effets du remplacement total et concomitant de la FP et l'HP pendant tout le cycle d'élevage sont donc peu connus, notamment les effets d'un tel remplacement sur les performances de reproduction, la qualité des œufs, la survie de la progéniture et les conséquences sur les capacités métaboliques des descendants. L'objectif principal des travaux de thèse a donc été de déterminer les effets d'une alimentation à base de plantes, complètement dépourvue de FP et HP, administrée à partir du premier repas et pendant tout le cycle d'élevage de la truite arc-en-ciel. Les effets d'un tel remplacement ont été étudiés à différents stades de développement, en incluant les géniteurs et les stades précoces. Dans ce contexte, les travaux réalisés ont eu pour but de répondre à trois questions majeures:

1. Les truites sont-elles capables de survivre, grandir et se reproduire lorsqu'elles sont nourries pendant tout le cycle de vie avec des aliments uniquement à base des matières premières végétales ? Quels sont les effets sur les ovules et la qualité de la reproduction ?

2. L'histoire nutritionnelle maternelle modifie-t-elle le transcriptome des alevins aux premiers stades de vie ? Les alevins issus de reproductrices nourries avec un aliment exclusivement à base de végétaux ont-ils des aptitudes particulières à utiliser les aliments végétaux ?

3. Quels sont les effets chez la truite arc-en-ciel d'une alimentation à long terme (et depuis le premier repas) exclusivement à base de matières premières végétales sur l'expression des gènes intestinaux et hépatique ? Les éventuels effets observés sont-ils persistants dans le temps ? En d'autres termes, les effets observés chez les alevins et les juvéniles sont-ils transposables aux stades ultérieurs ?

Chapter 1

Introduction and review of literature

Figure 1.1 Evolution of global per capita fish consumption during the last five decades (FAO 2015).

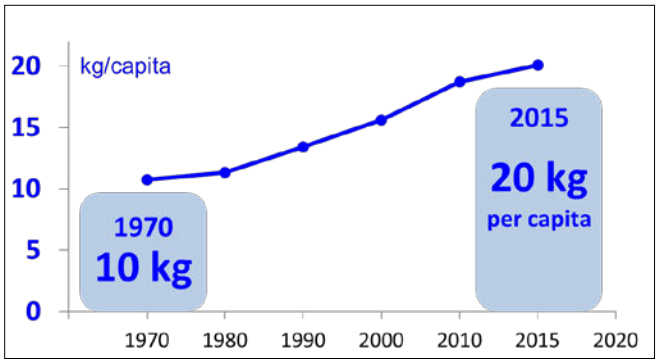
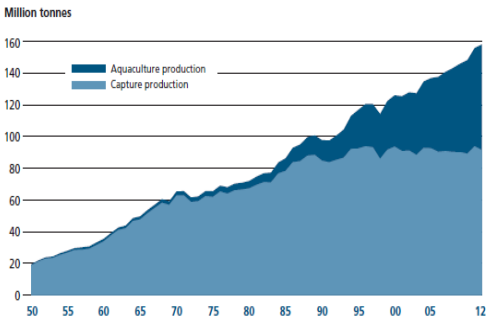
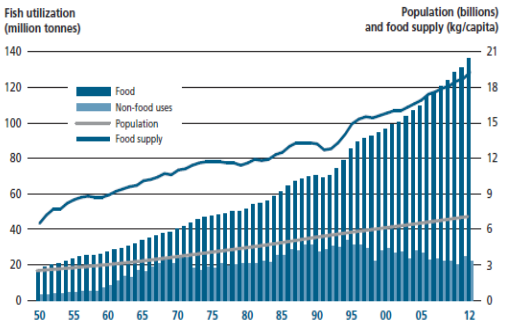


Figure 1.2 Aquaculture development: current status, trends and future prospects (FAO 2014).

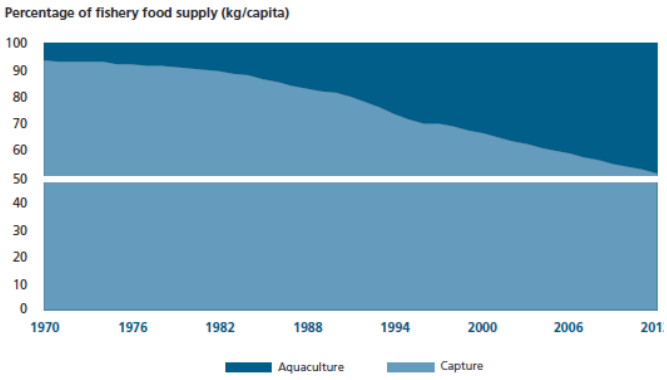
a) World capture fisheries and aquaculture production [Mt].



b) World fish utilization and supply [Mt].



c) Relative contribution of aquaculture and capture fish to food fish consumption.



1.1 Introduction

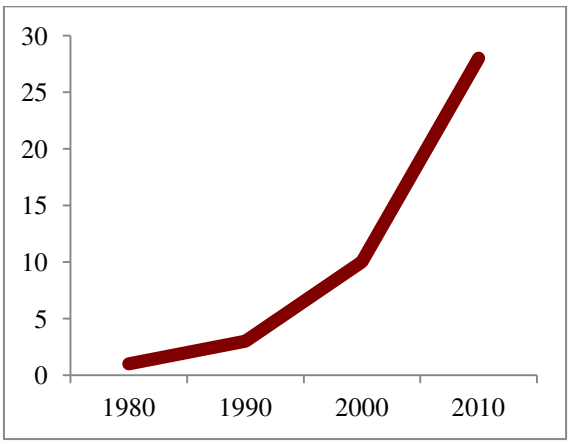
1.1.1 Aquaculture development and role in the global food system

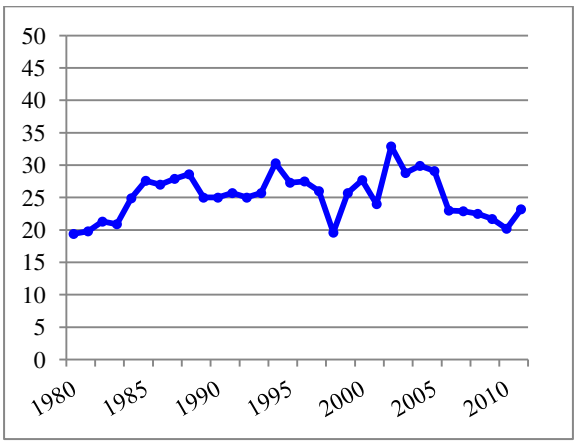
Fish have been a regular part of the human diet as long as 40 millennia ago and represent an important food commodity today. Fish currently account for 17% of animal derived protein and 6.5% of total human protein consumption globally (FAO 2014). Fish products, valued at 129 billion US$ in 2012, comprise one of the most widely traded segments of the world food economy (Troell, Naylor et al. 2014). During the past five decades, world per capita fish consumption displayed an impressive increase from an average of 10 kg in the 1970s to 19.2 kg in 2012 (FAO 2014), and around 20 kg per capita in 2015 (Figure 1.1). Indeed, fish are currently estimated to feed more than 2.9 billion people (FAO 2014). Fish do not only represent an excellent source of protein, but are also a unique and rich source of long chain polyunsaturated n-3 fatty acids (LC-PUFAs n-3), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Both fatty acids are key dietary components for human health. Indeed, these n-3 fatty acids have well-known and almost universally accepted beneficial effects on neuronal development in young children and in the prevention of a range of human pathologies, including cardiovascular and inflammatory disease, as well as neurological disorders (NRC 2011). Fish represent an important source of micronutrients (vitamins and minerals) and some less well-known nutrients such as taurine and choline. This unique blend of nutrients make fish a truly unique and important food for human consumption (Tacon and Metian 2013, FAO 2014).

Global fish production has grown steadily at an average annual rate of 3.2% over the last five decades (Figure 1.2 a – b), outpacing world population growth by 1.6% (FAO 2014). This increase in food fish supply has been facilitated by the dramatic increase in aquaculture production. Although capture fisheries provided most of the fish supply during the 1960-1970s, global capture production has leveled off since the end of the 1980s. In order to maintain a consistent supply of high-quality and sustainable seafood for a growing human population, aquaculture production has expanded by almost 12-fold since the 1990s (FAO 2014). In 2012, aquaculture industry set an all-time record, providing 67 million tons fish products, which accounts for about 49% of the fishery output destined for human consumption (Figure 1.2 c) (FAO 2014). Aquaculture is the fastest growing food production

Figure 1.3 Aquaculture growth increases needs for fish feed (FAO 2014).

a) Increase in aquafeed production [Mt]. b) Stable feed grade fisheries captures [Mt]**.**





These figures illustrate the increase in terms of aquafeeds production registered worldwide during the last three decades (a), and the concomitant stagnation in the feed grade fisheries captures in the same period (b).

sector, and it continues to expand alongside terrestrial crop and livestock production (Troell, Naylor et al. 2014). As a result of the impressive growth of aquaculture, the demand in terms of aquafeeds has also rapidly increased over the last years (Figure 1.3 a). Thus, global industrial compound aquafeeds production has increased almost four-fold from 7.6 million tons in 1995 to 29.2 million tons in 2008, and is expected to reach 71 million tons by 2020 (FAO 2014). This trend clearly indicates that the availability of quality aquafeeds, and therefore quality feed ingredients, is a major challenge to support the growing aquaculture production.

Aquaculture feeds have been traditionally based on fish meal (FM) and fish oil (FO) derived from industrial feed-grade fisheries (also called reduction fisheries) of small pelagic species such as anchovies, sardines, herring and mackerel. FM and FO supply essential amino acids and polyunsaturated fatty acids of the n-3 series (EPA and DHA), respectively, and are preferentially used because they mimic the food consumption of fishes in their natural habitat. However, reduction fisheries have reached their sustainable limits (Figure 1.3 b), and there is no realistic prospect of FM and FO production being increased in the future. Therefore, the strictly limited supply and ever-rising demand resulting in increasing prices, as well as the necessity to preserve wild fish populations, make it crucial to find efficient alternatives to FM and FO for aquaculture to continue to expand (Naylor, Goldburg et al. 2000, Tacon and Metian 2008, FAO 2014).

1.1.2 Towards a sustainable aquaculture

In the future, the amount of global aquaculture productions is expected to continue to expand to meet the growing consumer demands for seafood (Hardy 2010, Hixson 2014). To improve aquaculture sustainability, many issues require to be addressed, in particular those related to fish nutrition. As previously described, FM and FO have represented the most important ingredients for aquafeeds during the last decades. However, both are now a limited resource, and their prices, which have continually risen in the past three decades, are likely to increase further with continued growth in demand. Thus, a reduction in the use of FM and FO represents a major challenge for sustainable aquaculture.

The current situation of aquaculture has therefore forced a change from marine resources towards more sustainable ingredients in fish diets. Terrestrial plant ingredients represent a good alternative, mainly due to their abundance and the competitive market price (Gatlin, Barrows et al. 2007, Hardy 2010). Significant progress has been made during the last years in

partially replacing marine FM and FO by plant ingredients in nutritionally well-balanced diets. Nevertheless, the incorporation of plant products at high levels in fish diets is recognized to have some disadvantages, particularly related to the differences in amino acid (AA), carbohydrate, cholesterol and fatty acid (FA) composition compared to marine resources, but also to the presence of anti-nutritional factors in vegetables. Consequently, the replacement levels have to be adapted depending on the species and the developmental stages. These differences in composition can have metabolic and physiological consequences that deserve further investigation to provide adequate background for successful greater use of plant feedstuffs in aquafeeds.

The presented research was undertaken to identify the potential effects of a combined and total replacement of FM and FO by plant proteins and vegetable oils during the whole life cycle of rainbow trout (*Oncorhynchus mykiss*). Therefore, the following sections will firstly introduce basic concepts of fish nutrition, underlining the importance of lipid and protein metabolism in fish. Subsequently, the effects of FO, FM or combined FM and FO replacement in fish metabolism and physiology, at both the biochemical level and molecular-transcriptional level, will be discussed.

1.2 Lipid nutrition and metabolism

Lipids, together with proteins and carbohydrates, comprise the major macronutrients that are required to provide both essential nutrients for energy production and building blocks for cell and tissue development. In this context, they contribute to growth and maintenance of homeostasis in all organisms (Bell and Koppe 2011). Specifically, dietary lipid supply is crucial for different reasons in fish. First of all, lipids are vital to supply essential fatty acids (EFA). EFAs are defined as not being independently synthesized by the organism, but are necessary for the proper function of cellular metabolism (synthesis of prostaglandins and similar compounds), as well as for the maintenance of membrane structure integrity (via their fluidity). Secondly, they also mediate the intestinal absorption of different compounds, such as liposoluble vitamins and carotenoid pigments. Lastly, lipids play a major role in energy supply, a role which is of upmost importance in fish, since several fish, including rainbow trout, poorly digest carbohydrates (Guillaume 2001).

1.2.1 Lipids and essential fatty acid requirements in fish

Dietary lipid requirement is complicated to define for any fish species, because it is influenced by a variety of different factors. The factors include the chemical nature (ability to react in chemical meaning) and the different functional roles of lipids, the competition with other macronutrients (proteins and carbohydrates) as dietary energy sources, and the environmental factors, such as temperature (NRC 2011). Due to all these factors, the definition of the exact lipid requirements for fish is not particularly meaningful. However, it has long been accepted, that dietary lipid amounts ranging from 10-20% (dry weight basis) are sufficient to allow protein to be effectively used for fish growing, without resulting of the deposition of excessive lipids in fish tissues (Cowey and Sargent 1979, Watanabe 1982, Sargent, Henderson et al. 1989, Corraze 2001).

Another important aspect affecting the requirement of dietary lipid is linked to the fact that dietary lipids are necessary to supply EFAs. The fatty acids (FA) that are commonly termed 'essential' are not synthesized *de novo* in the organism, and must therefore be supplied by the diet (Glencross 2009). EFAs are important components of phospholipids, which are themselves the major constituents of cell membranes and transport lipoproteins. EFA are also implicated in the synthesis of a whole family of molecules, which have a hormonal function in *sensu lato*: the prostaglandins and similar compounds, such as leukotriens and thromboxanes (Sargent, Tocher et al. 2002). Moreover, some of these EFAs are known to be the precursors of an important class of signaling molecules named docosanoids. These molecules, which are made by oxygenation of twenty-two-carbon essential fatty acids (EFAs), especially docosahexaenoic acid (DHA), have been mainly studied in mammals and are known to possess both anti-inflammatory and protective properties.

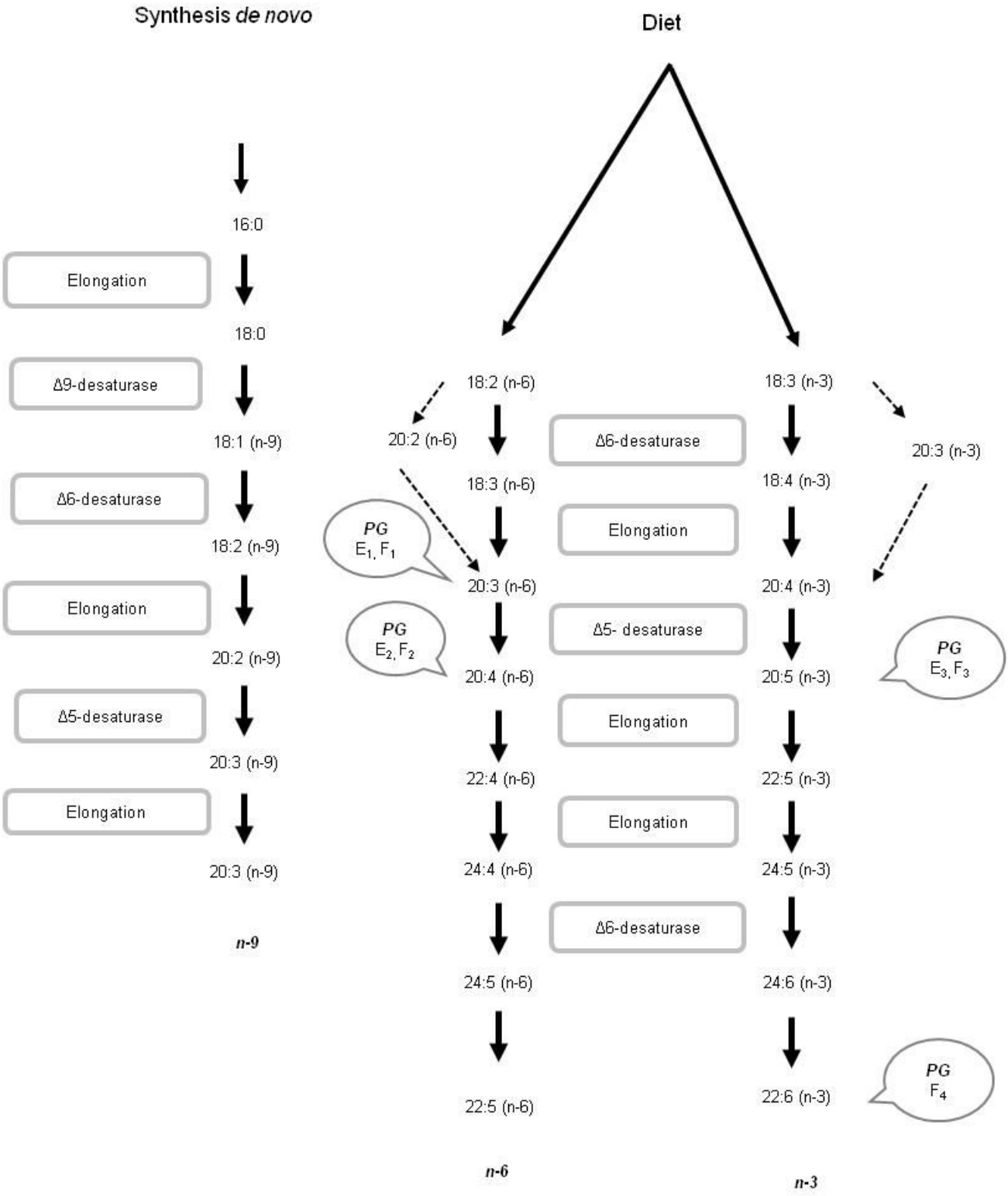
Table 1.1 Essential fatty acids (EFA, % dry diet) requirements of different fish species.

		EFA requirements			
Species	Scientific name	18:3 n-3	18:2 n-6	n-3 LC-PUFA	n-6 LC-PUFA
Freshwater and diadromous fish					
Arctic charr	*Salvelinus alpines*	1.0 - 2.0			
Atlantic salmon	*Salmo salar*	0.5 - 1.0		0.5 - 1.0	
Common carp	*Cyprinous carpio*	0.5 - 1.0	1.0		
Rainbow trout	*Onchorynchus mykiss*	0.7 - 1.0	0.8 - 1.6	0.2 - 1.0	
Tilapia	*Tilapia zilli*		1.0		
Marine fish					
European sea bass	*Dicentratux labrax*			1.0	
Gilthead seabream	*Sparus aurata*			0.5 - 1.9	
Turbot	*Psetta maxima*			0.6 - 1.3	0.3
Yellowtail flounder	*Pleuronectes ferrugineus*			2.5	

(Adapted from: Turchini, Torstensen et al. 2009, NRC 2011).
18:3 n-3, alpha-linolenic acid; 18:2 n-6, linoleic acid; n-3 LC-PUFA, n-3 long chian-polyunsaturated fatty acids; n-6 LC-PUFA, n-6 Long chain- polyunsaturated fatty acids.

In fish, the precise nature of EFA and their absolute dietary requirements are difficult to determine, and a high variability exists among species, in particular between freshwater and marine fish (Tocher 2003, Glencross 2009, NRC 2011). This difference is essentially related to the capacity of fish to bio-convert C_{18} FA into LC-PUFAs, and therefore to the distinct activity of two classes of enzymes implicated in this conversion process: elongases and desaturases. Elongases are responsible for the condensation of activated FAs with malonyl-CoA in the FA elongation pathway, while desaturases introduce a double bond in the fatty-acyl chain at the C_6 or C_5 position from the carboxyl group (Tocher 2003). This ability for elongation and desaturation is considered to be more effective in freshwater fish than in marine fish (Bell and Koppe 2011). The difference between marine and freshwater fish can generally be accounted for by considering the natural diets of the different species. Indeed, while marine food webs are generally characterized by high levels of LC-PUFAs of the series n-3 (n-3 LC-PUFA, which mainly came from the presence of microalgae and plankton), namely eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), freshwater food webs are not. On the other hand, freshwater food webs are rich in alpha-linolenic acid (18:3 n-3) and linoleic acid (18:2 n-6). Therefore, the widespread ability of freshwater fish to convert C_{18} PUFAs to the biologically active C_{20} and C_{22} PUFAs may be the result of an evolutionary adaptation and a high evolutionary pressure to maintain the capacity to endogenously produce LC-PUFA in freshwater species (Tocher 2003). Generally speaking, one can say that the only EFAs which are truly essential in freshwater fish are the two C_{18} (alpha-linolenic and linoleic acid), while the principally important EFAs for marine fish are PUFAs and, in particular EPA and DHA. However, it is important to keep in mind that this classification must be considered as a highly simplified classification, and that differences may exist even within the two classes. Indeed, although it is generally accepted that for freshwater fish 18:3 n-3 is the only EFA, the specific dietary requirements of this EFA can vary between 0.5 and 1% between species. EFA requirements can be variable, but requirement ranges for C_{18} PUFAs and LC-PUFAs for a certain number of species have been established, and the most important findings are summarized in Table 1.1.

Figure 1.4 Schematic representation of desaturation and elongation of FA to their LC-PUFA products.



This figure is adapted from: Tocher 2003 and Turchini, Torstensen et al. 2009. See text for further information. PG: prostaglandins; Δ9: delta-9-desaturases; Δ6: delta-6-desaturases; Δ5: delta-5-desaturases.

1.2.2 Endogenous synthesis and bioconversion of fatty acids

The FAs supplied by the diet and those endogenously synthesized by the animal can, to a certain extent, be bioconverted to FA with longer or more unsaturated chains (Corraze 2001).

In fish, the liver represents the main site for FA synthesis through the FA synthetase complex (Corraze 2001). Fish, as is the case for all organisms, can *de novo* synthesize the saturated fatty acids 16:0 (palmitic acid) and 18:0 (myristic acid) in different proportions, depending on the species (Cook 1996, Sargent, Tocher et al. 2002). All fish are then capable to convert these two *de novo* synthesized FA into monounsaturated FAs (MUFAs), such as 18:1 n-9, through the action of Δ9-desaturase. However, fish, as well as mammals and other vertebrates, lack both the Δ12-desaturase and Δ15-desaturase, which are necessary to convert 18:1 n-9 to 18:2 n-6 and 18:2 n-6 to 18:3 n-3, respectively (Tocher 2003). Thus, both 18:2 n-6 and 18:3 n-3 are EFAs, and must consequently be supplied by the diet (Sargent, Tocher et al. 2002, Tocher 2003). These two FAs must be further converted into their LC-PUFA bioactive products: arachidonic acid (ARA) of the n-6 series, and EPA and DHA of the n-3 series.

As mentioned previously, the capacity to convert C_{18} PUFA to LC-PUFA varies within fish species, being high in freshwater and low in marine fish species (Sargent, Tocher et al. 2002, Tocher 2003). Synthesis of ARA from its C_{18} precursor requires a first step of Δ6-desaturation followed by an elongation to produce 20:3 n-6, which is then desaturated to 20:4 n-6 by the action of Δ5-desaturase. The synthesis of ARA then requires additional elongation and Δ6-desaturation steps, followed by a chain-shortening step in the peroxisome (Sargent, Tocher et al. 2002). Synthesis of EPA is achieved by Δ6-desaturation of 18:3 n-3 to produce 18:4 n-3, which, in turn, is elongated to 20:4 n-3, followed by Δ5-desaturation (Cook 1996). The synthesis of DHA from EPA requires two further elongation steps, a second Δ6-desaturation and a peroxisomal chain-shortening step (Sprecher 2000). The biochemical pathway of LC-PUFA synthesis is depicted in Figure 1.4.

Freshwater fish, including rainbow trout, are able to convert C_{18} (linoleic acid, 18:2 n-6 and linolenic acid, 18:3 n-3), into PUFAs (Corraze 2001). In marine fish, the biosynthetic activity of the LC-PUFA synthesis pathway seems to occur at a much lower level, or may even be entirely absent, compared to freshwater fish. At the molecular level, previous studies have shown that Atlantic salmon (*Salmo salar*) possess separate genes for Δ5 and Δ6 fatty acyl desaturases (Hastings, Agaba et al. 2004, Zheng, Tocher et al. 2005, Monroig, Zheng et al. 2010). Distinct Δ6-desturase cDNA have been isolated from different fish species including

freshwater and marine species (Seiliez, Panserat et al. 2001, Seiliez, Panserat et al. 2003, Zheng, Seiliez et al. 2004, Tocher, Zheng et al. 2006). A bifunctional Δ5/Δ6-desaturases have been isolated from zebrafish, *Danio rerio* (Hastings, Agaba et al. 2001). For example, while marine fish appear to have some of the enzymatic steps required to biosynthesize DHA, in European sea bass (*Dicentrarchus labrax*) and Atlantic cod (*Gadus morhua*), either the Δ5-desaturase and/or the C_{18}-C_{20} elongation step appears to be blocked (Mourente, Good et al. 2005, Bell, Strachan et al. 2006, Tocher, Zheng et al. 2006) Thus, DHA must thus be supplied by the diet in marine fish.

1.2.3 Storage of lipids

Stored lipids are mainly triglycerides, which are synthesized in tissues from free circulating FAs liberated by the action of lipoprotein-lipase (LPL); stored lipids may additionally stem from endogenous *de novo* synthesis (Corraze 2001, Bell and Koppe 2011). As a consequence, both dietary lipid levels and the rate of newly synthesized FA contribute to fish whole body lipid content, although at different proportions. Feeding fish diets with high lipid levels undoubtedly contributes to increase body lipid contents (Sargent, Tocher et al. 2002, Tocher 2003).

The location and distribution of lipid depots varies between organisms, and while in mammals lipid storage is mainly restricted to a specific tissue (*i.e.* the adipose tissue), it can occur in different tissues, such as the liver, muscle, perivisceral adipose tissue or even subcutaneous tissue in fish (Corraze 2001). The localization of lipid storage is species-specific, and the lipid content of muscle can be used as criterion to differentiate between different categories of fish. Briefly, it is possible to distinguish between 'fat' fish, which have flesh lipid content greater than 10% (*i.e*. herring, *Clupea harengus*; mackerel, *Scomber scombrus*), 'lean' fish with muscle lipid content lower than 2% (*i.e.* cod) and an 'intermediate' group, for which the muscle lipid content is around 2.5 and 6% (*i.e*. trout).

In rainbow trout, as in other salmonids, lipid deposition mainly occurs in visceral adipose tissue, and to a lesser extent, in muscle (Corraze and Kaushik 1999). In marine species such as European seabass, lipids are mainly accumulated in liver and viscera (Corraze and Kaushik 1999), while in flat fish like turbot, Atlantic Halibut (*Hippoglossus hippoglossus*) and Senegalese sole (*Solea senegalensis*), subcutaneous fat deposition is also an important contributor to fish whole body lipid content (Regost, Arzel et al. 2001, Martins, Valente et al. 2007, Borges, Oliveira et al. 2009).

1.2.4 Mobilization and catabolism of lipids

Triacylglycerols (TAGs) are stored in fish tissues where they principally serve as long-term energy sources, which can be used when the energy requirement of the organism exceeds the energy available from the diet. This occurs in particular when the energy requirements for fish are very high, for example during reproduction or migration (Tocher 2003). During these highly energy- demanding periods, lipids are probably firstly mobilized from the main lipid storage tissue (*i.e.* adipose tissue in salmonids), and then from the secondary lipid storage sites (*i.e.* liver and muscle in salmonids) (Tocher 2003).

Lipid mobilization in mammals is dependent on a "hormone-dependent" TAG-lipase, which can hydrolyze TAG. In fish, the existence of a TAG lipase has been clearly demonstrated in trout, in particular in the liver and adipose tissue (Sheridan 1994). This enzyme, localized in the cytosol, is activated by a phosphorylation/ de-phosphorylation process, but less is known about its upstream hormonal regulation (Sheridan 1994). In fish, as in mammals, FAs liberated by the hydrolysis of TAG are then oxidized, mainly in the mitochondria and, to a lesser extent, in peroxisomes, leading to the provision of energy through successive rounds of β-oxidation (Médale and Guillaume 2001, Sargent, Tocher et al. 2002).

Generally speaking, β-oxidation involves the sequential cleavage of two-carbon units, released as acetyl-CoA through a cyclic series of reactions catalyzed by several distinct enzyme activities (Tocher 2003). Briefly, activated FAs are transported into the mitochondrion in the form of fatty acylcarnitine esters formed through the action of carnitine acyltransferases, converted back into fatty acyl-CoA derivatives, that then undergo a round of dehydrogenation, hydration, second hydrogenation and cleavage steps to produce acetyl-CoA and NADH. Acetyl-CoA can then be metabolized via the tricarboxylic cycle to produce more NADH, which can subsequently provide metabolic energy in the form of ATP through the process of oxidative phosphorylation (Figure 1.5). β-oxidation occurs mainly in the mitochondrial matrix, but it can also occurs at the level of peroxisome. Peroxisomal β-oxidation mainly acts to shorten LC-PUFAs, such as 22:1 n-9 and DHA. The shorter chain derivatives are then transferred to the mitochondria for oxidation (Sargent, Tocher et al. 2002)). It is generally accepted that mitochondria are more abundant than peroxisomes in most animal cells, and that they contribute to more than 90% of cellular oxidation of FAs under normal physiological conditions (Mannaerts, Debeer et al. 1979). On the other hand, in fish, the peroxisomal β-oxidation is preponderant in the liver (Nanton, Lall et al. 2003).

Previous studies with different fish species, including haddock (*Melanogrammus aeglefinus*), rock cod (*Lotella rhacina*) and Atlantic salmon, have shown that peroxisomal β-oxidation could amount to as much as 30 to 100% of the overall hepatic oxidation of FAs (Crockett and Sidell 1993, Frøyland, Lie et al. 2000, Nanton, Lall et al. 2003). Peroxisomal β-oxidation, however seems to be limited in white and red muscle, where mitochondrial β-oxidation is largely predominant (Crockett and Sidell 1993, Frøyland, Madsen et al. 1998, Nanton, Lall et al. 2003). The most active tissues for β-oxidation are red muscle, heart, and liver, but due to the large mass present, white muscle often represents the major site of β-oxidation in fish (Nanton, Lall et al. 2003, Stubhaug, Frøyland et al. 2005).

As mentioned before, body lipid reserves are actively mobilized when the energy demand is elevated. A particular example is the fish reproduction period, because the production of gametes, in particular the eggs, is very energy intensive (Sargent, Tocher et al. 2002).

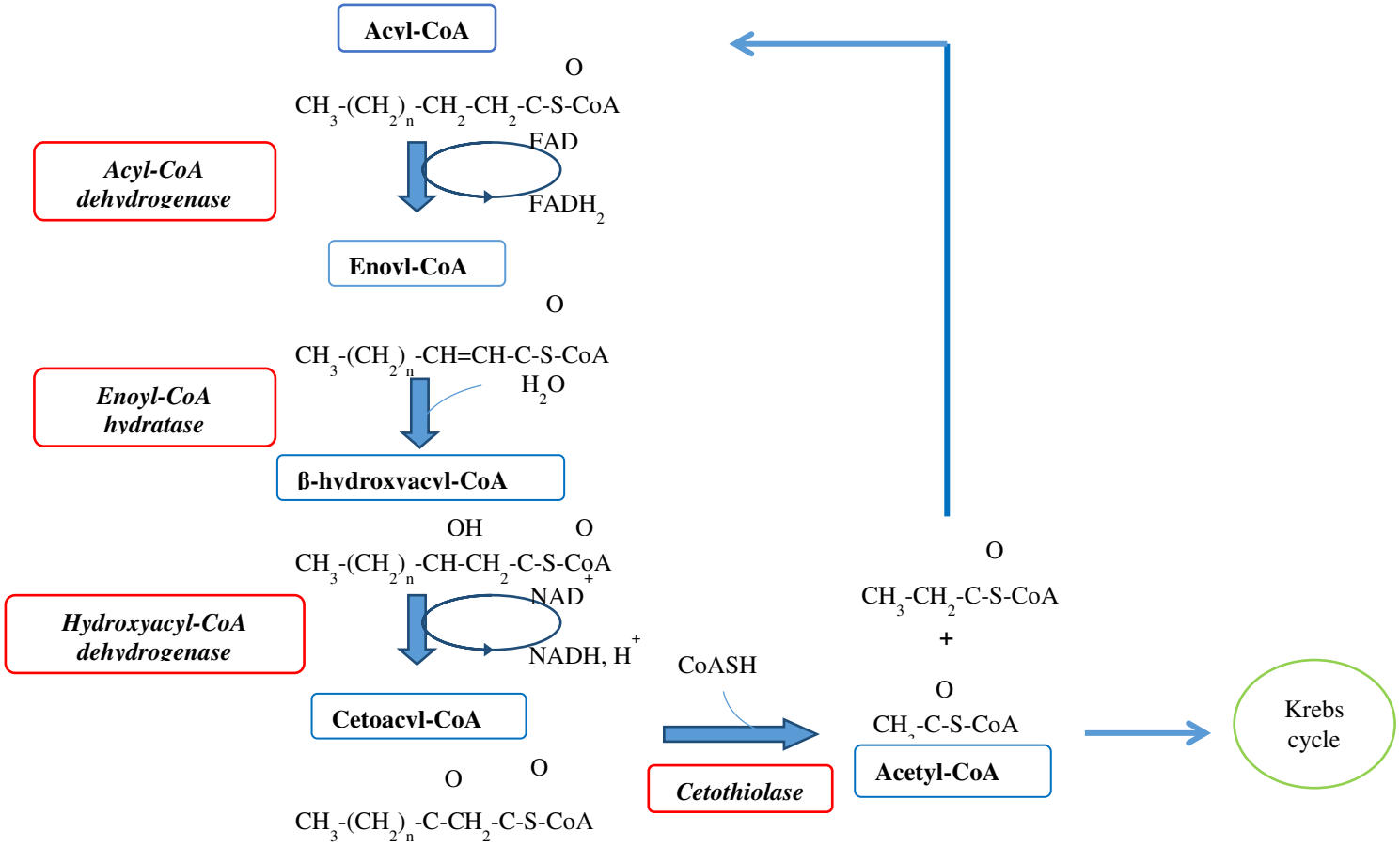


Figure 1.5 Schematic representation of the principal steps of mitochondrial β-oxidation.

1.2.5 Lipids and fatty acids in fish reproduction

It is generally accepted, that despite differences among species, the quality of broodstock diet, and in particular the lipid and fatty acid composition, is a major factor in determining successful reproduction and survival of offspring (Izquierdo, Fernandez-Palacios et al. 2001). In particular, fatty acids represent the major source of metabolic energy for fish reproduction (Tocher 2003), which is necessary not only to support the requirement of parent fish, but also the future requirement of progeny.

Nutritional deficiencies in broodstock diets are known to affect fish fecundity, one of the parameters commonly used to define the egg quality of fish (Fernandez-Palacios, Izquierdo et al. 1995). Fecundity represents the total number of eggs produced by each fish, and it could be expressed either in terms of eggs/spawn or eggs/body weight (Izquierdo, Fernandez-Palacios et al. 2001). Previous studies have show a reduced fecundity in several marine species, which could have be due either to a nutrient imbalance on the brain–pituitary–gonad endocrine system or to the restriction in the availability of a biochemical component for egg formation. For example, in diets for rabbitfish broodstock, the elevation of the lipid content from 12% to 18% resulted in an increased fecundity and hatching. However, this effect could be also related to the fact that the increase in lipid content in turns results to a gradual increase in the dietary PUFAs content. Indeed, the dietary FA composition of broodstock diet is recognized as a major nutritional factor affecting reproductive performance in fish (Izquierdo, Fernandez-Palacios et al. 2001). For example, in gilthead seabream and other sparids, a significant increase in fecundity was observed with an increase in dietary LC-PUFAs n-3 levels. However, in a study with Nile Tilapia (*Oreochromis niloticus*), the reproductive performance (number of females that spawn, spawning frequency, number of fry per spawning and total fry production) over a 24-week trial was much higher when fish were fed a diet supplemented with soybean oil, which is rich in n-6 FA (essential for this species) (Watanabe 1982) and relatively low in fish fed a diet supplemented with cod liver oil, which in turn is rich in n-3 FA (Santiago and Reyes 1993).

In many fish species, sexual maturation is an energy and nutrient demanding physiological process that alters growth efficiency and compromises muscle quality. In several fish species, with the exception of salmonids and turbot, lipids stored in the muscle are used in the process of maturation of ovaries (Lie, Mangor-Jensen et al. 1993). On the contrary, previous studies in salmonids, and specifically in rainbow trout, have shown that during sexual maturation, lipids

are initially mobilized from visceral adipose tissue (Nassour and Léger 1989). Mobilization from secondary storage sites (*e.g.* muscle) occurs only in the long term (Tocher 2003). Moreover, visceral adipose tissue appears to be the primary source of energy for vitellogenesis (Manor, Weber et al. 2012), a crucial step in the female reproductive cycle. After mobilization of adipose fatty acids, this process ensures subsequent hepatic synthesis and export of lipoproteins (vitellogenin). These lipoproteins, together with lipids and vitamins, are finally taken up by the ova through endocytosis and provide energy reserves for ovum and offspring development. During this process, n-3 LC-PUFAs such as EPA and DHA are preferentially incorporated into ova, typically at a ratio of 2:1 (Bromage and Roberts 1995). EPA and DHA, as well as n-6 PUFA (ARA), are recognized as determining factors in egg quality of several species (Harel, Tandler et al. 1994, Cerdá, Zanuy et al. 1995, Abi-Ayad, Mélard et al. 1997, Navas, Bruce et al. 1997, Takeuchi 1997, Rodriguez, Cejas et al. 1998, Salze, Tocher et al. 2005) and in offspring development (Leray, Nonnotte et al. 1985). In addition to meeting energy requirements of early ontogenesis, a specific role of n-3 LC-PUFAs, in particular DHA, is its incorporation into forming membranes in order to maintain their fluidity (Sargent 1995).

1.3 Protein nutrition and metabolism in fish

Protein is the single most important and expensive dietary component of fish diet, especially for salmonids and marine fish, which tend to have higher dietary protein requirements than the other fish species (Wilson 2002). Dietary intake of proteins is essential for animal growth and development (Tremblay, Perreault et al. 2007). The ingested proteins are digested or hydrolysed to release tri- or di-peptides and free amino acids (AAs), which are absorbed from the intestinal tract and distributed by the blood to the organs and tissues. A regular intake of protein or AAs is required because they are used continuously by the fish to either synthesize new protein, especially during growth and reproduction, or to replace existing proteins. Inadequate dietary protein level results in reduction or cessation of growth and a loss of weight. These effects are due to the withdrawal of protein from less vital tissues to maintain the function of more vital tissues. On the other hand, if the dietary protein level is too high, only a part of it will be used to make new proteins and the remaining part will be converted to energy.

1.3.1 Protein and amino acids requirement

Proteins and AAs are critical molecules because of the role they play in cell membrane structure and metabolism of all living organisms. Fish, as well as other animals, cannot synthesize all AAs, and must therefore acquire specific AAs with their diet, either through the consumption of proteins or, albeit to a lesser extent, the consumption of mixtures of AAs.

Protein requirements

In fish, as well as in others animals, the optimal dietary protein level is influenced by the optimal dietary protein-to-energy balance, the AA composition and digestibility, as well as the amounts of non-protein energy sources in the diet (Wilson 2002). Fish total protein requirements appears to be very high and data reviewed by Wilson (2002) indicate that the protein requirements of fish are two or four times higher than those of other vertebrates. This difference may be related to the fact that fish are ammonotelic. Indeed, the great majority of teleost fish excrete around 80% of their nitrogen from nitrogenous catabolism in the form of ammonia, which represents a minimal cost for discharging nitrogenous waste. In contrast, mammals and birds excrete urea and uric acid respectively, the biosynthesis of which is energetically much more costly (Mambrini and Guillaume 2001).

Several factors are known to affect protein requirements of fish, including fish size and age. Generally, the protein requirement of fish decrease with increasing size and age. In very young salmonids, for example, the optimal dietary protein level is around 45-50% of the diet, while the level decreases in juveniles (40%) and is even lower (35%) in yearlings (Hilton and Slinger 1981, Hardy 1989). The same trend is also observed for other species, such as channel catfish (*Ictalurus punctatus*) and tilapia (*Oreochromis niloticus*). The channel catfish fry require around 40% protein, while fingerlings need 30 to 35%, and larger fish require only 25 to 35% proteins (Page and Andrews 1973, Wilson 1991). In Nile tilapia, the protein requirements decrease from 50% in fry to 35% in juveniles (Lim 1989). Another factor, which induces changes in protein requirement of fish is water temperature. Indeed, changes in water temperature have been shown to alter protein requirements in some fish species, whereas no changes were found for others species. For example, striped bass (*Morone saxatilis*) were found to require 47% protein when reared at 20°C, and about 55% protein when reared at 24°C (Millikin 1982, Millikin 1983). On the contrary, when rainbow trout were fed practical diets containing 30%, 40% and 45% crude protein at water temperature ranging from 9°C to 18°C, no differences in protein requirement could be observed (NRC 2011).

Essential amino acids (EAA) requirements

The AAs that fish are not able to synthesise are termed essential amino acids, or EAAs. The dietary protein input must cover the requirements in EAAs in order to avoid deficiencies. EAAs for fish are well-established and are common to other farmed animals. They are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. They can be classified into three different categories (Mambrini and Guillaume 2001), depending on the way they are synthesized:

- the first category includes EAAs that are synthesized within the framework of intermediate metabolism (arginine and histidine);
- the second category comprises of EAAs which are synthesized by transamination, but at an insufficient rate because of the lack of substrate (isoleucine, leucine, tryptophan, valine, methionine and phenylalanine);
- the third category is represented by those EAAs that cannot be synthesized from any intermediate metabolism, due to the absence of the required transaminases (lysine and threonine).

Specific requirements for the main aquaculture species are summarized in Table 1.2 (NRC 2011). In addition fish have a requirement for a well-balanced mixture of essential (or indispensable) and nonessential (or dispensable) amino acids.

Table 1.2 Amino acid (% dry diet) requirements of different freshwater and marine species.

	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Triptophan	Valine	Taurine
Freshwater fish											
Salmo salar	1.8	0.8	1.1	1.5	2.4	0.7	0.9	1.1	0.3	1.2	NR
Cyprinus carpio	1.7	0.5	1.0	1.4	2.2	0.7	1.3	1.5	0.3	1.4	NR
Labeo rohita	1.7	0.9	1.0	1.5	2.3	0.7	0.9	1.7	0.4	1.5	NT
Oreochromis spp.	1.2	1.0	1.0	1.9	1.6	0.7	1.1	1.1	0.3	1.5	NT
Ictalaurus punctuatus	1.2	0.6	0.8	1.3	1.6	0.6	0.7	0.7	0.2	0.8	NR
Onchorynchus mykiss	1.5	0.8	1.1	1.5	2.4	0.7	0.9	1.1	0.3	1.2	NR
Onchorynchus spp.	2.2	0.7	1.0	1.6	2.2	0.7	0.9	1.1	0.3	1.2	NT
Marine fish											
Lates calcarifer	1.8	NT	NT	NT	2.1	0.8	NT	NT	NT	NT	R
Dicentratux labrax	1.8	NT	NT	NT	2.2	NT	NT	1.2	0.3	NT	0.2
Seriola spp.	1.6	NT	NT	NT	1.9	0.8	NT	NT	NT	NT	R

(Adapted from: NRC 2011).

R, Required in the diet but quantity not detrmined; NR, Not Required under practical conditions (*e.g.*, diets containing ingredients from marine and land animal proteins and fish oil and water of at least medium hardness); NT, Not Tested.

1.3.2 Utilization of dietary proteins

Ingested proteins are broken down during digestion through the hydrolysis to free AAs, dipeptides, and tripeptides by digestive enzymes secreted into the gastrointestinal tract. In fish, protein digestibility is often high (90%), although it depends on the AA composition and the absence of anti-protease factors in the diet (Kaushik 1999). The products of protein digestion are absorbed by the mucosal cells where intracellular digestion of small peptides occurs; thus mainly AAs appear to be released into the portal vein following protein digestion (Murai, Ogata et al. 1987). The intestinal absorption of AAs is highly efficient in fish.

The free AA pool can thus be supplied in the organism by three different ways: intestinal absorption of the products of hydrolysed food proteins (exogenous source), *de novo* synthesis and inter-conversions, and breakdown of body proteins (endogenous sources) (Mambrini and Guillaume 2001). From this p+ool, free AAs can be used:

- for synthesis of body protein or other nitrogenous compounds (*i.e.* nucleic acids, amines, peptides,…);
- as precursors for other compounds by providing carbon backbones for intermediate metabolism. Some of them serve as precursors for endogenous production of glucose through gluconeogenesis. In fish AAs are also efficient substrates for lipogenesis, contrarily to what is observed in mammals and birds where the glucose is the preferred substrate (Hillgartner, Salati et al. 1995);
- for oxidation to provide energy. The breakdown of AAs occurs in two steps. The first is generally deamination, and includes the removal of the amino group, which is either converted to ammonia or transferred to become the amino group of a glutamic acid molecule. The second stage is the conversion of the carbon skeletons (the α-keto acids produced by deamination) to citric acid cycle intermediates (Cowey and Walton 1989).

Remaining proteins are kept in the muscle with high efficiency: 50-70% compared to 25-40% in mammals (Mambrini and Guillaume 2001). This efficiency, associated with the low energy requirements linked to the poikilothermic nature of fish, makes fish efficient converters of proteins, despite the high oxidative catabolism.

1.3.3 Proteins and amino acids in fish reproduction

Dietary proteins, as well as lipids, vitamins (E-C), and carotenoids, are known to be the major nutrients influencing different reproduction parameters, such as fecundity, fertilization, hatching and larval development (Izquierdo, Fernandez-Palacios et al. 2001). One of the most important contributions of dietary protein towards broodstock performance is the diet effect on brood size itself (Chong, Ishak et al. 2004). A positive correlation between the dietary protein content, broodstock size and fry production have already been demonstrated in several fish species (De Silva and Radampola 1990, El-Sayed, Mansour et al. 2003). Besides promoting growth, protein dietary intake is also important for gonadal maturation and eventually the formation of ova. In teleosts, all the essential components of eggs must be incorporated during oocyte development, since ovulated eggs take up very little to no nutrients (Chong, Ishak et al. 2004). In contrast to dietary lipid requirements, specific quantitative and qualitative protein requirements are much less studied with regard to reproduction, egg quality and survival. Little information exists on the specific AA composition of fish eggs (Brooks, Tyler et al. 1997), and dietary protein requirements adequate for growth in juveniles and adults are generally considered appropriate for reproductive stages in fish. However, a significant gap in knowledge exists in the effects of FM replacement by plant proteins on reproduction in finfish aquaculture species, and rainbow trout in particular.

1.4 Replacement of fish oil (FO) and fishmeal (FM) by plant-ingredients in aquafeeds

A large body of studies has been undertaken to analyze the consequences of the replacement of FO by VOs and that of FM replacement by plant protein sources in fish diets. In most of the trials, either FO was replaced by VOs while maintaining FM as protein source, or FM was replaced by plant protein ingredients while maintaining FO as major dietary lipid source. These experimental designs allowed distinguishing the respective consequences of substitution of FO or FM. However, in commercial aquafeeds, FO and FM are replaced concomitantly. This results in quantitative and qualitative changes in many nutrients, which induce multiple physiological responses. Application of "*omics*" methods such as transcriptomics and proteomics has allowed a "*without a priori*" integrative approach of the metabolic pathways affected, reflecting the multiple physiological responses to dietary changes.

1.4.1 FO replacement by vegetable oils

1.4.1.1 Characteristics of vegetable oils

As mentioned before, global FO production is at its sustainable limit. Further expansion of aquaculture requires suitable alternatives, with VOs being prime candidates. VOs represent a source of energy comparable to that of FOs. However, the composition of VOs differs from that of FOs, which is particularly linked to the fact that VOs are generally rich in C_{18} PUFAs, but lack in long chain n-3 PUFAs abundant in FOs (Sargent, Tocher et al. 2002).

The major difference between FO and VO is fatty acid composition (Table 1.3). The FA composition of the majority of VOs is dominated by 16:0, 18:0, 18:1 n-9 and 18:2n-6, although the content of the major FA varies. Alpha-linolenic acid (18:3 n-3) is much less common in VOs. Despite the limitations in terms of FA composition, VOs are commonly used as lipid sources for aquaculture diets. In particular, it is now a common practice to use of a blend of VOs in aquafeeds, in order to provide an overall amount of FA classes closely resembling the proportion of FA classes found in FO (Turchini, Ng et al. 2010). Moreover, when FO is replaced by VOs in diets containing fishmeal, the LC-PUFAs are supplied by lipids contained in fishmeal.

Another important difference between oils of marine (fish) origin and VOs is the fact that VOs contain high levels of phytosterols (Liland, Espe et al. 2013) and no cholesterol, while fish oils contain large amounts of cholesterol (from 3.5 to 7.7 g $kg^{-}1$) (Tocher, Bendiksen et al. 2008, Moffat 2009, Bell and Koppe 2011). Therefore, the increasing substitution levels of FO by VOs in diets for farmed fish are not only responsible for the modification of the FA composition in feed, but are also responsible for reduced levels of cholesterol.

Is it important to notice that VOs, compared to FOs, are also poorer in vitamins A, D and E (Guillaume 2001). On the other hand, they do not contain organic pollutants that often contaminate FOs. One group of contaminants, which have received much attention with regard to possible biological effects, are persistent organic pollutants (POPs), a class of pollutants, which are predominantly anthropogenic (manmade), and are often globally dispersed in the environment (Pickova and Mørkøre 2007). Dioxins and PCBs (Polychlorinated Bisphenols) are the main persistent POPs. Dioxins, in particular, are unwanted by-products of a wide range of industrial processes, such as paper manufacturing and oil refining. PCBs are also used as technical mixers and in the manufacture of plastic, adhesives and flame retardants (Pickova and Mørkøre 2007). Their persistent, fat-soluble and semi-volatile proprieties cause them to readily accumulate along the aquatic food chain, particularly in fatty fish because of the lipophilic nature of POPs. Marine feed ingredients are usually derived from pelagic fisheries, and FOs are considered the main sources of POPs in farmed fish (Easton, Luszniak et al. 2002, Jacobs, Covaci et al. 2002). The increased use of VOs has been associated with a decrease in PCB and dioxins levels in Atlantic salmon and rainbow trout (Bell, McGhee et al. 2005, Berntssen, Lundebye et al. 2005, Drew, Ogunkoya et al. 2007, Friesen, Ikonomou et al. 2008). For example, in Atlantic salmon fed a diet in which all FO was replaced by vegetable oil over the whole production cycle, the authors observed a reduction of 84% in the total dioxins and dioxin like-PCB load in fish, compared to those fish fed a FO-based diet (Berntssen, Lundebye et al. 2005).

Table 1.3 Fatty acid composition (% total FA) of fish oils and vegetable oils.

	SAT	MUFA	18:2 n-6	**20:4 n-6 (AA)**	PUFA n-6	18 :3 n-3	**20:5 n-3 (EPA)**	**22 :6n-3 (DHA)**	PUFA n-3
Fish oils									
Anchovy oil	28.8	24.9	1.2	0.1	1.3	0.8	17.0	8.8	31.2
Capelin oil	20.0	61.7	1.7	0.1	1.8	0.4	4.6	3.0	12.2
Menhaden oil	30.5	24.8	1.3	0.2	1.5	0.3	11.0	9.1	25.1
Herring oil	20.0	56.4	1.1	0.3	1.4	0.6	8.4	4.9	17.8
Cod liver oil	19.4	46.0	1.4	1.6	3.0	0.6	11.2	12.6	27.0
Vegetable oils									
Crude palm oil	48.8	37.0	9.1	-	9.1	0.2	-	-	0.2
Soybean oil	14.2	23.2	51.0	-	51.0	6.8	-	-	6.8
Canola/rapeseed oil	4.6	62.3	20.2	-	20.2	12.0	-	-	12.0
Sunflower oil	10.4	19.5	65.7	-	65.7	-	-	-	0.0
Cottonseed oil	45.3	17.8	51.5	-	51.5	0.2	-	-	0.2
Groundnut oil	11.8	46.2	32.0	-	32.0	-	-	-	0.0
Corn oil	12.7	24.2	58.0	-	58.0	0.7	-	-	0.7
Linseed oil	9.4	20.2	12.7	-	12.7	53.3	-	-	53.3

(Adapted from: Turchini, Torstensen et al. 2009).

SAT, Saturated fatty acid; MUFA, monounsaturated fatty acid; 18:2 n-6, linoleic acid; 20:4n-6, arachidonic acid; PUFA n-6, polyunsaturated fatty acid of n-6 series; 18 :3 n-3, alpha-linolenic acid ; 20 :5 n-3, eicosapentaenoic acid; 22 :6 n-3, docosahexaenoic acid; PUFA n-3, polyunsaturated fatty acid of n-3 series.

1.4.1.2 Consequences of dietary FO replacement by VOs

Effects on feed intake

To guarantee the maximization of feed intake and minimization of feed waste, both of which assure optimal growth and feed efficiency, aquafeeds must be agreeable in flavor and acceptable in taste to the fish. Although determining flavor perception and preferences in fish is difficult (Lamb 2001), feed palatability can be estimated by quantifying differences in amounts of feed eaten (Jobling, Covès et al. 2001). As previously reviewed by Turchini et al. (2009), lipids seem to play a minor role in determining the palatability of the diet. Indeed, it is known that fish are primarily attracted by a variety of nitrogen-containing compounds, that are non-volatile, amphoteric and water soluble, with low molecular weight, *i.e.* free AAs, nucleotides and nucleosides, as well as quaternary ammonium bases (De la Higuera 2001, Kasumyan and DÖving 2003). However, due to the fact the raw materials used in feed production are rarely purified, it is possible that different lipid sources contain some of the aforementioned compounds that could positively or negatively impact feed palatability and consequently feed intake (Glencross 2009). This field has been poorly investigated, however a study with rainbow trout showed that fish are capable of distinguishing between a FO- and a VO-based diet, and in particular between diets containing linseed oil, sunflower oil or camelina/rapeseed oil (Geurden, Cuvier et al. 2005). Specifically, fish showed higher preference for the diet containing FO (Geurden, Cuvier et al. 2005), which resulted in a higher feed demand (Geurden, Corraze et al. 2007). This distinction could also be linked to the nutritional qualities of these diets, since postprandial feedbacks could play an important role in the regulation of appetite in rainbow trout (Cuenca and De la Higuera 1993, Sánchez–Vázquez, Yamamoto et al. 1999), and other species, such as seabream (Paspatis, Kentouri et al. 1997) and seabass (Brotons Martinez, Chatzifotis et al. 2004).

Effects on growth performance

Despite the great number of studies carried out to date, the effects of substitution of FO by VO on growth performance are not completely clear. Indeed, even if it has been stated that when EFA requirements are met, high proportions (around 60-75%) of FO can be replaced by VO in diets for almost all farmed fish species (Turchini, Torstensen et al. 2009), many factors are recognized to potentially affect the results in substitution trials. Among these factors,

species is a major one. Based on the biochemistry of EFAs, fish can be essentially divided into two groups: those which require LC-PUFA (marine fish), and those whose requirements can be satisfied by the C_{18} FAs (freshwater fish) (Tocher 2010).

Based on the data available today, the overall conclusion is that in salmonids, including rainbow trout, 100% of FO can be replaced by VO (or blends of VOs), without affecting growth performance and feed efficiency (Turchini, Ng et al. 2010).
With most marine species, the situation is slightly more complicated, and the maximum level for FO replacement without deleterious effects on growth has been fixed at the range of 60-75% (NRC 2011). This difference in maximum replacement levels between freshwater and marine fish can mainly be explained by the difference in LC-PUFA bioconversion capacities of these two 'groups' of fish (Tocher, Dick et al. 2010).
Nevertheless, it is important to notice that the majority of these data on both freshwater and marine species were obtained in trials, where a large proportion of dietary protein was supplied by FM. It is known that a dietary level of 40% FM would generally supply lipid to at least 4% total diet and n-3 LC-PUFA to around 1-1.5% of diet, sufficient to satisfy EFA requirements (NRC 2011).

Effects on whole body and flesh composition

A large number of studies have shown that partial or total replacement of FO with VO, like rapeseed oil, linseed oil, palm oil or soybean oil, or with a blend of these VOs, has no adverse consequences on whole body and muscle lipid content in several freshwater and marine species (Bell, McGhee et al. 2003, Bell, Tocher et al. 2003, Bendiksen, Berg et al. 2003, Torstensen and Stubhaug 2004, Izquierdo, Montero et al. 2005, Montero, Robaina et al. 2005, Richard, Kaushik et al. 2006, Richard, Mourente et al. 2006, Nanton, Vegusdal et al. 2007). However, lipid classes may be differentially affected by VO-containing diets. For example, while the substitution of FO with VO, characterized by largely different FA composition, did not change body lipid storage in any major way (Nanton, Vegusdal et al. 2007), it did result in drastic changes with regard to triacylglycerol:phospholipids ratios. Specifically, the triacylglycerol:phospholipids ratio showed a decreasing trend in both visceral lipid stores and myosepta in Atlantic salmon fed a 100% VO-replaced diet.

Along the same lines, when considering the effect of VO replacement on specific FA composition in fish, strong changes in FA composition can be observed in fish, irrespective of

whole body lipid levels. This is because the final FA composition of fish is the direct consequence of dietary input and endogenous metabolism. Therefore changes in dietary lipids sources (marine *vs* VOs) highly affect final FA composition of fish (Torstensen, Lie et al. 2000, Caballero, Obach et al. 2002, Izquierdo, Obach et al. 2003, Nanton, Lall et al. 2003, Torstensen, Frøyland et al. 2004, Torstensen, Frøyland et al. 2004, Corraze, Larroquet et al. 2006). The precise changes of FA composition depend on several factors: the source of VOs used, species, tissue, and the proportion of tissue neutral and polar lipids.

Irrespective of the species, replacement of FO by a single VO source almost invariably results in increased proportions of C_{18} FA (18:1 n-9, 18:2 n-6 and 18:3 n-3), which are particularly abundant in VOs, and decreased proportions of LC-PUFA (ARA, EPA and DHA), since none of the VOs contain these FAs (Rosenlund, Corraze et al. 2011). The main reason behind the choice of using multiple VOs instead of a single VO-source is to mimic the proportion of the different FA classes found in FO as much as possible. Consequently, dietary inclusion of VO blends tends to modify tissue composition less, than diets containing a single VO source (Izquierdo, Montero et al. 2005, Torstensen, Bell et al. 2005). However, when using dietary VOs blends, differences exist between tissue profiles of EPA and DHA. Changes in tissue FA composition are less pronounced in DHA and ARA, compared to EPA (Izquierdo, Obach et al. 2003, Menoyo, Izquierdo et al. 2004, Izquierdo, Montero et al. 2005). This effect is especially evident in neutral lipids (Izquierdo, Montero et al. 2005). Differences in fish tissue FA composition are generally less pronounced than those of the diets. This is due to endogenous metabolism of FA in fish, particularly FA catabolism and bioconversion, which may partially compensate for dietary induced changes (Torstensen, Espe et al. 2008).

Considering species, a difference exists mainly between freshwater and marine species, with lower effects in organs of freshwater fish, mainly due the high bioconversion ability in the latter (see paragraph 2.2). Tissues are also known to be differentially affected (Bell, Dick et al. 2001, Bell, McGhee et al. 2003, Caballero, Izquierdo et al. 2003, Izquierdo, Obach et al. 2003, Torstensen, Frøyland et al. 2004). As previously described, depending on the species, fish are able to accumulate lipids in different tissues, such as liver for Atlantic cod or abdomen and flesh for Atlantic salmon and rainbow trout. Generally, FA changes are higher in those organs or tissues containing high amounts of neutral lipids because of their high turnover (Ganga, Bell et al. 2005, Caballero, Gallardo et al. 2006). In contrast, organs with higher ratio of phospholipids reflect the inclusion of VO to a lower extent (Brodtkorb, Rosenlund et al. 1997, Olsen and Henderson 1997, Benitez-Santana, Masuda et al. 2007),

because of their lower turnover. These observations have been combined into a model proposed by Robin and colleagues (Robin, Regost et al. 2003). This model has been validated in several studies with Atlantic salmon (Jobling 2003, Jobling 2004), Murray cod (*Macchullocella peeli)* (Turchini, Francis et al. 2006) and gilthead sea bream (Benedito-Palos, Navarro et al. 2009).

Effects on digestibility and lipid metabolism

Changes in FA composition of the diet may also directly influence lipid metabolism in fish and the lipid composition of the tissues at multiple levels: in addition to affecting lipid transport and the regulation of circulating lipid levels in the plasma, dietary FA also exert an influence on lipogenesis, FA bioconversion and catabolism in various tissues.

FA digestibility has been reported to be affected by changes in dietary FA composition when FO is replaced by VO. In particular, the amount of saturated fatty acids (SAT) is known to influence digestibility of lipids, particularly at low water temperature (Olsen, Løvaas et al. 1999, Torstensen, Lie et al. 2000, Caballero, Obach et al. 2002). For example, the inclusion of rapeseed oil in replacing FO in diets for rainbow trout and Atlantic salmon resulted in a decrease of SAT, and a concomitant increase in monounsaturated fatty acids (MUFA), which, in turn, resulted in increased lipid digestibility at lower temperatures (Caballero, Obach et al. 2002, Karalazos, Bendiksen et al. 2007). The increase in liver lipid stores and increased protein productive value reported at low water temperature in VO-fed fish may be linked to such increased digestibility (Bendiksen, Berg et al. 2003, Torstensen, Bell et al. 2005).

Replacing FO by VO in aquafeeds is also known to affect plasma lipids and lipogenesis. The observed changes depend on the VO sources. For example, a diet in which FO was totally replaced by oleic acid-enriched sunflower oil, rapeseed oil, and soybean oil, resulted in a slight increase in liver total lipid storage in Atlantic salmon (Torstensen, Lie et al. 2000, Bell, McEvoy et al. 2001), while no changes were found when FO was replaced by palm oil or rapeseed/linseed oil mix (1:1) (Tocher, Bell et al. 2001). This difference may be linked to LPL hepatic activity. Indeed, it is known that dietary FAs differentially regulate LPL expression in fish liver and adipose tissue (Liang, Ogata et al. 2002). A decrease in total and LDL cholesterol in plasma has been reported in Atlantic salmon and rainbow trout fed VO, in addition to a down-regulation of hepatic LDL receptor expression in trout (Jordal, Lie et al. 2007, Richard, Kaushik et al. 2006). However, a functional genomics study with Atlantic salmon fed VO showed that fish appear to compensate for the lower dietary cholesterol intake

by enhancing the endogenous hepatic cholesterol synthesis (Leaver, Villeneuve et al. 2008). Studies investigating gene expression in salmonids using microarray analysis of hepatic transcriptome have provided mechanistic consequences for this effect at the gene expression level. It has been shown that several genes implicated in cholesterol synthesis pathway are up-regulated in fish fed diets containing VO compared to those fed FO (Leaver, Villeneuve et al. 2008, Panserat, Kolditz et al. 2008). For example, in a study with European seabass the authors found up-regulation of 3-hydroxy-3methylglutaryl-CoA reductase (HMGCR), a transmembrane enzyme involved in the rate-limiting step of sterol biosynthesis, when fish were fed a diet in which FO was replaced by VOs (Geay, Ferraresso et al. 2011). In a study with Atlantic salmon an increase in cholesterol biosynthetic genes was also observed, with up-regulation of lanosterol 14-alpha demethylase (CYP51A1) in fish fed a VO-based diet. Because tissue levels of cholesterol were not reduced, it can be assumed that the up-regulation of cholesterol biosynthesis effectively compensates for the lower intake (Leaver, Villeneuve et al. 2008). The trigger affecting gene expression could be reduced dietary cholesterol, but may also be the presence of VO-derived phytosterols, which are abundant in plant-based ingredients (Liland, Espe et al. 2013).

The activity of fatty acid synthase (FAS), which represents the rate-limiting enzyme in hepatic FA synthesis, was also found to be affected by FO replacement by VO. Indeed, in a previous study with rainbow trout juveniles, the authors found decreased levels of FAS mRNA in the liver of fish fed a totally VO-based diet (Panserat, Kolditz et al. 2008), correlating with the significantly decreased FAS activity after 16 months of feeding VO blends (Jordal, Lie et al. 2007).

Replacing FO with VO in fish diets is known to affect different metabolic pathways, as well as expression of genes encoding for proteins involved in lipid metabolism. In particular, it has been recognized that FA bioconversion is highly affected by the inclusion of VO in the diets for fish (Tocher 2003, Tocher, Dick et al. 2010). The desaturase-elongase pathway has been shown to be up-regulated with high dietary levels of C_{18}, while it is down-regulated by increasing dietary levels of the pathway's end products, EPA and DHA (Bell, Tocher et al. 1997, Tocher 2003). Therefore, feeding fish diets containing VO, which are rich in C_{18}, induced enhanced expression of genes involved in the FA biosynthetic pathway, while FO-based diets, which are rich in LC-PUFA n-3, had the opposite effect (Bell and Koppe 2011). In a previous study with Atlantic salmon, the authors observed increased expression of Δ6- and Δ5-desaturase in liver, adipose tissue and red muscle of fish fed a diet containing high

levels of FO (Zheng, Tocher et al. 2005, Zheng, Torstensen et al. 2005). A more recent study with Atlantic salmon confirmed the up-regulation of these two desaturases in liver of fish fed a diet, in which FO was totally replaced by VO (Leaver, Villeneuve et al. 2008). An enhanced expression of Δ5-desaturase was also found in the intestine of cod fed diets with partial replacement (*c.a.* 30%) of FO by VO (Morais, Edvardsen et al. 2012). Several other transcriptomic studies investigating the effects of FO replacement by VO have shown that, regardless of oil employed, lipid metabolism, and in particular LC-PUFA biosynthesis, is highly affected in Atlantic salmon (Morais, Pratoomyot et al. 2011, Betancor, Sprague et al. 2015), sea bream (Calduch-Giner, Sitjà-Bobadilla et al. 2012) and Japanses flounder (*Paralichys olivaceus*) (Limtipsuntorn, Haga et al. 2014). These studies confirmed the overall up-regulation of the LC-PUFA biosynthetic pathway.

With regard to FA catabolism, it is well known that the main metabolic pathway through which fish produce energy is the β-oxidation pathway (see paragraph 2.4). Changes in FA profile can affect FA catabolism at both molecular and cellular levels. Considering the effects at the molecular level, it has been demonstrated that feeding Atlantic salmon a totally VO-based diet resulted in a decreased expression of carnitine palmitoyl transferase-II (CPT-II) in white muscle. This enzyme is responsible for the conversion of acyl-carnitine into acyl-CoA inside the mitochondrial matrix. In addition, long chain-acyl-CoA-dehydrogenase was found to be down-regulated in white muscle of Atlantic salmon fed VO-based diets, while no changes were observed in liver, heart or red muscle (Torstensen, Nanton et al. 2009). This enzyme has been associated with mitochondrial β-oxidation of unsaturated fatty acids (Le et al., 2000). The expression of Acyl-CoA oxidase (ACO), which is thought to be the rate-limiting step in the peroxisomal β-oxidation system (Inestrosa, Bronfman et al. 1979), was also found to be slightly down-regulated in white muscle of Atlantic salmon fed VO-based diet, while no changes were observed in liver, heart, red muscle and visceral adipose tissue (Torstensen, Nanton et al. 2009).

Previous studies with Atlantic salmon have shown that monoenes, such as 18:1 n-9 and 22:1 n-11, as well as 18:2 n-6 and 18:3 n-3, are readily oxidized when are present at high concentrations (Bell, McGhee et al. 2003, Bell, Tocher et al. 2003, Torstensen and Stubhaug 2004, Stubhaug, Lie et al. 2006). Furthermore, EPA and DHA were also highly β-oxidated in tissues when in dietary surplus during high growth periods (Stubhaug, Lie et al. 2007). Depending on the dietary FA composition, different FA will serve as the predominant energy substrate. For example, salmon fed VO-based diets with limited amounts of EPA and DHA,

will preferentially retain EPA and DHA (70%) instead of using these substrates for β-oxidation. This is in contrast to fish fed FO based diets, which retain only 30% of these FA, suggesting increased β-oxidation of these substrates (Stubhaug, Lie et al. 2007).

Effects on reproduction

As described in the previous section, broodstock nutrition has long been recognized to have significant effects upon fish gonad growth and fecundity (Watanabe 1985, Mourente and Odriozola 1990, Harel, Tandler et al. 1994, Rainuzzo, Reitan et al. 1997) Moreover, among the nutritional constituents of broodstock diets, lipids are the component which mostly affects egg composition (Izquierdo, Fernandez-Palacios et al. 2001), and in particular the dietary EFA content (Watanabe, Arakawa et al. 1984, Watanabe, Ohhashi et al. 1984).

An important function of EPA and DHA is their involvement in reproductive processes, particularly with regard to egg quality and progeny development (Bell, Henderson et al. 1986, Tandler, Watanabe et al. 1989, Harel, Tandler et al. 1994, Fernández-Palacios, Izquierdo et al. 1995, Izquierdo 1996). Indeed, deficiency in the n-3 LC-PUFA content of broodstock diets has been identified as an important factor negatively affecting fish reproductive performances (Izquierdo, Fernandez-Palacios et al. 2001). Therefore, one of the major consequences when replacing FO by vegetable oil can be related to the changes in EFA dietary profile of broodstock, which in turn will affect the deposition of these fatty acids in ova, resulting in lower quality spawning and eggs and consequently affect offspring development.

PUFAs are also known to potentially affect reproduction through the regulation of eicosanoids production, particularly prostaglandins, which are involved in several reproductive processes (Moore 1985). This includes the production of steroid hormones, gonadal development and ovulation. ARA is the major precursor for eicosanoids in fish (Tocher 2003). Thus, the use of VOs, which lack in ARA, could also affect fish reproductive performance. However, since VOs are normally rich in linoleic acid, the ARA precursor, the effects may be less pronounced in freshwater fish.

1.4.2 FM replacement by plant proteins

1.4.2.1 Characteristics of plant proteins

FM is an ingredient which has excellent nutritional properties. Indeed, it is rich in highly digestible proteins that supply adequate levels of essential AAs, and is also a source of n-3 PUFAs and minerals. However, the development of aquaculture and the limited amount of FM available does not allow for any large-scale use of FM in feeds for farmed fish.

Various plant protein sources are now commonly used in aquaculture feeds, including meals from oilseed (soybean, rapeseed/canola, sunflower, and cottonseed), grains (wheat and corn glutens) and legumes (peas, beans, peanut and lupins). When formulating feeds with high levels of plant meals, two main factors have to be considered: energy density and AAs content. Firstly, energy density is linked to the fact that some meals have high carbohydrate content, like non-starch polysaccharides, which are of little nutritional values for carnivorous species like rainbow trout (NRC 2011). This is because carnivorous fish have evolutionarily adapted their anatomy, physiology and metabolism according to their natural diet that contains very little or no carbohydrates from starch or other sources (Buddington, Krogdahl et al. 1996, Kaushik 2001, NRC 2011). Consequently, after a carbohydrate-rich meal, most carnivorous fish are not able to clear glucose influx efficiently, resulting in a prolonged hyperglycaemia (Wilson 1994, Kaushik 2001, Stone 2003, Polakof, Panserat et al. 2012), suggesting that carbohydrate do not represent a major source of energy in these fish.

Secondly, AA composition of plant meals is generally deficient in some EAAs, such as lysine and methionine (Gatlin, Barrows et al. 2007). These EAAs must therefore be supplied by diets in order to meet nutritional requirements (NRC 2011). Evidence from replacement trials has shown that a combination of plant-derived feed ingredients is required to successfully replace FM (Gatlin, Barrows et al. 2007). For example, corn, an alternative for FM, is known to contain lower levels of lysine than other grains and oilseed. Therefore, combining corn with soybean meal, which contains greater amounts of lysine, results in a blend containing lysine at a nearly acceptable level (Gatlin, Barrows et al. 2007).

Moreover, a wide range of antinutritional factors are present in plant feedstuff, and must be controlled (Hardy and Barrows 2002, Krogdahl, Penn et al. 2010, NRC 2011). Anti-nutritional factors are often secondary plant metabolites, which play a role in plant physiology, or defend plants against predators. In fish, they can act at different levels. For example they may limit the utilization of specific nutrients by decreasing the efficiency of the digestive

process or affecting appetite. However, even if the presence and the impact of the certain antinutritional compounds have been confirmed in fish (Kaushik, Flos et al. 1990, Francis, Makkar et al. 2001, Gatlin, Barrows et al. 2007), the precise mode of action of additional antinutritional factors still remains relatively poorly understood.

1.4.2.2 Consequences of dietary FM replacement by plant proteins

Effects on growth performance

Several studies carried out to date have investigated the effect of dietary FM replacement by plant ingredients on fish growth performances. In this context, the replacement levels, the ingredients used, and the possible interaction between components in plant protein blends may result in different growth outcomes.

Previous studies with rainbow trout and Atlantic salmon have shown that the partial replacement of FM with alternative plant sources is possible without deleterious effects on growth, nutrient utilization and without compromising fish health (Kaushik, Cravedi et al. 1995, Espe, Lemme et al. 2006, Espe, Lemme et al. 2007). In rainbow trout, the threshold level for the dietary replacement of FM without any detrimental effects on growth has been determined to be as high as 90%. Globally however, no negative effects on growth generally occur at FM replacement of 10 to 80% in salmonids (Gomes, Rema et al. 1995, Médale, Boujard et al. 1998, Glencross, Evans et al. 2004, Thiessen, Maenz et al. 2004, Morris, Gallimore et al. 2005, Pierce, Palti et al. 2008).

To the contrary, other studies carried out in both salmonids and non-salmonid species which investigated the effects of FM replacement by plant protein have shown negative effects on fish growth performance (De la Higuera, Garcia-Gallego et al. 1988, De Francesco, Parisi et al. 2004, Glencross, Evans et al. 2004, Drew, Racz et al. 2005, Barrows, Gaylord et al. 2007). In particular, studies with gilthead seabream, Atlantic salmon, and rainbow trout reported decreased growth rates when 50%, 75%, or 100% of FM was replaced with plant protein sources (Gómez-Requeni, Mingarro et al. 2004, Mundheim, Aksnes et al. 2004, Panserat, Hortopan et al. 2009, Alami-Durante, Médale et al. 2010). One of the possible reasons behind the reduced growth with plant-based diets could be related to a reduction of feed intake, as observed in seabream fed diets with increasing levels of plant ingredients ranging from 50% to 100% (Sitjà-Bobadilla, Peña-Llopis et al. 2005). Other studies have shown a reduction in feed intake especially at the beginning of the feeding trial with total (De Francesco, Parisi et al. 2004, Espe, Lemme et al. 2006) or partial replacement (Gomes, Rema et al. 1995, Kaushik, Coves et al. 2004), which finally resulted in lower growth. In salmonids, reduced growth at high dietary inclusion levels of plant proteins has been associated with various factors including an increase digestible and indigestible carbohydrate levels (starch/fibre levels) (Opstvedt, Aksnes et al. 2003), reduced feed palatability, the presence of antinutritional

factors (Francis, Makkar et al. 2001), and an imbalanced dietary AA profile (Espe, Lemme et al. 2006, Espe, Lemme et al. 2007).

Effects on whole body and tissues composition

A large body of literature describes the effects of substitution of FM with alternative plant sources on whole body and tissue composition. However, the results, particularly those regarding muscle composition, are somewhat contrasting. On the one hand, studies with European sea bass (Gouveia and Davies 2000) and gilthead sea bream (Pereira and Oliva - Teles 2003) have reported that inclusion of plant ingredients in the diets (from 10% to 30%) does not affect whole body lipid content. On the other hand, Robaina et al. (1998) observed a decrease in muscle total lipid content in sea bream fed a diet containing 30% of soy byproducts. Kissil et al. (2000) reported a decrease in whole body lipid content in sea bream fed a 100% of fish meal substitution diets based either on soybean or rapeseed protein concentrates. In contrast to these results, an increase in whole body lipids in fish fed diets containing increasing level of plant ingredients was observed in trout (Burel, Boujard et al. 2000) and in sea bass (Kaushik, Coves et al. 2004). The inclusion of plant ingredients in diets for European seabass reduced liver and visceral fat content, while a higher total lipid content was found in dorsal muscle (Dias, Alvarez et al. 2005). These findings are in accordance with observations previously reported in gilthead sea bream (Mourente, Díaz-Salvago et al. 2000, Montero, Robaina et al. 2001).

When considering the FA profile in detail, a slight variation of PUFA levels has been observed in association with FM replacement (Médale and Kaushik 2009). The replacement of 75% of FM by plant proteins in diets for sea bream resulted in high levels of n-6 PUFA, particularly 18:2 n-6, and lower levels of MUFA and n-3 PUFA (De Francesco, Parisi et al. 2007). However, the PUFA content in meals is generally low compared to FO, and therefore has a comparatively low impact on fish FA profile.

Effects on lipid metabolism

With regard to metabolic pathways particularly affected by dietary plant proteins, literature points to lipogenesis and cholesterol metabolism as principal targets.

The effect of dietary protein sources on hepatic lipogenesis has been studied to a much lesser extent than macronutritional effects of lipids or carbohydrates in general. It is however known, that changes in dietary protein and AA imbalances can affect fish metabolic pathways

(Gómez-Requeni, Mingarro et al. 2003, Gómez-Requeni, Mingarro et al. 2004, Dias, Alvarez et al. 2005, Sitjà-Bobadilla, Peña-Llopis et al. 2005), including lipid metabolism.

In a previous study, Dias et al. (2005) have shown that FM dietary replacement by soybean meal affects seabass liver lipogenesis by decreasing enzymatic activity of glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME) and fatty acid synthetase (FAS). Hepatic lipogenic activity was down-regulated in European seabass (Dias, Alvarez et al. 2005) fed soybean meal and in gilthead seabream (Gómez-Requeni, Mingarro et al. 2003) fed soybean meal supplemented with glutamate. Still, it is difficult to clearly distinguish whether these effects on lipogenesis are simply due to dietary AA, or to some antinutritional factors present in soybean meal such as trypsin inhibitors, saponins, phytoestrogens, phytosterol and isoflavones, all of which are suggested to affect cholesterolemia and hepatic lipogenic enzymes (Anderson and Wolf 1995, Kaushik, Cravedi et al. 1995, Potter 1995, Gómez-Requeni, Mingarro et al. 2003, Dias, Alvarez et al. 2005). Lipid metabolism seems to be equally affected by FM replacement, mainly through a decrease in plasma cholesterol level in both rainbow trout (Kaushik, Cravedi et al. 1995) and seabream (Gómez-Requeni, Mingarro et al. 2004). One of the possible reasons of this decrease may be related to the high proportion of oleic and linoleic acids (Dietschy 1998) or phytosterols contained in plants used in fish diets, particularly in rapeseed oil (Phillips, Ruggio et al. 2002). Phytosterols may compete with cholesterol and therefore decrease cholesterol absorption. Other studies also showed a decrease in lipid oxidation when FM was replaced by plant-proteins either partially (39%, Alvarez, Lopez-Bote et al. 1999) or totally (100%, Ustaoglu and Rennert 2002).

More recent studies have also focused their attention to underlying mechanisms by addressing molecular effects of FM replacement by plant-proteins. In a study with Atlantic salmon, the expression of genes involved in lipid metabolism, including genes involved in LC-PUFA bioconversion (*i.e.* Δ6- and Δ5-desaturase, Elovl5), was increased in the mid intestine of fish fed a 50% plant protein-based diet, compared to those fed a FM-based diet (Tacchi, Secombes et al. 2012). In the same study, genes involved in cholesterol metabolism, and in particular in mobilization and transport of cholesterol to the intestine (apolipoproteins), were also up-regulated. The authors suggest that this altered regulation could possibly be seen as an adaptive response of fish to lower dietary cholesterol intake. Other studies on Atlantic salmon have also reported that hepatic sterol biosynthesis and lipid transport was up-regulated by the inclusion of plant ingredients in the diet, including soybean meal (Kortner, Gu et al. 2013, Gu, Kortner et al. 2014). In a recent study investigating the effects of air-classified faba bean protein concentrate (BPC) and soy protein concentrate (SPC) in diets for Atlantic salmon (De

Santis, Ruohonen et al. 2015), the authors found evidence of impaired metabolism in the liver in response to high BPC and SPC, including a significant decrease in the expression of genes involved in the lipogenic pathways. The authors hypothesized that high levels of dietary BPC or SBM caused a degree of oxidative stress resulting in impaired mitochondrial function.

Effects on reproduction

Dietary proteins are nutrients which potentially influence reproductive processes at the level of fecundity, fertilization, hatching and larval development (Izquierdo, Fernandez-Palacios et al. 2001). One of the most important contributions of dietary protein towards broodstock performance is the diet effect on brood size itself (Chong, Ishak et al. 2004), which is known to affect fry production in several fish species (De Silva and Radampola 1990, El-Sayed, Mansour et al. 2003). One of the possible consequences of replacing FM by plant proteins in diets for fish may be a reduction in terms of growth, as previously described in different fish species (De Francesco, Parisi et al. 2004, Glencross, Evans et al. 2004, Drew, Racz et al. 2005, Barrows, Gaylord et al. 2007, Panserat, Hortopan et al. 2009, Alami-Durante, Médale et al. 2010). Therefore, since bigger broodstock are known to produce bigger sized eggs (Sargent, Taylor et al. 1987), the decrease in growth with plant ingredients could result in the production of small sized eggs, which in turn contains less reserves (vitellus), thus limiting offspring development.

Besides promoting growth, dietary protein intake is also important for gonadal maturation and eventually, the formation of ova. However, in contrast to dietary lipids, specific quantitative and qualitative protein requirements have been much less studied with regard to reproduction, egg quality and survival. Little information exists on specific AA composition of fish eggs (Brooks, Tyler et al. 1997), and little is known about the reproductive effect of FM replacement by plant protein sources on egg quality and offspring development. In female rainbow trout, negative effects of total FM replacement by cottonseed meal were observed on fertility and plasma testosterone levels (Lee, Rinchard et al. 2006). Another study in male rainbow trout fish suggests no adverse effects of FM replacement by cotton-seed based plant proteins on male reproductive capacity assessed by sperm quality and offspring development (Dabrowski, Rinchard et al. 2000).

1.4.3 Combined replacement of FO and FM by plant sources

In the previous section, I have reviewed the main effects of different levels of FO or FM replacement in diets for farmed fish. However, due to the high necessity of replacing both marine ingredients, few recent studies have begun to investigate the effects of combined replacement of FM and FO. These studies focused on different aspects of fish responses, ranging from feed intake and growth performances, to metabolic and molecular effects mediated by alterations in gene expression. In the following section I will summarize the effects of the concomitant replacement of FO and FO on the basis of the results obtained through these studies.

Effects on feed intake and growth performance

As mentioned before, the introduction of both plant proteins and vegetable oils in diets for aquaculture seems to affect feed intake, which is probably related to changes in diet palatability which seem to be mostly linked to FM replacement, rather than FO (Turchini, Torstensen et al. 2009). In the first study investigating the concomitant replacement of both FO and FM by plant ingredients in the diet, Panserat et al. (2009) observed highly decreased feed intake in rainbow trout fed diets containing exclusively plant proteins and vegetable oils, compared to fish fed a marine-based diet. This suggests that the reduced feed intake was mainly linked to the FM replacement and not to fish oil substitution. However, in a study with Atlantic salmon fed diets with combined replacement of FM and FO with plant ingredients, a decrease in feed intake was observed only when FM and FO were both replaced at high levels (80% FM and 70% FO replacement), but not when FM exclusively was replaced at high levels (80% FM and 35% FO replaced) (Torstensen, Espe et al. 2008). These last results therefore suggest a synergistic effect of high replacement of both the lipid and protein fractions on the regulation of feed intake. Feeding fish with diets containing plant protein and vegetable oils as substitutes of FM and FO respectively may additionally impact fish growth, through alternative mechanisms related to fish health.

Effects on protein and lipid metabolism

Effects of the concomitant replacement of FM and FO by plant ingredients have mostly been investigated in intestine and liver, because of their high responsiveness and sensitivity to nutritional changes. The intestine is the first organ in contact with the feed, while the liver serves as the central hub of metabolism. As reviewed in the previous sections, dietary FM replacement has mainly been associated with intestinal protein turnover and inflammation processes, while in the liver glucose and lipid metabolism are mainly affected. FO replacement on the other hand is mainly associated with gene expression changes in LC-PUFA biosynthesis and cholesterol metabolism.

Relatively few studies have investigated the concomitant replacement of FM and FO on fish metabolic responses at molecular level. Panserat et al. (2009) found changes in the expression of genes related to protein metabolism, however their results were not as clear-cut as those they had previously obtained when only FM was replaced (Panserat, Kolditz et al. 2008). This suggests a combined effect of low EFA level (mainly related to FO replacement) along with a change in AA profile (mainly related to FM replacement). Additionally, a recent study investigating the effects of the inclusion of camelina products (meal and oils) on the hepatic transcriptome of Atlantic salmon found an up-regulation of long chain fatty acid-CoA ligase 4 (*facl4*) in fish fed diets containing vegetable sources, confirming previous results obtained in rainbow trout (Panserat, Ducasse-Cabanot et al. 2008, Panserat, Kolditz et al. 2008, Xue, Hixson et al. 2015). Protein metabolism was also affected in the liver of European sea bass, showing an up-regulation of genes involved in proteolysis when fish were fed a totally plant-based diet (Geay, Ferraresso et al. 2011). In the same study, the main effects of the combined replacement were found in lipid and sterol metabolism, with an increased expression of genes involved in long chain fatty acids elongation and/or desaturation and in cholesterol biosynthesis. The total replacement of FO and FM resulted in a diet completely devoid of LC-PUFAs and cholesterol. Therefore, due to the role that both lipid classes play in membrane structure, permeability and fluidity, the increase in long chain elongation/ desaturation and cholesterol biosynthesis may represent a mechanism of how fish restore membrane properties, which are affected by total FO and FM replacement.

Effects on reproduction

As discussed in the previous sections, dietary changes in broodstock nutrition, due to the replacement of FM and FO by plant ingredients, can affect reproductive performance of fish. The main effects on fish reproduction related to FO replacement concern the drastic modification of broodstock dietary LC-PUFA. This, in turn, impacts the incorporation of these FA in ova, therefore affecting egg quality and progeny development, as well as the production of prostaglandins, which are involved in reproductive processes. Less is known about the effects of FM replacement by plant proteins in reproduction: the main reproductive effects seem to be related to a reduction of broodstock size.

When considering the replacement of both FM and FO, we can hypothesize a combined effect of the concomitant substitution of these ingredients. Indeed, in concomitant FM and FO replacement, the reduction in terms of dietary LC-PUFA is higher compared to the replacement of only FM or FO, particularly when the replacement of FM and FO by plant ingredients is total (100% plant based diet) the dietary intake in terms of EPA and DHA is nil. Therefore, the only way for fish to produce these FA is through bioconversion of dietary precursors, particularly present in VO. This bioconversion therefore becomes essential to assure successful reproduction. Moreover, since a combined metabolic effect of the replacement of both FM and FO at high levels have been described (Torstensen, Espe et al. 2008), we can also hypothesize that replacing both FM and FO may increase the negative effects on growth, and therefore amplify the consequences on egg production and consequent progeny development.

To date, a significant gap in knowledge exists in the effects of concomitant FM and FO replacement by plant protein and VOs in finfish aquaculture species, and rainbow trout in particular.

1.5 Objectives of the thesis and major questions

Most of the studies carried out to date have investigated the effects of FM and/or FO on portions of the fish life cycle, mainly through relative short-term trials focusing on the fish growing period. This period represents the longest phase of the breeding process for aquaculture fish production, so that the highest amount of feed is consumed during this period. The current aquaculture practice is to use aquafeeds containing plant ingredients mainly during the on-growing period. Current knowledge of the effects of replacement of FM and FO by plant ingredients in the diets of cultured fish, including rainbow trout, during the entire life cycle, is scarce. Very few studies have investigated the impact of such dietary changes on reproductive performance, egg quality, offspring survival and subsequent (intergenerational) consequences. The overarching aim of this PhD thesis was therefore to determine the effects of feeding rainbow trout from first-feeding and during the whole life cycle, a diet in which FM and FO were both totally replaced by plant ingredients. The effects of such dietary changes were studied across different developmental stages, including broodstock and early stages. Within this framework, three major questions were addressed, with specific underlying hypotheses.

Question 1

Are rainbow trout capable to survive, grow and reproduce when reared on a totally plant-based diet during the whole life cycle? What are the effects on ova and offspring?

Given the known importance of dietary lipid supply on egg quality and reproductive success in fish, we wondered whether female rainbow trout fed on a completely plant-based diet (total FM and FO replacement) from first feeding up to reproduction would be capable to produce ova and viable progeny. We hypothesized, that successful rearing of female rainbow trout with diets devoid of marine resources over the entire life cycle (3-year feeding trial, including two spawning events) was dependant on the capacity to synthesize LC-PUFAs. The question of whether rainbow trout are capable of synthesizing sufficient amounts of n-6 and n-3 LC-PUFAs from dietary precursors not only to survive, but also to successfully reproduce was addressed by measuring the incorporation of n-3 and n-6 PUFAs into ova and offspring. Additionally, reproductive performance parameters, offspring survival and growth (body weight) were assessed. Thus, we addressed, for the first time in rainbow trout, the effect of an

entirely plant-based diet in female broodstock on reproductive capacity and intergenerational consequences in offspring, particularly with regard to lipid metabolism (Publication 1).

Question 2

Does maternal dietary history affect progeny transcriptional response at early stages? Does maternal dietary history influence the metabolic response of alevins fed different first feeding diets?

After the determination of reproductive capacity and offspring survival from maternal broodstock reared on entirely plant-based diet across the entire life cycle, the subsequent question focused on potential intergenerational effects of such a dietary regime. Based on recent knowledge about metabolic programming by nutritional maternal history in mammals (Moghadam, Mørkøre et al. 2015), we hypothesized that maternal broodstock diet might affect metabolic capacity of progeny and program the first generation offspring to differentially respond to diets. In particular, we wondered whether progeny of females fed a plant-based diet would be more efficient in utilizing first-feeding diets containing plant ingredients than progeny from females fed a marine resources-based diet. Since no knowledge exists in literature about which pathways, if any, may be modified by the maternal feeding, we choose a transcriptomic *"without a priori*" approach, which allowed us to obtain a snapshot of molecular changes induced by broodstock nutritional history. The transcriptome of whole alevins from both groups of females (fed either the marine- or the plant-based diet) was investigated before first feeding; then after three weeks feeding diets made of either marine resources, a mix of marine resources and plant ingredients or only plant ingredients without any marine resources. The objective was to assess the potential effect of broodstock nutritional history on the capacity of progeny to adapt to a plant-based diet (publication 2).

Question 3

Long-term feeding rainbow trout plant-based diets since first feeding: Are there any major diet-induced changes on intestinal and hepatic gene expression? Are transcriptional changes temporally persistent?

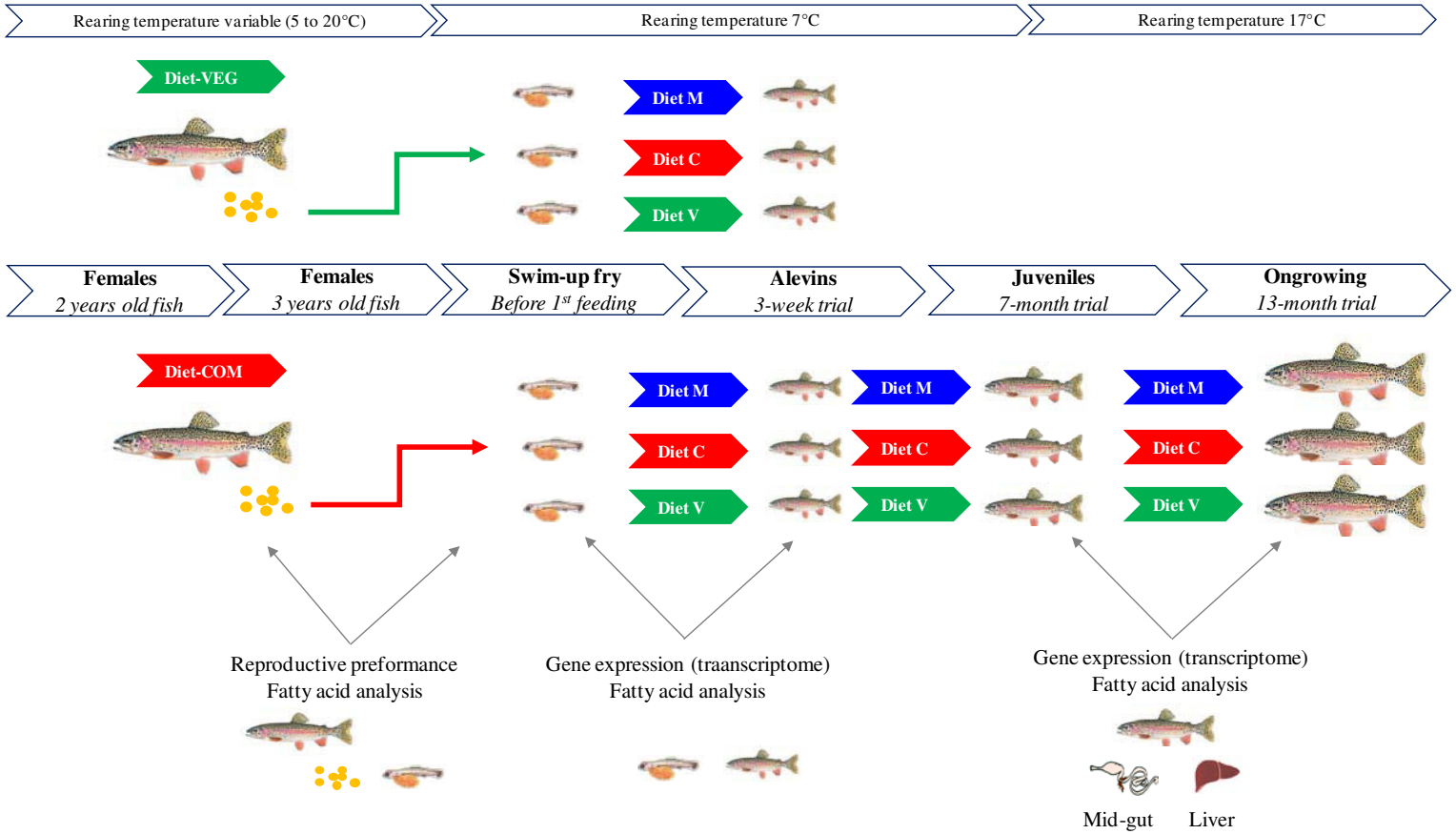
The third part of the PhD work focused on the long term effect of diets with different levels of marine resources and plant ingredients (zero, half or total FM&FO replacement). We wondered whether rainbow trout were capable of adaptations to diets of different composition

when given since first-feeding, and whether such adaptations were stable in the long-term across the life-cycle. The hypothesis was that diet-induced changes might vary according to fish developmental stages. We therefore investigated the effects of experimental diets with different levels of FM, FO and plant ingredients on the responses of juveniles (10g) after 7 months of feeding, as well as those of ongrowing fish (250-350g) after 13 months of feeding. In addition to phenotypic parameters, such as survival, growth and plasma metabolites of trout fed the experimental diets since first-feeding, we analysed gene expression in two target tissues of juveniles and ongrowing fish. Target tissues were firstly the intestine, important in diet absorption and transport, and secondly the liver, which is the centre of intermediate metabolism in fish (publication 3).

Chapter 2

Material and methods

Figure 2.1. Experimental design and feeding protocol of the entire thesis work.



Ingredients and composition of the diets used during the feeding trials are given in details in the following section of Materials & Methods chapter (section 2.1). More details about the sample size, sampling protocols, and specific tissues studied and the analyses performed are given in the respective articles (Chapter 3, Results).

2.1 Experimental trials

Animals, feeding trial and sampling procedures

Rainbow trout used in the first part of the study were from a mixed sex population, which was produced at INRA fish facilities located in Brittany (PEIMA, Sizun, France). Fish were reared under natural photoperiod and temperature conditions, from first feeding throughout a three-year life cycle, including two reproduction events. Just prior to first feeding, trout were divided into two groups and fed one of the two following diets : a commercial (COM) diet from "*Le Gouessant"* (Lamballe, France) containing a mix of FM, FO and plant ingredients, and an experimental plant-based diet (VEG), completely devoid of FM and FO, which were replaced by a blend of plant ingredients. The latter diet was formulated by UR NuMeA (INRA, Saint-Pée sur Nivelle, France) and manufactured at the INRA experimental facilities of Donzacq (France). Ingredients and compositions of the diets are presented in Table 2.1. Briefly, in the COM-diet, 45% of FM and 50% of FO were replaced by plant ingredients. During the feeding trial, the producer of the commercial diet (*Le Gouessant*) changed the origin of FO, so that fish of the COM group received diets with slightly different FA composition during the first two years (COM_1) and the third year (COM_2) of rearing. The VEG-diet contained only plant protein sources (corn and wheat gluten, soybean meal, white lupin, dehulled peas) and a blend of vegetable oils (50% rapeseed oil, 30% linseed oil, 20% palm oil). The protein sources were chosen to bring a high level of protein and essential AAs. Nevertheless, it was necessary to add lysine and arginine in order to meet rainbow trout requirements (NRC 2011). The blend of vegetable oils was chosen in order to provide an overall amount of FA classes resembling proportions of FA classes found in fish oil. The proportions of the main FA of the two diets are provided in Table 2.2. A complete profile of all detected FA is given in Annex 1.

At the moment of first spawning (2 year-old females), ten females from each dietary treatment were sacrificed by benzocaine overdose, weighed and measured. The ova, liver, viscera and carcasses (whole gutted fish) were collected from each female and weighed. Three pools of 50 ova from each female were weighed, and the average weight (mg) of a single ovum was calculated. Remaining fish were reared for one more year at the PEIMA fish facility. At the second spawning event (3 year-old females), ova were sampled following the same protocol as described for the first one. At each spawning, approximately 400 of the collected ova per female

were fertilized with a pool of sperm collected from males fed a commercial diet, and the survival rate of progeny was recorded at different stages. At the second spawning event, eggs from the two broodstock groups (COM and VEG-fed females) were transferred to our experimental hatchery (INRA, Lees Athas, France), where the water temperature is around 7°C all year long.

Fry from both cohorts were subsequently split into three groups. Each group (4 replicates) received one of the three experimental diets from first feeding: a marine (M) diet, based on marine resources (no replacement), a commercial-like (C) diet, containing both marine and plant-based ingredients (replacement of 46% FM and 69% of FO), and a completely plant-based diet (V), with total replacement of marine FM and FO by plant-based proteins and VOs. The ingredients and composition of the three diets are provided in Table 2.3. In order to obtain total replacement of fish products, only plant protein sources and vegetable oils (7% rapeseed oil, 7% linseed oil and 4% palm oil) were used in the V-diet, whereas the M and C-diets contained FO (12% and 8%, respectively). Consequently, the V-diet contained no n-3 LC-PUFAs, whereas it contained a high level of 18:3 n-3, mainly derived from linseed oil, compared to the other two experimental diets (Table 2.4; see Annexe 2 for the complete profile of all detected FA). Just prior to first feeding (62 days post-fecundation), whole body samples of some fry were collected. After 3 weeks of feeding, survival rates and body weights of alevins were recorded, and whole body alevin samples were collected for analysis of body lipid composition and for transcriptome analysis.

Remaining alevins from COM-fed broodstock were reared until juvenile stage in the same fish facility and fed the same experimental diets M_1, C_1 or V_1. After 7 months of feeding, some fish were sacrificed by benzocaine overdose and samples of whole body fish, liver and intestine (mid gut) were collected (n=8 per dietary treatment).

In order to investigate the responses at very long term under conditions that stimulate the growth rate, remaining juveniles were then transferred to INRA fish facilities of Donzaq, where they were reared at a constant water temperature of 17°C under natural photoperiod for six more months (ongrowing fish). Fish were fed on the same kind of experimental diets (marine-based, mix or plant-based, 3 replicates per dietary group), however pellet size and formula of the diets were adapted to the fish developmental stage and size (M_2, C_2, V_2 – Table 2.3 and 2.4; see Annexe 2 for the complete profile of all detected FA). At the end of the 13-month feeding trial,

samples of whole body fish, liver and intestine (mid-gut) were collected (n=8 per dietary treatment).

All the samples of collected tissues, ova and whole body of swim-up fry and alevins were immediately frozen in liquid nitrogen and stored at -80°C until analysis. Whole fish (juveniles and ongrowing fish) collected for whole body composition analysis were stored at -20°C until analysis.

Table 2.1 Ingredients and composition of the experimental broodstock diets.

Diets	**COM $_{1-2}$**	**VEG**
Ingredients (g/Kg)		
Fish meal *	**434**	**0**
Corn gluten	0	170
Organic soybean meal	163	200
Wheat gluten	0	250
Organic durum wheat	100	49.8
White lupin	0	57.2
Organic dehulled pea	86	30
Fish oil **	**105**	**0**
Rapeseed oil	0	62
Linseed oil	0	37
Palm oil	0	24
Organic soybean oil	105	20
L-lysine	0	15
L-arginine	0	10
CaHPO4.2H20 (18%P)	0	35
Binder	0	20
Min.-Vit. Premix[a]	7	20
Composition (% DM)		
Crude protein	40	44.8
Crude fat	28	23.3
Energy kJ/g DM	24.5	23.6

*Origin co-fishery products - all species; ** Origin co-fishery products – sardine; COM: commercial diet *Le Gouessant;* VEG: experimental 100% plant-based diet.

[a] Detailed composition of Vit-Min premix (UPAE, INRA) used in the experimental diet is given in the Annex 3.

Table 2.2 Proportions of the main fatty acids (% of total FA) of broodstock diets.

	COM $_1$	**COM $_2$**	**VEG**
Saturated	23.7	25.8	16.1
MUFA	22.5	38.3	39.8
18:2 n-6	5.7	8.5	21.7
20:2 n-6	0.2	0.4	0.05
20:3 n-6	0.2	0.2	0
20:4 n-6	1.3	0.6	0
22:2 n-6	0.4	0.1	0.2
22:4 n-6	0.1	*nd*	0
PUFA n-6	8.1	10.0	21.9
18:3 n-3	1.5	2.7	20.1
18:4 n-3	2.5	1.9	0
20:3 n-3	0.1	0.2	0
20:4 n-3	0.9	0.7	0
20:5 n-3	17.6	6.9	0
22:4 n-3	0.3	*nd*	0
22:5 n-3	1.9	1.1	0
22:6 n-3	11.5	6.4	0
PUFA n-3	36.3	20.2	20.1

COM: commercial diet *Le Gouessant*; VEG: experimental 100% plant-based diet; MUFA: monounsaturated fatty acid; PUFA n-3: polyunsaturated fatty acids n-3; PUFA n-6: polyunsaturated fatty acids n-6; *nd*: not detected

Table 2.3 Ingredients and composition of the experimental diets.

	alevins – juveniles			***ongrowing fish***		
Diets	**M_1**	**C_1**	**V_1**	**M_2**	**C_2**	**V_2**
Ingredients (%)						
Fish meal *	**65.2**	**30.0**	**0.0**	**54.3**	**30.0**	**0.0**
Corn gluten	0.0	13.2	24.0	0.0	10.2	18.0
Soybean meal 48	0.0	6.1	2.0	0.0	6.3	4.3
Wheat gluten	0.0	10.0	22.0	0.0	5.0	12.1
Soy protein concentrate	0.0	10.2	20.0	0.0	3.5	18.1
White lupin	0.0	0.4	2.5	0.0	6.5	5.0
Peas	0.0	4.1	0.0	0.0	6.9	2.4
Rapseed meal 00	0.0	6.2	2.3	0.0	6.3	9. 8
Extruded whole wheat	21.1	1.3	0.0	30.1	7.2	2.8
Fish oil **	**11.7**	**8.1**	**0.0**	**13.6**	**8.0**	**0.0**
Rapeseed oil	0.0	8.1	6.7	0.0	8.0	7.3
Linseed oil	0.0	0.0	6.7	0.0	0.0	7.3
Palm oil	0.0	0.0	3.6	0.0	0.0	3.0
Vit- Min premix[a]	2.0	2.0	2.0	2.0	2.0	2.0
Soy lecithin	0.0	0.0	2.0	0.0	0.0	2.0
L-lysine	0.0	0.3	1.5	0.0	0.1	1.5
L-Methionine	0.0	0.01	0.3	0.0	0.0	0.3
CaHPO4.2H20 (18%P)	0.0	0.0	2.9	0.0	0.0	2.6
Attractant Mix	0.0	0.0	1.5	0.0	0.0	1.5
Composition (% DM)						
Dry Matter (DM, %)	94.3	95.3	95.5	93.8	95.2	95.0
Crude protein	48.9	53.3	52.9	44.4	46.3	47.2
Crude fat	21.5	22.1	21.8	22.0	24.2	24.5
Starch	20.5	11.5	8.2	20.0	11.5	8.0
Energy (kJ/g DM)	23.0	24.2	24.1	23.9	24.3	25.1
Cholesterol	0.70	0.55	0.36	0.68	0.51	0.41

*Origin co-fishery products - all species; ** Origin co-fishery products – sardines.
M: marine FM-FO-based diet; C: commercial-like FM-FO & plant-based diet; V: 100% plant-based diet.

[a] Detailed composition of Vit-Min premix (UPAE, INRA) used in the experimental diets is given in the Annex 3.

Table 2.4 Proportions of the main fatty acids (% of total FA) in experimental diets.

	alevins – juveniles			***ongrowing fish***		
	M_1	**C_1**	**V_1**	**M_2**	**C_2**	**V_2**
Fatty acid						
Saturated	30.8	20.9	18.5	26.6	18.2	17.6
MUFA	33.2	41.9	38.3	28.9	42.9	37.9
18:2 n-6	3.2	12.5	21.5	2.9	12.0	21.4
20:4 n-6	0.7	0.4	0.0	0.8	0.4	0.0
PUFA n-6	4.3	13.1	21.5	4.3	12.8	21.4
18:3 n-3	1.1	4.8	21.3	0.8	4.5	22.7
18:4 n-3	2.1	1.2	0.0	2.3	1.3	0.0
20:5 n-3	11.1	6.7	0.0	14.7	8.3	0.0
22:5 n-3	1.1	0.7	0.0	1.7	1.1	0.0
22:6 n-3	6.7	4.2	0.0	9.9	5.5	0.0
PUFA n-3	23.3	18.1	21.3	30.8	21.4	22.7

M: marine FM-FO-based diet

C: commercial-like FM-FO & plant-based diet

V: 100% plant-based diet

MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid

2.2 Analytical methods

Chemical composition of the diets

The chemical composition of the diets was analysed using the following procedures: dry matter (DM) after drying at 105°C for 24h, lipid content by petroleum ether extraction (Soxterm, Gerhardt, Konigswinter, Germany), protein content (Nx6.25) by the Kjeldahl method after acid digestion, gross energy in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany), ash content by incinerating the samples at 600°C for 6h.

Plasma metabolites

Plasma metabolite levels (glucose, cholesterol, triglycerides and phospholipids) were determined using commercial kits adapted to microplates, according to the recommendations of the manufacturer.

Total lipids and fatty acid analysis

Samples of female trout (carcass, viscera and liver), ova and whole body alevins were analysed for lipid content and fatty acid profile. Total lipids were extracted according to Folch et al. (1957), using dichloromethane instead of chloroform and quantified gravimetrically. FA composition was determined on the total lipid extract. Fatty acid methyl esters (FAME) were prepared by acid-catalyzed transmethylation of total lipids using boron trifluoride (BF3) in methanol (14%) according to Shantha and Ackman (1990). They were analysed in a Varian 3900 gas chromatograph (Varian, les Ulis, France), equipped with a fused silica DB Wax capillary column (30m x 0.25 mm internal diameter, film thickness 0.25 µm; JW Alltech, France). The injection volume was 1 µl, using helium as carrier gas (1 ml/min). The temperatures of the injector and the flame ionization detector were set at 260°C and 250°C, respectively. The thermal gradient was as follows: 100-180°C at 8°C/min, 180- 220 °C at 4°C/ min and a constant temperature of 220°C for 20min. FAs were identified by references from a known standard mixture (Sigma, St Louis, MO, USA) and peaks were integrated using Varian Star Chromatography Software (Star Software, version 5).

Microarray and RT-qPCR analysis

RNA extraction

Total RNA was extracted from individual whole body swim-up fry, alevins, as well as from liver and intestine (mid-gut) of juveniles and on-growing fish (n=8/treatment), using the TRIzol® reagent method (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The quantity and quality of extracted RNA were analysed using a spectrophotometer (ND-1000, NanoDrop) and a Bioanalyzer (Agilent Technologies, Kista, Sweden), respectively.

Microarray analysis

Microarray analysis, including cRNA synthesis, labeling and purification, as well as microarray hybridization and scanning, were performed at the INRA genomic analysis platform at Rennes.

cRNA synthesis, labelling and purification

Cyanine 3-CTP (Cy3) labelled experimental cRNA samples were generated using the Agilent "One-Color Microarray-based Gene Expression Analysis" (Low Input Quick Amp Labeling-LIQA) kit, following the manufacturer's instructions. The method uses T7 RNA Polymerase Blend, which simultaneously amplifies target material and incorporates Cy3. 150ng of total RNA was used for each sample to generate fluorescent cRNA. Agilent Spike-In was included in each reaction. After a denaturation step (10min in circulating water bath at 65°C) and a cRNA synthesis step (2h at 40°C), the reactions were incubated at 70°C for 15 min to inactivate the AffinityScript enzyme. To perform the labeling reaction, cRNA samples were each mixed with 6 µl of Transcription Master Mix cocktail, containing Cy3-dye, and then incubated at 40°C for two hours. Purification was performed using Quiagen RNeasy mini spin columns, eluting in 30µl of RNase-free water.

Microarray hybridization and scanning

Cy3-labeled cRNA samples yield (>0.825µg cRNA) and specific activity (> 6pmol of Cy3/µg of cRNA) were verified using a NanoDrop ND-1000. 600ng of Cy3-cRNA were fragmented and hybridized on a sub-array, following the LIQA kit instructions. During the experiment, samples were randomized, preventing samples from the same dietary treatment being overrepresented in a particular batch, in order to avoid unintentional biases. The hybridization reactions were allowed to occur for 17h in a rotating hybridization oven (65 °C), prior to washing according to the manufacturer's instructions.

Slides were scanned with an Agilent Scanner (Agilent DNA Microarray Scanner, Agilent technologies, Massy, France) using the standard parameters for a gene expression 8x60K oligoarray (3µm–20bits). Data were then obtained with the Agilent Feature Extraction software (10.7.1.1), according to the appropriate GE protocol (GE1_107_Sep09).

Table 2.5 Primer sequences of genes selected for analysis by RT-qPCR (alevins).

Gene	*Primer 5'-3' (FW)*	*Primer 5'-3' (RV)*	*Annealing temperature, °C*
Broodstock nutritional history			
Muscle growth and contraction			
ACTA1	AAAACAGGCCAGGGACAACA	CCTGGTATTGCTGCCCGTAT	60
CKM	TGCGTTGGTCTGAAAAGGATTGA	TCTCCTCAAACTTGGGGTGTGT	60
MYBPC1	CCAGCATCCAGAACCATCCT	TACACTGGGGAAGGTCGACA	60
MYBPC2	GTGAGTGTCCGTTTGTTGCC	CTGCCAAGTGAGACTGACGT	60
Carbohydrate/energy metabolism			
PYGM	TGCAATGTGTGTCGGTGTTG	AAGTTCCCTGGAGACCACGA	60
PYGL	AACCGACACCTCCACTTCACC	CCTGCATCTTCCTCCATCTC	60
6PFKM	GAGGGCGAAGATGAAGCTTG	GGGACCTCGAGATGAACGTA	60
SDHA	TGGTGTTTGGACGTGCCTGC	AACACAGCGGCGTGGTTCTG	60
GPD1	CTTCGCCCGGATATTCTGCA	GACCCTGGAGCTTCTGCCCA	60
PGK1	TTCGGCACAGCACACAGAGC	AAAGGGCCTGGCTGGTTTCTCC	60
First feeding diets			
Carbohydrate metabolism			
GCK	GCACGGCTGAGATGCTCTTTG	GCCTTGAACCCTTTGGTCCAG	60
HK2	CGCCGTGGTCGATAAGAT	TGATGAGAGCCGCCCCCTTT	60
LDHA	ATGCGTGCTGGGCAACAGTG	GCTGATAAATTAACCCTCCGC	60
Lipid/cholesterol metabolism			
HMGCR	GAACGGTGAATGTGCTGTGT	GACCATTTGGGAGCTTGTGT	60
HMGCS1	AGTGGCAAAGAGAGGGTGTG	TTCTGGTTGGAGACGAGGAG	60
Amino acids/protein metabolism			
DARS	GACCTGGCGGACATTGTGAA	GAGAGGGCCATTCACCACAA	60
EPRS	GTCGTCTGATGCCCTCTTGA	TGAAGCAGGGTCAGTGTGTG	60
IARS	ACATCGTGACTCGCTTCGCC	CTACAACCGTCAGATACGCGG	60
LARS	CGGCAGTGACATGAATGCAG	CCACTGGCCACAATGCTTTC	60
Reference gene			
EF1α	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	59

Quantitative RT-PCR (RT-qPCR)

Six individual RNA samples per experimental condition, chosen based on homogeneity and purity of signals obtained by microarray analysis, were used as biological replicates. Total RNA (1 µg) was reverse-transcribed to cDNA with the SuperScript III RNase H reverse transcriptase (Invitrogen, Carlsbad, CA, USA), using oligo dT Primers. Real-time PCR (RT-PCR) was performed in the iCycler iQ TM (BIO-RAD, Hercules, CA, USA). Quantitative PCR (q-PCR) analyses for gene expression were performed, using the Roche Lightcycler 480 system (Roche Diagnostics, Neuilly-sur-Seine, France). The assays were performed using 2 µl of diluted cDNA mixed with 3 µl of Light cycler 480 SYBR® Green I Master mix in a total volume of 6 µl, using forward and reverse primers at a final concentration of 400 nM.

Primers design was performed using the Primer 3 software. Specific primer pairs were designed with an overlapping intron, when possible, using known trout sequences in nucleotide databases (GeneBank and INRA-Sigenae). In order confirm their specificity, amplicons obtained with the designed primer pairs were sent for sequencing. Database accession numbers and the sequences of forward and reverse primers used to test each gene are provided in Table 2.5 and Table 2.6.

The PCR protocol was initiated at 95 °C (10 min) for hot-start iTaq TM DNA polymerase activation. Forty-five cycles of PCR were performed, each one consisting of a heating step at 95 °C (15 sec) for denaturing, a second step at 60 °C (10 sec) for annealing, and third extension step at 72 °C (15 sec). Following the final cycle of the PCR, melting curves were systematically monitored (with a gradient of 0.5 °C/10 s from 55 °C to 94 °C), to ensure that only one target fragment was amplified. Each PCR run included replicate samples (duplicate of reverse transcription and PCR amplification, respectively) and negative controls (reverse transcriptase and RNA-free samples, respectively).

PCR reaction efficiency was calculated for each run based on the slope of a 5-point standard curve obtained with a serial dilution of pooled sample cDNAs. mRNA levels of target genes were normalized with the housekeeping gene α-elongation factor 1 (Ef1α), when its expression was found to be stable (alevins). In this case, the expression levels were calculated according to threshold cycle method (ΔΔCT) (Pfaffl 2001). When no reference genes (Ef1α, 18S or β-actin)

were found to be stably expressed between experimental groups (juveniles and ongrowing fish), mRNA levels of target genes studied were normalized following the method proposed by Matz et al. (2013), since the conditions of applicability of this method were satisfied by our dataset. The results were analysed using the R-package MCMC.qpcr, which implements generalized linear mixed model analysis of RT-qPCR data, based on the lognormal-Poisson model.

Table 2.6 Primer sequences of genes selected for analysis by RT-qPCR (juveniles and ongrowing fish).

Gene	*Primer 5'-3' (FW)*	*Primer 5'-3' (RV)*	*Annealing temperature, °C*
Protein catabolism			
CTSZ	GGAGCCCTTCATCAACCACA	TTGTTGGTCCACTGCCTGTT	60
CTSS	TTTGCCTCATTGCGTGTTCC	GTCTTTCATCAGCTGGCCCT	60
Carbohydrate metabolism			
PFKFB3	CTCCATGGCGTCTGTCTTGT	TGCAGTCTTTGATGCCACCA	60
Fatty acid catabolism			
FAAH	TCCCTGTCTCCACGGTAACA	AACAGCCTCTCCACCTCTCT	60
Cholesterol metabolism			
CYP51A1	CCCGTTGTCAGCTTTACCA	GCATTGAGATCTTCGTTCTTGC	60
HMGCR	GAACGGTGAATGTGCTGTGT	GACCATTTGGGAGCTTGTGT	60
DHCR7	GTAACCCACCAGACCCAAGA	CCTCTCCTATGCAGCCAAAC	60
Energy pathways and electron transport			
MDH2	TTGACATTGCCCACACACCT	AGATCATCACGGGTCATGCC	60
COX5b	AGATCACTGCCACGACACTATG	CTTTCCTTTCTTCAGTGCCTGC	60
Fatty acid bioconversion			
Elovl2	TGTGGTTTCCCCGTTGGATGCC	ACAGAGTGGCCATTTGGGCG	59
Elovl5	GAACAGCTTCATCCATGTCC	TGACTGCACATATCGTCTGG	59
Δ6-desaturase	AGGGTGCCTCTGCTAACTGG	TGGTGTTGGTGATGGTAGGG	59
Reference genes			
EF1α	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	59
18S	CGGAGGTTCGAAGACGATCA	TCGCTAGTTGGCATCGTTTAT	56
β-actin	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59

2.3 Statistical analysis and data mining

Data of fish weight, fish survival, and content in lipid and fatty acids of whole body or tissue are presented as mean ± standard deviation (SD). Data were statistically analysed using the R-software (version 2.14.0) and the Rcmdr package. The normality and the homogeneity of variance of the variables were tested with Shapiro-Wilk's and Levene's test, respectively. Data for broodstock (tissues), ova, and swim-up fry collected before the first feeding were analysed by an independent sample t-test to assess the effect of the different broodstock nutritional history, when both conditions were satisfied. The variables with non-parametric distribution were either normalized with an arcsin transformation or, if the criteria were still not met, compared using a non-parametric paired Wilcoxon test. Data for alevins collected after 3 weeks of feeding were analysed using a 2-way ANOVA (p-value <0.05) to assess the effects of the nutritional broodstock history and the first feeding diets. The variables with non-parametric distribution were normalized with an arcsin transformation.

Data from microarray analysis were normalized and analysed statistically using GeneSpring software (12.6, Agilent). Data were scale-normalized using the median value of each array to identify differentially expressed genes between conditions. An unpaired t-test was performed to determine the effects of the nutritional broodstock history on the transcriptome of alevins collected before first feeding (Benjamini Hochberg FDR correction, p-value cut-off 0.05). For analysis of whole body alevins collected after 3 weeks of feeding, differentially expressed genes were obtained by 2-way ANOVA, with the different broodstock nutritional histories and first feeding diets as independent variables (Benjamini-Hochberg correction, p-value cut-off 0.05). For juveniles, differentially expressed genes were obtained by a 1-way ANOVA (diet, p-value<0.05). For all genes found to be differentially expressed in both trancriptomic assays, Gene Ontology (GO) annotations (biological process, cellular component, molecular functions) were obtained using the Expression Analysis Systematic Explorer (EASE) software version 2.0. Significant enrichment of GO was tested by using EASE software and the Benjamini correction (score < 0.05). All data were deposited in GO databases.

Gene expression data obtained by RT-qPCR were tested for normality and homogeneity of variances with Shapiro-Wilk's test and Levene's test, respectively. When variances were not normally distributed, a logarithmic transformation was performed. To assess the effects of the nutritional broodstock history and the first feeding diets, gene expression was analysed by two-way ANOVA (p-value <0.05). Post-hoc comparisons were made using Tukey's range test, and differences were considered statistically significant at p-value<0.05. To assess the diet effect in juveniles and ongrowing fish, data from RT-qPCR were analysed by one-way ANOVA (diet, p-value<0.05), followed by a Tukey's post-hoc test (p-value<0.05).

Chapter 3

Results

The data generated from the experiments performed in this PhD thesis have been the subject of three peer-reviewed publications: the first one is published, the second one has been accepted for publication (in press), while the third one will be submittedsoon. Therefore, in this section, the original scientific articles are presented chronologically, following the order of the questions addressed in the thesis.

Publication 1

Viviana Lazzarotto, Geneviève Corraze, Amandine Leprevost, Edwige Quillet, Mathilde Dupont-Nivet and Françoise Médale. 2015. Three-year breeding cycle of rainbow trout (Oncorhynchus mykiss) fed a plant-based diet, totally free of marine resources: consequences for reproduction, fatty acid composition and progeny survival. *PlosOne,* 2015, vol. 10, no 2. *(DOI: 10.1371/journal.pone.0117609).*

Publication 2

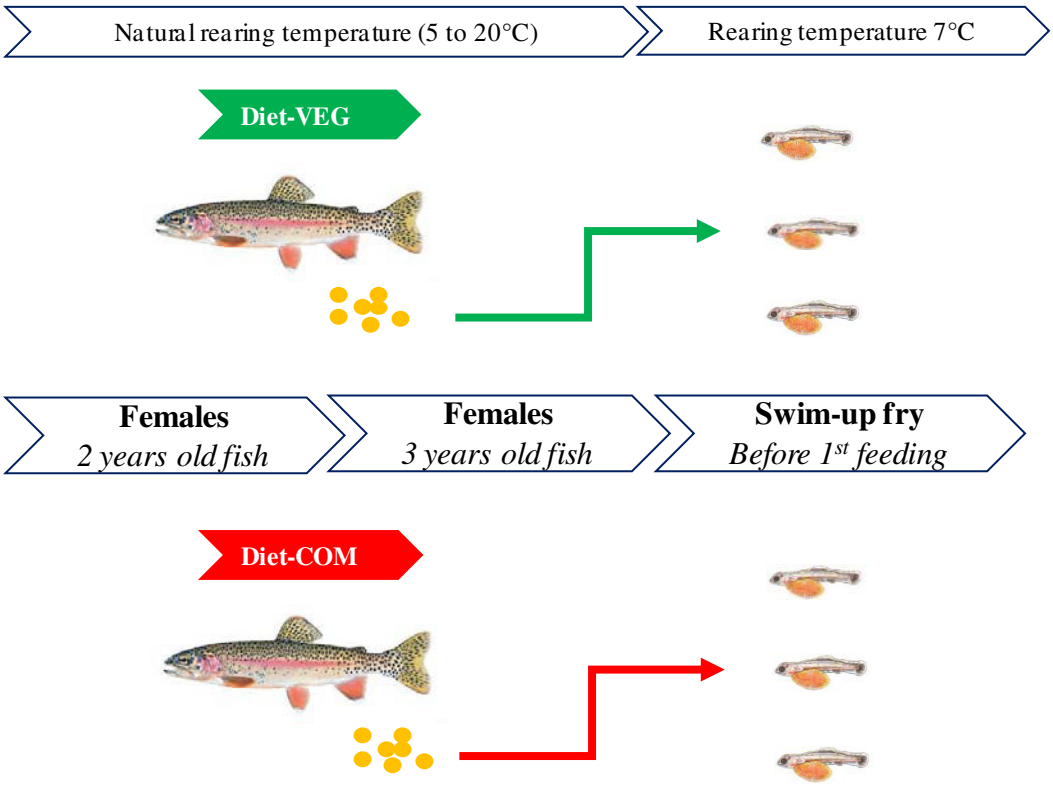
Viviana Lazzarotto, Geneviève Corraze, Laurence Larroquet, David Mazurais and Françoise Médale. Does broodstock nutritional history affect the response of progeny to different first-feeding diets? A whole-body transcriptomic study of rainbow trout alevins. (In press: *The British Journal of Nutrition).*

Publication 3

Viviana Lazzarotto, Françoise Médale, Laurence Larroquet and Geneviève Corraze. Replacement of fish meal and fish oil by plant products in diets for rainbow trout: long-term effects with focus on gene expression in the intestine and liver (*The British Journal of Nutrition, will be submitted soon*).

Publication #1

Figure 3.1 Experimental design of Publication 1.



Presentation of the article

Objective

In the present study we aimed to assess, for the first time, if rainbow trout are able to survive, grow, and reproduce when fed a totally plant-based diet free from FM and FO during the whole life cycle (from first feeding to broostock stage). Focusing on females, we therefore studied the reproductive performance of trout after a complete cycle of breeding, including two spawning events, while consuming a diet totally devoid of marine ingredients, and thus of n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFAs), that play a major role in the formation of ova.

Experiment & analysis

Two groups of rainbow trout were fed right from first feeding and during the whole life cycle (3-year feeding trial) either a commercial diet-C, containing both marine and plant ingredients, or a 100% plant-based diet-V, containing a blend of plant proteins and VOs. At spawning (2-years old fish), reproductive performance was assessed, and ova, liver, digestive tract, and carcasses were sampled from ten females of each dietary treatment. Rearing of the remaining fish continued with the two diets up to the following reproduction event (3-years old fish), when ova were collected from females of each group. At each spawning, ova were fertilized with a mix of sperm from different males fed the commercial diet, and the survival rate of progeny was measured at eyed stage, hatching and swim-up stage (before first feeding). All collected tissues, ova and swim-up fry were analysed for lipid and FA profile.

Results and Conclusions

The results of the study prove for the first time, that rainbow trout can achieve a three-year breeding cycle including two spawning events, in spite of being fed a plant-based diet totally free of marine resources from first feeding onwards.

Higher perivisceral lipid deposition was found in V-fed females, while no differences in lipid content were found in ova and swim-up fry from either broodstock groups. Lipid content was lower in swim-up fry (5%) than in ova (11%). This decrease reflected the utilization of ovum-

stored nutrients by developing embryos, and was observed in offspring from both broodstock groups.

Although the V-diet was devoid of n-3 LC-PUFAs, relatively high amounts of EPA and DHA were found in livers and ova, demonstrating efficient bioconversion of alpha-linolenic acid into EPA and DHA, and selective deposition of these n-3 LC-PUFAs into the ova. However, this bioconversion was not enough to compensate for the diet-induced changes in FA profile of fish tissues in broodstock.

Concerning the survival of offspring, the mean survival rate of progeny originating from V-fed females was lower than that of progeny from COM-fed females at year-2 spawning only. No significant differences in the survival rate were found at the year-3 spawning event.

In conclusion, trout which were reared on plant-based diets completely devoid of LC- PUFAs throughout the entire life cycle (3 years) were able to survive and produce viable ova and progeny. This was probably linked to the fact that the female rainbow trout were able to synthesize ARA, EPA and DHA from dietary precursors. These neo-synthesized LC-PUFAs were preferentially incorporated into ova, which in fish represent the main sources of nutrients utilized by the embryo prior to exogenous feeding.

PLOS ONE

RESEARCH ARTICLE

Three-Year Breeding Cycle of Rainbow Trout (*Oncorhynchus mykiss*) Fed a Plant-Based Diet, Totally Free of Marine Resources: Consequences for Reproduction, Fatty Acid Composition and Progeny Survival

Viviana Lazzarotto[1], Geneviève Corraze[1], Amandine Leprevost[1], Edwige Quillet[2], Mathilde Dupont-Nivet[2], Françoise Médale[1,2]*

1 INRA, UR 1067 "Nutrition, Métabolisme, Aquaculture", Aquapôle, 64310 Saint-Pée-sur-Nivelle, France, **2** INRA, UMR 1313 "Génétique Animale et Biologie Intégrative", 78352 Jouy-en-Josas, France

* medale@st-pee.inra.fr

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Abstract

Terrestrial plant resources are increasingly used as substitutes for fish meal and fish oil in fish feed in order to reduce the reliance of aquaculture on marine fishery resources. Although many studies have been conducted to assess the effects of such nutritional transition, no whole breeding cycles of fish fed diets free from marine resources has been reported to date. We therefore studied the reproductive performance of trout after a complete cycle of breeding while consuming a diet totally devoid of marine ingredients and thus of n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFAs) that play a major role in the formation of ova. Two groups of female rainbow trout were fed from first feeding either a commercial diet (C, marine and plant ingredients), or a 100% plant-based diet (V, blend of plant proteins and vegetable oils). Livers, viscera, carcasses and ova were sampled at spawning and analyzed for lipids and fatty acids. Although the V-diet was devoid of n-3 LC-PUFAs, significant amounts of EPA and DHA were found in livers and ova, demonstrating efficient bioconversion of linolenic acid and selective orientation towards the ova. Some ova were fertilized to assess the reproductive performance and offspring survival. We observed for the first time that trout fed a 100% plant-based diet over a 3-year breeding cycle were able to produce ova and viable alevins, although the ova were smaller. The survival of offspring from V-fed females was lower (-22%) at first spawning, but not at the second. Our study showed that, in addition to being able to grow on a plant-based diet, rainbow trout reared entirely on such a diet can successfully produce ova in which neo-synthesized n-3 LC-PUFAs are accumulated, leading to viable offspring. However, further adjustment of the feed formula is still needed to optimize reproductive performance.

Introduction

Aquaculture is currently the fastest growing animal production sector and has been expanding continuously for the last 25 years to meet the increasing demands in terms of fish as food that fisheries cannot meet. Aquaculture now contributes to 40% of the total fish production for human consumption and is expected to reach 60% by 2020 [1,2]. This supposes a growth rate of aquaculture that cannot be achieved by using fish meal (FM) and fish oil (FO) as major dietary sources. Their use in fish production is more than ever limited because of their decreasing availability and high cost. Carnivorous fish species, such as salmonid species, remain among the highest consumers of fish oil (51% of the market share) and the third highest of fish meal (19.5% of the market share) [3]. Among salmonids, rainbow trout (*Oncorhynchus mykiss*) farming represents the main freshwater production in Europe [3], and intense research efforts are being focused on replacing marine feedstuffs (FM and FO) with more easily available plant-based ingredients [4] in their diets.

Replacement of FM by alternative terrestrial plant products has been explored for more than twenty years [4–6]. Although substantial reduction in dietary levels of FM can be achieved, there are still several difficulties to be overcome with regard to total replacement of FM by plant ingredients, even in salmonids. Some previous studies have shown that the complete replacement of FM by plant sources of protein leads to lower growth performance in rainbow trout [5], possibly linked to decreased feed intake [7]. Moreover, poorer digestibility of carbohydrates [8], that are abundant in plant-based diets, and the presence of antinutritional factors [9] can also be responsible for poorer growth performance. With regard to FO, several studies carried out in salmonids (rainbow trout, brown trout-*Salmo trutta*-, Atlantic salmon-*Salmo salar*) have shown that it is possible to replace FO totally by individual or mixtures of vegetable oils, without affecting growth or feed efficiency [10,11], provided that n-3 LC-PUFA requirements are met by the lipids contained in fish meal. A major problem when replacing both FM and FO by plant sources is the lack of n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids (FA) are important in the fish life-cycle, most notably for their roles in flesh quality [12] and reproduction, egg quality and offspring development [13,14]. In both salmonids and many cultured fish, sexual maturation and gonad development occur at the expense of stored energy, including lipids [15]. Previous studies in rainbow trout have shown that during sexual maturation lipids are initially mobilized from visceral adipose tissue [16], and mobilization from secondary storage sites (e.g. muscle) occurs only in the long term [17]. Moreover, visceral adipose tissue appears to be the primary source of energy for vitellogenesis [18], a crucial step in the female reproductive cycle. After mobilization of adipose fatty acids, this process ensures subsequent hepatic synthesis and export of lipoproteins (vitellogenin) which, together with lipids and vitamins, is finally taken up by the ova through endocytosis, providing energy reserves for ovum and offspring development. During this process, n-3 LC-PUFAs such as EPA and DHA are preferentially incorporated into ova, typically at a ratio of 2:1 [19]. EPA and DHA, as well as n-6 PUFA arachidonic acid (ARA), are recognized as determining factors in egg quality of several species [13,20–25] and in offspring development [26]. In addition to meeting energy requirements of early ontogenesis, one specific role of n-3 LC- PUFAs, in particular DHA, is incorporation into forming membranes and maintaining their fluidity [27]. Salmonids fed a diet lacking in n-3 LC-PUFAs are capable of biosynthesizing DHA via the desaturation and elongation of linolenic acid (available in plant ingredients, such as linseed oil) [17]. However, it has also been demonstrated that such bioconversion is not sufficient to compensate for the lack of dietary n-3 LC-PUFAs, resulting in a significant reduction of these FA in fish tissues [28]. While several studies have examined the effects of a plant-based diet on growth, metabolism

and fish flesh quality, the consequences of supplying a plant-based diet devoid of FM and FO across an entire life-cycle on reproductive performance and offspring survival have not been studied to date. This constitutes a significant gap in knowledge, since successful reproduction, egg quality and offspring development are key elements in fish farming [29].

We investigated whether rainbow trout reared on a plant-based diet from first feeding to reproduction were capable of synthesizing sufficient amounts of n-6 and n-3 LC-PUFAs from precursors to survive and reproduce. Furthermore, in addition to reproductive performance the incorporation of n-3 and n-6 PUFAs into ova and the subsequent trans-generational effects on offspring survival were also studied, in order to assess whether female trout exclusively reared on a plant-based diet can produce viable ova and offspring.

Materials and Methods

Ethics

The experiment was carried out in strict accordance with EU legal frameworks relating to the protection of animals used for scientific purposes (Directive 2010/63/EU) and guidelines of the French legislation governing the ethical treatment of animals (Decree no. 2001-464, May 29th, 2001). It was approved by the ethics committee of INRA (INRA 2002-36, April 14, 2002). The INRA experimental facility is certified for animal services under the permit number B29-277-02 by the French veterinary services, which is the competent authority and the scientist in charge of the experimentation received training and personal authorization (N°B64 10 003). During the experiment absence or occasional presence of dead (opaque, whitish) or unfertilized (transparent) eggs was daily checked, as well as the eggs shape and size. Dead, unfertilized or not normally shaped/sized eggs were carefully removed with a pipette by competent person in charge of the experiment fish rearing. As well as for the eggs, fish were daily monitored during the study. If any clinical symptoms (i.e. morphological abnormality, restlessness or uncoordinated movements) were observed, fish were sedated by immersion in 2% benzocaine solution and then euthanized by immersion in a 6% benzocaine solution (anesthetic overdose) during 3 minutes, to be certain that death was achieved.

Diets

The experiment was conducted with two different diets: a commercial (C) diet from "*Le Gouessant*" (Lamballe, France) containing a mix of FM, FO and plant ingredients, and an experimental plant-based diet (V), completely free from FM and FO, which were replaced by a blend of plant ingredients. The latter diet was formulated by UR NuMeA (INRA, Saint- Pée sur Nivelle, France) and manufactured at the INRA experimental facilities (Donzacq, France). Ingredients and compositions of the diets are presented in Table 1. In the C-diet 45% of FM and 50% of FO were replaced by plant ingredients. During the feeding trial, the producer of the commercial diet (*Le Gouessant*) changed the origin of the ingredients (mainly FO), so that fish of the C group received diets (C_1 and C_2) with slightly different FA composition over the three breeding years. The V-diet contained only plant protein sources and a blend of vegetable oils (50% rapeseed oil, 30% linseed oil, 20% palm oil). This blend was chosen in order to provide an overall amount of FA classes closely resembling the proportions of FA classes found in fish oil. No n-6 or n-3 long chain poly-unsaturated fatty acids (LC-PUFAs), such as eicosapentaenoic acid (EPA), docosahexaenoic acid (EPA) or arachidonic acid (ARA), were present in the V diet, whereas it contained high levels of 18:3 n-3 (alpha-linolenic acid, ALA) and 18:2 n-6 (linoleic acid, LA) compared to the C diet. The fatty acid composition of the two diets is provided in Table 2.

Table 1. Ingredients and composition of diets.

Diets	C	V
Ingredients (g/Kg)		
Fish meal *	434	0
Corn gluten	0	170
Soybean meal	163	200
Wheat gluten	0	250
Durum wheat	100	49.8
White lupin	0	57.2
Dehulled peas	86	30
Fish oil **	105	0
Soybean oil	105	0
Rapeseed oil	0	62
Linseed oil	0	37
Palm oil	0	24
Soy lecithin	0	20
L-lysine	0	15
L-arginine	0	10
CaHPO4.2H20 (18%P)	0	35
Binder	0	20
Min.-Vit. Premix	7	20
Composition (% DM)		
Crude protein	40	44.8
Crude fat	28	23.3
Energy kJ/g DM	24.5	23.6

*Origin co-fishery products—all species

** Origin co-fishery products—sardines

C: commercial diet *Le Gouessant*, V: experimental 100% plant-based diet

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Fish and experimental design

Female rainbow trout were produced at the INRA fish facilities (PEIMA, Sizun, France). Throughout the experiment, fish were reared under natural photoperiod and temperature conditions. Fish were randomly divided into two groups that were reared either on the commercial diet (C) or on the plant-based FM/FO-free diet (V). The dietary treatment was applied from first feeding until the end of the trial. Female fish of the V-diet group were fed twice a day, until apparent satiety. In order to avoid wide differences in body weight at the time of analysis, feed ration of C-group was adjusted to that of V-group, because it is known that feeding rainbow trout with plant-based diets free of marine resources leads to reduced feed intake [5,7], which results in reduced weight gain in large size rainbow trout [30].

At the moment of first spawning (2 years old females), ten females from each dietary treatment were sacrificed by benzocaine overdose, weighed and measured. The ova, liver, digestive tract (intestine with perivisceral adipose tissue) and carcasses (whole gutted fish) were collected from each female and weighed. Three pools of 50 ova from each female were weighed and the average weight (mg) of a single ovum was calculated. Absolute fecundity was measured as number of ova/female and gonadosomatic index (GSI) was calculated as: (gonad weight/total female body weight) x 100.

Table 2. Fatty acid composition (% of total fatty acids) of diets.

Diets	C_1	C_2	V
Fatty acid			
Saturated	23.7	25.8	16.1
MUFA	22.5	38.3	39.8
Σ n-6	8.1	10.0	21.9
18:2 n-6 (LA)	5.7	8.5	21.7
20:2 n-6	0.2	0.4	0.05
20:3 n-6	0.2	0.2	0.0
20:4 n-6 (ARA)	1.3	0.6	0.0
22:2 n-6	0.4	0.1	0.2
22:4 n-6	0.1	nd	0.0
Σ n-3	36.3	20.2	20.1
18:3 n-3 (ALA)	1.5	2.7	20.1
18:4 n-3	2.5	1.9	0.0
20:3 n-3	0.1	0.2	0.0
20:4 n-3	0.9	0.7	0.0
20:5 n-3 (EPA)	17.6	6.9	0.0
22:4 n-3	0.3	nd	0.0
22:5 n-3	1.9	1.1	0.0
22:6 n-3 (DHA)	11.5	6.4	0.0

C_{1-2}: commercial diet *Le Gouessant*, V: experimental 100% plant-based diet
C_1: fed until year-2 spawning; C_2: fed between year-2 and year-3 spawning
MUFA: monounsaturated fatty acids
nd: not detected

doi:10.1371/journal.pone.0117609.t002

Rearing of the remaining fish continued with the two dietary treatments (C_2 and V) up to the next reproduction (3 years old females) when ova were sampled following the same protocol as described for the first.

At each spawning approximately 400 of the collected ova per female were fertilized with a pool of sperm collected from males fed a commercial diet and the survival rate of progeny was measured at the eyed stage, at hatching, and at the swim-up fry stage (before 1st feeding).

All collected tissues, ova and swim-up fry sampled were stored at -80°C until analysis.

Lipid and fatty acid analysis

Total lipid content of female tissues, ova and swim-up fry was quantified gravimetrically after extraction by dichloromethane/methanol (2:1, v/v), containing 0.01% of butylated hydroxytoluene (BHT) as antioxidant, according to Folch *et al.* [31]. Neutral (NL) and polar lipid (PL) fractions were separated on silica cartridges (Sep-Pak, Waters, Ireland), according to Juaneda and Roquelin [32].

Fatty acid methyl esters (FAME) were prepared by acid-catalyzed transmethylation, using boron trifluoride according to Shantha & Ackman [33]. FAME were then analyzed in a Varian 3900 gas chromatograph equipped with a fused silica DB Wax capillary column (30m x 0.25 mm internal diameter, film thickness 0.25 μm; JW Alltech, France). Injection volume was 1 μl, using helium as carrier gas (1 ml/min). The temperatures of the injector and the flame ionization detector were 260°C and 250°C, respectively. The thermal gradient was as follows: 100–180°C at 8°C/min, 180–220°C at 4°C/ min and a constant temperature of 220°C for 20min.

Fatty acids were identified with reference to a known standard mixture (Sigma, St Louis, MO, USA) and peaks were integrated using Varian Star Chromatography Software (Star Software, version 5). The results for individual FA were expressed as percentage of total identified FA methyl esters and as quantities (g/100g tissue) for ARA, EPA and DHA.

Statistical analysis

Data were analyzed statistically using the R software version 2.14.0 and the Rcmdr package. The normality of distribution and the homogeneity of variance of the variables were tested with Shapiro-Wilk's and Levene's test, respectively. When both conditions were satisfied, an independent sample t-test was performed to assess the effects of the dietary treatment; the variables with non-parametric distribution were either normalized with an arcsin transformation or, if the criteria were still not met (some fatty acids), a non-parametric test (paired Wilcoxon test) was used for analysis.

Results

Biometric parameters and reproductive performance

Biometric parameters (Table 3) of fish of both groups were measured at the spawning of year-2 and year-3. At the year-2 spawning the body weights of females fed the V-diet were higher (+18%) than that of females fed the C-diet, indicating that limitation of food supplied to the C-fed group may have been too restrictive, but no such significant difference was found at the year-3 spawning. Female body lengths were similar at both spawnings irrespective of the dietary treatment. In terms of reproductive parameters (Table 3), total spawn weight from both C and V-fed females increased at the year-3 spawning, compared to the year-2. At the first spawning, the absolute fecundity (ova/female) of the V-fed females was higher (+17%) than that of the C-fed females. Absolute fecundity was higher at the year-3 spawning than at the year-2 for both the C-fed and V-fed females, and at that time no significant difference was detected between the two groups. Average ovum weight was significantly lower in V-fed fish at both spawnings (year-2: -17%, year-3: -12%). One female among the fish fed the V-diet did not

Table 3. Biometric parameters, reproduction performance and survival rates.

	Year-2 spawning			Year-3 spawning		
Diets	**C**	**V**	**p-value**	**C**	**V**	**p-value**
Biometric parameters						
Fish weight (g)	1185 ± 253	1446 ± 205	<0.05	3453 ± 727	3166 ± 539	ns
Fish length (mm)	395 ± 41	406 ± 20	ns	570 ± 51	558 ± 43	ns
Whole spawn weight (g)	224 ± 45	222 ± 43	ns	495 ± 140	349 ± 84	<0.05
Ova weight (mg)	53 ± 7	44 ± 7	<0.05	65 ± 9	57 ± 6	<0.05
Reproduction and survival performance						
Gonadosomatic index (%)	18.9 ± 2	15.3 ± 1.4	<0.05	14.3 ± 2.3	11.1 ± 2.4	<0.05
Absolute fecundity (ova female^{-1})	4243± 589	5113 ± 994	<0.05	7680 ± 2538	6334 ± 1700	ns
Eyed stage survival (%)	91 ± 4	69 ± 30	<0.05	90 ± 7	84 ± 12	ns
Hatching survival (% of eyed)	90 ± 5	56 ± 29	<0.05	87 ± 10	82 ± 14	ns
Swim-up fry survival (% of hatched)	85 ± 5	50 ± 32	<0.05	84 ± 7	78 ± 13	ns

Data are presented as mean ± SD. *p-values* were produced by independent sample t-test. *ns*: not significant

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produce any viable ova at the year-2 spawning. The gonadosomatic index of the V-fed females was significantly lower than that of the C-fed females, at both spawnings (-19% year-2 spawning; -28.8% year-3 spawning). A lower gonadosomatic index was observed for both groups at the year-2 spawning compared to the year-3.

Rates of progeny survival (Table 3) were assessed as percentage of fertilized ova that reached the eyed stage and that were then able to hatch and to develop into swim-up fry. The survival rates for progeny from the first spawning of V-fed females were lower than for progeny of broodstock fed the C-diet, and wide inter-individual variability was observed for the V-fed group (Fig. 1). However, at the year-3 spawning, no such significant diet-induced differences were detected between alevins from the two treatment groups (Table 3).

Tissue lipid composition

Fish fed the V-diet exhibited significantly lower lipid content in the liver (-38%), while higher lipid content was found in the digestive tract (+51%) and carcass (+44%) (Fig. 2). The lipid content in the carcass and digestive tract was mainly composed of neutral lipids (NL) for both V- and C-fed groups (>90% and >88%, respectively). However, the lipid content of livers was principally composed of polar lipids (PL) in both V- and C-fed females (65% and 55%, respectively).

In ova from the year-2 and the year-3 spawning (Fig. 2) no significant differences were observed in total lipid content (11%) or in the proportions of NL and PL, which represented 62–65% and 38–35%, respectively, for both female diets.

Similarly, no significant differences were detected in terms of total lipid content (5% i.e. lower than ova), or neutral or polar lipid fractions (68–69% and 31–32%, respectively) in swim-up fry from either treatment group (Fig. 2).

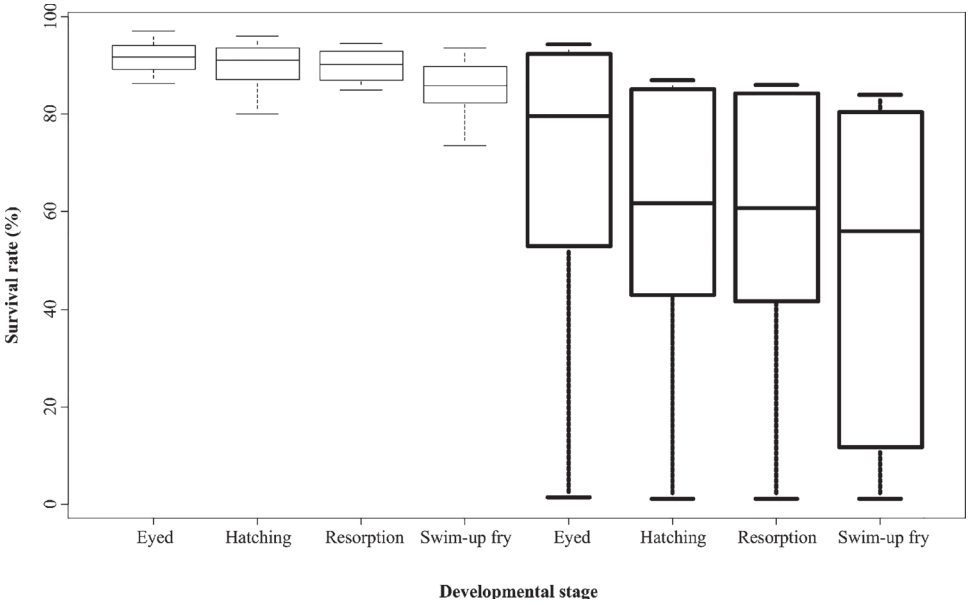

Fig 1. Individual variability of progeny survival rates at different stages from females fed the C and the V diet (in bold).

doi:10.1371/journal.pone.0117609.g001

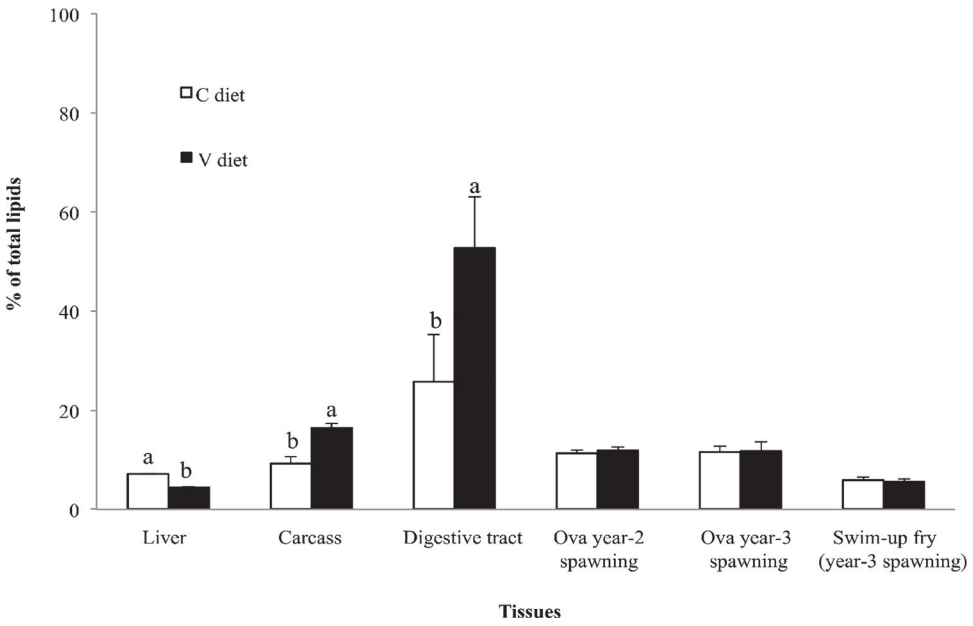

Fig 2. Total lipid content in female tissues, ova and swim-up fry. Data are presented as mean ± SD. p-values were produced by independent sample t-test. Different letters indicates significant differences (*p-value* <0.05).

doi:10.1371/journal.pone.0117609.g002

Fatty acid profile

NL and PL fractions of the tissues collected from sexually mature females, ova from the year-2 and year-3 spawning and alevins were analyzed for fatty acid composition. The proportions of FAs of all the tissues analyzed are presented in Table 4 and Table 5. A significant effect of the dietary treatment was observed for all fatty acids analyzed.

Saturated fatty acids (SAT) were mainly present in the PL fraction in all maternal tissues and in ova and swim-up fry, with lower values for the V-fed group (Table 4 and 5). In ova from the year-2 spawning and swim-up fry, no significant difference was detected in the PL fraction in terms of SAT content (Table 5), whereas percentages of SAT of the NL fraction were lower in the V-fed group than in the C-fed group (-24% and -21% in ova and swim-up fry, respectively), as well as in all the others tissues analyzed (Table 4).

With regard to monounsaturated fatty acid (MUFA) content, feeding the V-diet led to higher percentages of these FA in all tissues analyzed (+40%), in ova (+31–38%) and swim-up fry (+34%) in both the PL and NL fractions, with a more pronounced effect in the NL fraction. MUFAs were the most abundant FA class in the NL fraction of all maternal tissues, and ova and swim-up fry from V-fed fish.

Total n-6 PUFAs were higher in maternal tissues, and in ova and swim-up fry from the V-fed group (+10–15%). Linoleic acid (LA, 18:2 n-6) was mostly recovered in the NL fraction in all the tissues analyzed from both C- and V-fed groups, with significantly higher values when fed the V-diet. A higher proportion of both n-6 PUFA and LA was found in ova from C-fed females at the year-3 compared to the year-2, both in PL and NL fractions.

ARA was mainly present in the PL fraction of all tissues, and in ova and swim-up fry, irrespective of diet, with higher percentages observed for the V-fed group, except in the digestive tract. Lower percentages of ARA were found in the NL fraction of all maternal tissues with the V-diet (Table 4). Similar ARA content (g/100g tissue) was found in the carcasses and digestive tracts of

Table 4. Fatty acid composition (% of total FA) of livers, carcasses and digestive tracts (polar and neutral lipid fractions).

	Polar lipids			Neutral lipids		
Diets	**C**	**V**	**p-value**	**C**	**V**	**p-value**
Liver						
Saturated	26.6 ± 2.2	21.9 ± 2.4	<0.05	22.1 ± 1.9	14.1 ± 1.5	<0.05
MUFA	13.8 ± 1.4	16.5 ± 2.4	<0.05	23.3 ± 4.0	38.5 ± 5.5	<0.05
n-6 PUFAs	10.8 ± 0.8	20.1 ± 1.0	<0.05	10.6 ± 0.5	21.9 ± 0.6	<0.05
18: 2 n-6	3.0 ± 0.3	8.2 ± 0.9	<0.05	6.4 ± 0.9	16.8 ± 1.3	<0.05
ARA	6.9 ± 0.7	7.4 ± 1.0	ns	3.2 ± 0.7	1.8 ± 0.7	<0.05
n-3 PUFAs	47.1 ± 3.8	40.9 ± 4.7	<0.05	37.9 ± 6.1	23.5 ± 4.7	<0.05
18: 3 n-3	0.5 ± 0.1	3.4 ± 0.9	<0.05	1.5 ± 0.3	8.8 ± 0.8	<0.05
EPA	12.0 ± 1.3	6.1 ± 1.4	<0.05	12.6 ± 2.1	2.7 ± 1.1	<0.05
DHA	29.0 ± 3.8	24.8 ± 3.4	<0.05	16.2 ± 3.7	6.7 ± 3.3	<0.05
Carcass						
Satured	28.3 ± 1.0	23.6 ± 2.0	<0.05	25.1 ± 0.9	15.5 ± 0.8	<0.05
MUFA	15.7 ± 1.7	20.7 ± 2.0	<0.05	30.2 ± 1.6	47.5 ± 0.7	<0.05
n-6 PUFAs	6.2 ± 0.5	21.2 ± 0.8	<0.05	8.5 ± 0.4	21.7 ± 0.4	<0.05
18: 2 n-6	3.1 ± 0.3	14.0 ± 1.3	<0.05	7.1 ± 0.4	18.9 ± 0.3	<0.05
ARA	2.3 ± 0.1	3.9 ± 0.5	<0.05	1.1 ± 0.1	0.3 ± 0.1	<0.05
n-3 PUFAs	45.1 ± 2.8	32.1 ± 2.3	<0.05	31.4 ± 2.1	14.3 ± 0.7	<0.05
18: 3 n-3	1.0 ± 0.1	7.5 ± 0.7	<0.05	1.9 ± 0.1	9.9 ± 0.3	<0.05
EPA	11.9 ± 0.6	6.1 ± 0.8	<0.05	10.1 ± 1.0	0.7 ± 0.1	<0.05
DHA	27.1 ± 2.5	13.9 ± 2.2	<0.05	12.5 ± 0.9	0.9 ± 0.3	<0.05
Digestive tract						
Satured	31.7 ± 1.2	17.9	nc	17.9 ± 1.9	11.7 ± 0.8	<0.05
MUFA	26.8 ± 1.5	35.6	nc	30.9 ± 2.0	48.4 ± 0.9	<0.05
n-6 PUFAs	9.2 ± 0.4	21.4	nc	11.0 ± 0.6	23.0 ± 0.8	<0.05
18: 2 n-6	5.4 ± 0.7	15.2	nc	7.9 ± 0.6	19.7 ± 0.7	<0.05
ARA	2.5 ± 0.5	2.2	nc	1.4 ± 0.2	0.4 ± 0.1	<0.05
n-3 PUFAs	27.1 ± 2.1	22.6	nc	32.9 ± 3.5	15.4 ± 1.0	<0.05
18: 3 n-3	1.1 ± 0.2	6.1	nc	1.6 ± 0.3	8.9 ± 0.6	<0.05
EPA	6.5 ± 1.0	2.4	nc	7.5 ± 0.9	0.6 ± 0.1	<0.05
DHA	14.3 ± 1.7	8.5	nc	13.1 ± 1.7	1.7 ± 0.3	<0.05

Data are presented as mean ± SD; *p-values* were produced by independent sample t-test or equivalent non-parametric test (two-sample Wilcoxon test)
MUFA: monounsaturated fatty acids
nc: *p-value* not produced, PL for the V group pooled for practical reasons

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females from the two groups (Table 6), as well as in the ova at the year-3 spawning (Table 7). Significantly higher (or equal) percentages of ARA were detected in both NL and PL lipid fractions in ova and swim-up fry of the V-fed group compared to the C-fed group (Table 5).

Percentages of n-3 PUFAs were higher in the PL fraction compared to the NL fraction. It was the most highly represented FA class in maternal tissues (muscle, liver), ova and swim-up fry. Lower percentages of n-3 PUFAs were observed in all tissues for the V- fed group (-5 to -15%). Higher proportions of linolenic acid (ALA, 18:3 n-3) were detected in the V-fed group, particularly in the NL fraction. Percentages of EPA (20:5 n-3) and DHA (22:6 n-3) were lower in all maternal tissues, ova and swim-up fry from V-fed females (Table 5). Lower levels of EPA and DHA

Table 5. Fatty acid composition (% of total FA) of ova and swim-up fry (polar and neutral lipid fractions).

	Polar lipids			Neutral lipids		
Diets	**C**	**V**	**p-value**	**C**	**V**	**p-value**
Ova (year-2 spawning)						
Saturated	29.5 ± 3.1	28.0 ± 2.9	ns	17.0 ± 2.4	12.9 ± 0.8	<0.05
MUFA	11.3 ± 1.1	14.2 ± 1.1	<0.05	21.7 ± 1.6	34.7 ± 1.6	<0.05
n-6 PUFAs	5.9 ± 0.4	16.6 ± 0.8	<0.05	8.3 ± 0.5	19.9 ± 0.4	<0.05
18: 2 n-6	1.2 ± 0.1	6.0 ± 0.7	<0.05	5.1 ± 0.4	13.1 ± 0.7	<0.05
ARA	3.3 ± 0.2	5.2 ± 0.8	<0.05	1.9 ± 0.1	1.9 ± 0.3	ns
n-3 PUFAs	51.0 ± 3.9	39.6 ± 4.2	<0.05	46.4 ± 3.8	29.6 ± 1.7	<0.05
18: 3 n-3	0.3 ± 0.0	1.8 ± 0.3	<0.05	1.8 ± 0.2	7.7 ± 0.4	<0.05
EPA	12.2 ± 0.8	9.0 ± 1.2	<0.05	17.1 ± 1.7	4.2 ± 0.4	<0.05
DHA	33.3 ± 3.7	22.7 ± 3.8	<0.05	18.2 ± 2.1	9.7 ± 1.4	<0.05
Ova (year-3 spawning)						
Saturated	33.4 ± 2.2	31.3 ± 1.0	<0.05	20.5 ± 1.3	15.1 ± 0.8	<0.05
MUFA	12.2 ± 0.5	15.3 ± 0.7	<0.05	27.1 ± 1.4	39.4 ± 1.1	<0.05
n-6 PUFAs	9.1 ± 0.6	18.1 ± 1.2	<0.05	15.9 ± 0.9	19.7 ± 0.7	<0.05
18: 2 n-6	3.0 ± 0.4	6.2 ± 1.2	<0.05	12.5 ± 0.8	13.8 ± 1.0	<0.05
ARA	3.2 ± 0.1	6.1 ± 1.4	<0.05	1.3 ± 0.1	1.9 ± 0.6	<0.05
n-3 PUFAs	43.3 ± 1.9	33.6 ± 1.8	<0.05	32.1 ± 2.2	23.5 ± 0.8	<0.05
18: 3 n-3	0.4 ± 0.0	1.4 ± 0.3	<0.05	2.5 ± 0.2	7.4 ± 1.0	<0.05
EPA	10.1 ± 0.3	8.7 ± 0.8	<0.05	9.4 ± 1.1	3.4 ± 0.6	<0.05
DHA	29.0 ± 1.4	19.7 ± 1.8	<0.05	14.3 ± 1.4	6.9 ± 1.1	<0.05
Swim-up fry (year-3 spawning)						
Saturated	31.1 ± 2.5	28.3 ± 1.5	ns	18.7 ± 1.2	14.7 ± 0.7	<0.05
MUFA	13.0 ± 0.4	15.1 ± 0.6	<0.05	24.1 ± 0.7	36.4 ± 0.7	<0.05
n-6 PUFAs	8.2 ± 0.4	13.7 ± 0.6	<0.05	15.5 ± 0.2	20.3 ± 0.3	<0.05
18: 2 n-6	3.2 ± 0.1	4.3 ± 0.2	<0.05	11.4 ± 0.3	13.0 ± 0.2	<0.05
ARA	3.3 ± 0.2	6.4 ± 0.6	<0.05	1.7 ± 0.1	2.4 ± 0.2	<0.05
n-3 PUFAs	44.4 ± 2.7	38.6 ± 2.6	<0.05	37.1 ± 1.9	25.9 ± 1.0	<0.05
18: 3 n-3	0.5 ± 0.0	1.3 ± 0.4	<0.05	2.3 ± 0.2	6.6 ± 0.1	<0.05
EPA	9.0 ± 0.7	7.7 ± 0.5	<0.05	9.9 ± 0.6	3.8 ± 0.2	<0.05
DHA	32.5 ± 2.4	28.5 ± 2.8	ns	18.3 ± 1.1	9.6 ± 0.7	<0.05

Data are presented as mean ± SD. *p-values* were produced by independent sample t-test or equivalent non-parametric test (two-sample Wilcoxon test)
MUFA: monounsaturated fatty acids
ns: not significant

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(g/100g tissue) were found in the digestive tracts, carcasses and ova of females fed the V-diet compared to those of the C-fed females (Table 6 and Table 7). In swim-up fry, the DHA content of the PL fraction was in the same range, irrespective of the broodstock dietary treatment (Table 5).

Discussion

The results of this study prove, for the first time to our knowledge, that rainbow trout can achieve a 3-year breeding cycle including two spawnings events, despite being fed a plant-based diet totally free from marine resources. Our study focused on characterization of the dietary effects on the lipid content and fatty acid profile of female tissues, ova and progeny across

Table 6. ARA, EPA and DHA content (g/100g tissue) in carcasses and digestive tracts of C- and V-fed females.

	Diets		
	C	V	*p-value*
Carcass			
ARA	0.12 ± 0.03	0.11 ± 0.03	*ns*
EPA	1.05 ± 0.3	0.22 ± 0.1	*<0.05*
DHA	1.44 ± 0.4	0.31 ± 0.1	*<0.05*
Digestive tract			
ARA	0.01 ± 0.01	0.02 ± 0.01	*ns*
EPA	0.06 ± 0.03	0.03 ± 0.01	*<0.05*
DHA	0.10 ± 0.05	0.07 ± 0.03	*<0.05*

Data are presented as mean ± SD. *p-values* were produced by independent sample t-test
ns: not significant

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two spawning events, because these parameters are known to be key determinants of reproductive success and progeny survival [17, 19].

Several studies have been carried out to investigate the possibility of using plant-based diets in rearing fish but very few of them challenged fish with diets in which both FM and FO were substituted [34]. The use of plant-based diets in aquaculture-raised fish species has revealed limitations, such as reduction in feed-intake [35–38]. The concomitant replacement of dietary FM and FO is known to result in lower growth performance [5,39], probably linked to a combination of lower feed intake and lower feed efficiency [5]. This poorer growth effect of a totally plant-based diet is believed to be mainly related to the replacement of FM and not to the FO substitution in rainbow trout [40,41], European seabass [42] and gilthead sea bream [43]. Pereira *et al*. observed that the replacement of FM by plant proteins both reduced feed intake and resulted in poorer reproductive performance in rainbow trout [7], but it was difficult to be certain that the reproductive performance was impaired by FM replacement or by the reduced feed intake. In the present study we adjusted the feed intake of the C-group to that of the V-group, in order to avoid differences in growth and the potential subsequent effects on spawn [44]. Although at the first spawning this feed restriction was slightly too high, we reached our goal at the spawning of the year after.

Table 7. ARA, EPA and DHA content (g/100g ova) in ova from second- and third-year spawning.

	Year-2 spawning			Year-3 spawning		
Diets	**C**	**V**	**p-value**	**C**	**V**	**p-value**
Fatty acid						
ARA	0.6 ± 0.1	0.8 ± 0.2	<0.05	1.2 ± 0.2	1.3 ± 0.5	ns
EPA	3.9 ± 1.1	1.6 ± 0.3	<0.05	5.8 ± 1.4	2.1 ± 0.5	<0.05
DHA	6.1 ± 1.0	3.8 ± 0.8	<0.05	6.2 ± 1.8	2.7 ± 0.7	<0.05

Data are presented as mean ±SD. *p-values* were produced by independent sample t-test
ns: not significant

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V-diet induces perivisceral and carcass lipid deposition

In teleost fish, stored fat supplies energy in periods of low feed availability and, more specifically, in periods of particularly high energy demand, such as reproduction and smoltification [17]. Depending on the species, fish are able to accumulate fat in different tissues [45,46], e.g. the liver in Atlantic cod [38] and perivisceral adipose tissue and flesh in Atlantic salmon and rainbow trout [5,38]. In the present study, females from both groups had significantly higher lipid content in the digestive tract (Fig. 2) than in the carcass and liver. This was is in accordance with the fact that perivisceral adipose tissue is the main lipid storage site in rainbow trout [47], while the liver does not function as a fat store, despite its important role in FA metabolism [48].

The lipid content in the digestive tract was significantly higher in trout fed the V-diet than in those fed the C-diet, mainly due to the NL fraction. This result confirmed results from previous nutritional studies carried out on salmonids [5,38], thus indicating that feeding a 100% plant-based diet leads to greater lipid deposition in perivisceral fat. Previous studies also demonstrated that maternal dietary lipids and adipose tissue lipids mobilized during previtellogenic and vitellogenic development are transported to oocytes via the circulatory system [27,49,50]. Interestingly, in the present study the total lipid content of ova was similar in both dietary groups (11%) at both spawnings and, furthermore, no significant difference in lipid content was found between swim-up fry (5%) originating from the two dietary treatments. The decrease in terms of total lipid content (from 11% in ova to 5% in swim-up fry) is due to the fact that prior to exogenous feeding the embryo utilizes nutrients derived from the ovum, including lipids [51]. In conclusion, the fat content of ova and fry was not related to the stored maternal body fat.

V-diet affects FA profile in maternal tissues, ova and offspring

One of the main problems of the concomitant replacement of FM and FO by plant sources in fish feed is modification of the fatty acid (FA) profile, that results in decrease in n-3 PUFAs in the fish body [52–54]. Indeed, vegetable oils do not contain EPA and DHA, and the FA profile of fish tissue is known to mirror the FA composition of the diet [36,38]. Our results were consistent with these previous findings; we observed higher concentrations of MUFA, linolenic acid and n-6 PUFA in NL and PL fractions of all maternal fish tissues investigated, reflecting their abundance in the V-diet. In particular, feeding fish the latter diet led to similar or even higher concentrations of ARA in the PL fraction of maternal tissues, although the dietary intake of ARA was zero. When calculating the quantities of ARA (g ARA/100g tissue) we found higher (or equal) values for the V-fed group in the carcasses, digestive tracts and ova from both spawnings. These results can be explained by the higher proportion of n-6 PUFA in the V-diet (22%) compared to the C-diet (8% and 10%), mainly due to the high linoleic acid content, the ARA precursor. On the other hand, the levels of EPA and DHA, a characteristic component of dietary FO, were lower in all tissues of fish fed the V-diet, as were the concentrations of saturated FAs. The bioconversion of 18:2 n-6 into ARA in the V-fed group seems therefore to be greater than the bioconversion of 18:3 n-3 into EPA and DHA. However, measurable concentrations of both EPA and DHA were found in NL and PL fractions of all maternal tissues of trout reared on the V-diet that was devoid of these FAs. Several previous studies reported that the synthesis of n-3 LC-PUFAs, such as EPA and DHA, as measured by the desaturation and elongation of linolenic acid 18:3n-3, was increased in salmonids fed diets in which dietary FO was replaced by vegetable oils [55–57], although the replacement of FO by vegetable oils resulted in reduced levels of EPA irrespective of the species [36]. The present study is the first in which rainbow trout were fed across the whole life cycle (3 years) without dietary intake of

EPA and DHA (concomitant replacement of FM and FO). The fact that non-negligible quantities of these two FA were found, indicated that female rainbow trout are capable of synthesizing EPA and DHA from dietary linolenic acid and to store these two fatty acids in both NL (reserve) and PL (membrane) fractions. However, the EPA and DHA content of tissues, ova and swim-up fry was significantly lower in V-fed trout than in those fed the C-diet (Table 4 and 5), meaning that the biosynthesis of EPA and DHA was not enough to overcome the total absence of these two FAs in the diet.

The degree of modification of the FA composition as a result of dietary FO replacement was significantly different between lipid storage sites (adipose tissue, muscle) and other tissues. Irrespective of the diet, EPA and DHA were mainly stored in the PL fraction of all maternal tissues, confirming selective retention of n-3 PUFAs, particularly DHA, in the membranes [58,59].

Broodstock nutrition is known to play a major role in quality of eggs and larvae, and lipid and fatty acid composition has in particular been identified as the main factor that determines successful reproduction and survival of offspring [60]. Moreover, the lipids derived directly from the dietary intake of broodstock in the period preceding gonadogenesis determine the essential fatty acids vital for early survival and development of newly hatched progeny [27].

With regard to n-6 PUFAs in ova, higher or equal concentrations of ARA were found in NL and PL fractions of ova and alevins from V-fed trout and C-fed trout, suggesting that neo-synthesized ARA from the 18:2 n-6 precursor (LA) is stored in ova. This is important, since ARA plays a major role in the reproductive process [13,61]. Moreover ARA is considered to be the major precursor of eicosanoids in fish [62] and eicosanoids play an important role in ovulation and are probably involved in embryogenesis, hatching and early larval development [63,64].

Our study also revealed increased percentages of n-3 LC-PUFA, EPA and DHA in ova compared to maternal tissues in both C-fed and V-fed fish, suggesting active maternal transfer of these neosynthesized FAs from linolenic acid to the ova. The amounts of EPA and DHA found per 100g of tissue strongly suggested preferential mobilization from perivisceral lipid reserves, rather than from the carcass. As mentioned above, perivisceral fat storage was increased in females fed the V-diet so that sufficient stores were available for the vitellogenic process. The amounts of EPA and DHA found in ova supported the preferential incorporation of these FAs in ova.

EPA and DHA were stored in the reserves (NL fraction) in ova and swim-up fry in addition to the membranes (PL fraction), which are the main sites of deposition. Increased levels of EPA and DHA have previously been described in fish eggs [60,61], supporting the important role that DHA plays in egg and subsequent offspring development. n-3 PUFAs are stored in the reserves for potential use during development [17] or to be catabolized for energy after hatching [65]. For example, DHA is mainly incorporated into the phospholipid-rich vitellogenin which is synthesized in the liver, then transferred via the serum to the developing ova [27]. The accumulation of DHA in the PL fraction of ova has a major biological role as structural phospholipids [27] assuring the fluidity of cell membranes. Our results also showed a greater increase in DHA content from ova to alevins for both groups. This indicates that rainbow trout alevins are able to retain DHA selectively, mainly in the polar lipid fraction.

Measurements of absolute fecundity indicate that the V-diet does not diminish the quantity of ova produced, though ova from V-fed females were smaller. Moreover, with regard to offspring development, feeding the V-diet resulted in a significant decrease (>22%) in survival rate at all stages at first spawning (year-2), confirming previous studies [26,66]. Interestingly, no such significant difference in survival occurred in alevins developing from eggs from the year-3 spawning. It is well known that bigger broodstock produce greater number of eggs and also bigger sized eggs [67]. In our study, comparing the two spawning events, we observed an

increase of female weight at the second spawning and a concomitant increase of the ova weight for both females groups. The lower reproductive performance observed for the V-fed group at the first spawning, is possibly linked to the smaller size of eggs, in comparison with those from females fed the C-diet. Indeed, at the year-3 spawning, V-fed females produced ova with a weight comparable with that of ova produced by the C-fed females at the first spawning. We also initially hypothesized that differential accumulation of total lipids or specific changes in the FA profile of ova might be responsible for the differences in survival rate observed between the two spawns of the V-fed females. In this group, ova spawned by 3-years old females had higher ARA (1.3 *vs* 0.8 g/100g ova) and EPA (2.1 *vs* 1.6 g/100g ova) content than those spawned by the 2-years old females. Given the importance of these FAs in reproduction performance [68,69], the higher survival rates observed for offsprings developing from ova spawned by 3-years old females might be linked to the greater ARA and EPA content in ova. However, we also found a lower DHA content, in ova from V-fed females at the year-3 spawning compared to the year-2 spawning. Taking into account the crucial role of DHA in reproduction and embryo development [60], it seems that the higher survival rate observed for offsprings from V-fed females at the year-3 spawning is mostly linked to the egg size and energy reserves than to differences in FA profile of ova.

In conclusion, trout which were reared on plant-based diets completely devoid of long chain PUFAs throughout the entire life cycle (3 years) were able to produce viable ova and progeny. This was probably linked to the fact that the female rainbow trout were remarkably able to synthesize ARA, EPA and DHA from dietary precursors. These neo-synthesized LC-PUFAs were preferentially incorporated into ova, which in fish represent the main sources of nutrients utilized by the embryo prior to exogenous feeding [51]. However, further adjustments of the feed-formula are still needed to optimize reproductive performance, especially at the first spawning.

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Author Contributions

Conceived and designed the experiments: FM MDN EQ. Performed the experiments: VL AL FM GC MDN EQ. Analyzed the data: VL AL GC FM. Wrote the paper: VL GC FM.

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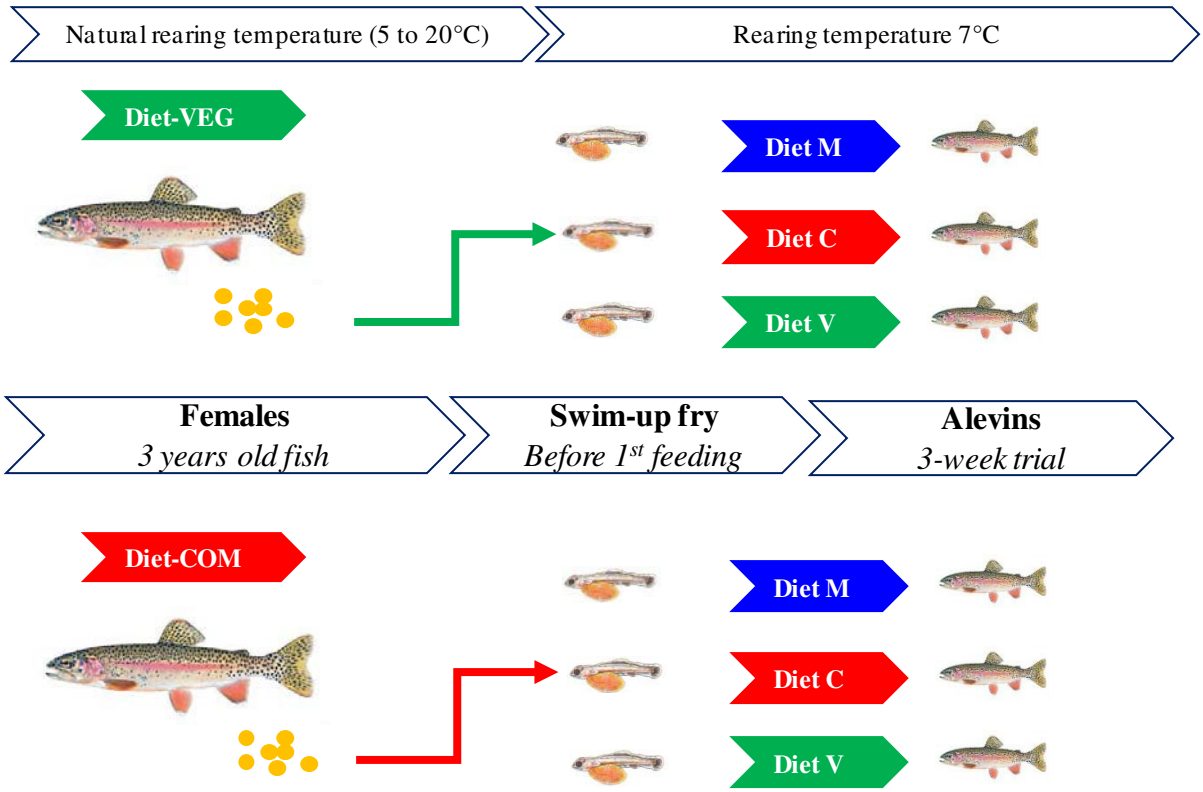
Publication #2

Figure 3.2 Experimental design of Publication 2.



Presentation of the article

Objective

Based on the findings that rainbow trout are able to survive and reproduce when reared on a totally plant-based diet during the whole life cycle (Publication 1), we aimed to investigate the effects of broodstock nutritional history on metabolic capacity of progeny before first feeding, as well as the potential effects that broodstock nutrition might have on the progeny's capacity to respond to different first-feeding diets. With this purpose, we performed a transcriptomic analysis, focusing on two critical stages of trout development: *(i)* before first-feeding (end of endogenous feeding period) and *(ii)* after 3 weeks of feeding (exogenous feeding period).

Experiment & analysis

Rainbow trout females were fed either a commercial diet (COM, mix of marine and plant ingredients) or an experimental totally plant-based diet (VEG, only plant proteins and VOs) from first feeding and throughout a three-year period. At spawning, ova from 3 year-old females of both broodstock groups were fertilized. 62 days post-fecundation (just before first-feeding), body weights and survival rates of alevins were recorded, and whole body samples of fry were collected. The remaining alevins from both groups were subsequently split into three groups, and each group received one of the three experimental diets, *i.e.* diet-M (marine diet), diet-C (mix marine-plant ingredient) or diet-V (plant-based diet) from first feeding and during 3 weeks. Alevins were monitored daily, and if any clinical symptoms were observed, alevins were sedated by immersion in 2% benzocaine solution and then euthanized by benzocaine overdose (6% benzocaine solution during 3 minutes). After 3 weeks of feeding, survival rates and body weights of alevins were recorded and samples of whole body alevins were collected. Lipid content and FA profile were analysed at both developmental stages and RNA was extracted to characterize the whole body transcriptome, using microarray analysis.

Results and Conclusions

No significant differences were found in survival of alevins from different experimental groups, either before the first feeding or after the 3-week feeding period. However, feeding fish the

completely plant-based diets resulted in lower alevin body weight, irrespective of maternal nutritional history. No differences in whole body lipids were found between dietary treatments, and tissue FA profile strongly reflected that of the respective broodstock or first-feeding diets.

Maternal diet history was not associated with any significant changes in alevins gene expression before first feeding. However, an effect of maternal diet history on whole body alevins did become evident after 3 weeks of feeding. In summary, gene involved in muscle growth and contraction processes were down regulated in progeny from VEG-fed broodstock. Conversely, expression of genes involved in carbohydrate and energy metabolism was increased in the same progeny. A significant effect of first feeding diet on whole alevin transcriptomes was evident, irrespective of maternal nutritional history. Briefly, alevins fed plant-based diets (C and V) from first feeding onwards, exhibited an increase in the expression of genes with functions in AA/protein and cholesterol metabolism. Differential regulation of genes related to carbohydrate metabolism was identified between experimental groups. Surprisingly, expression of genes involved in FA bioconversion was not affected probably because the reserves of n-3 LC-PUFAs supplied by vitellus were not totally exhausted yet.

In conclusion, the replacement of marine sources by plant-based ingredients in both broodstock and first-feeding diets resulted in significant effects on the transcriptome of whole body alevins after 3 weeks of feeding. However, the relatively low-fold changes in gene expression found in this study suggest that the modifications induced by the diets, and therefore the metabolic consequences of the dietary replacement, are not very drastic. Nevertheless, feeding female broodstock with a plant based diet did not result in improved capacity of progeny to adapt to diets rich in plant ingredients.

Overall, these results improve the understanding of mechanisms and pathways activated by concomitant FM and FO replacement in first-feeding diets for rainbow trout. These findings open up avenues for further reduction of the use of marine resources for aquaculture, by allowing an extension of feeding trout with diets low in marine ingredients to the broodstocks and alevins.

Does broodstock nutritional history affect the response of progeny to different first-feeding diets? A whole-body transcriptomic study of rainbow trout alevins

Viviana Lazzarotto[1], Geneviève Corraze[1], Laurence Larroquet[1], David Mazurais[2] and Françoise Médale[1§]

[1]INRA - UR 1067 "Nutrition Métabolisme Aquaculture", Aquapôle, 64310 Saint-Pée-sur-Nivelle, France

[2] IFREMER – LEMAR UMR 6539, CNRS-UBO-IRD-Ifremer, ZI de la Pointe du Diable, CS 10070, 29280, Plouzané, France

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Abstract

The whole-body transcriptome of trout alevins was characterized to investigate the effects of long-term feeding of rainbow trout broodstock females a diet free of fishmeal and fish oil on the metabolic capacities of progeny. Effects were studied before first feeding and after 3 weeks of feeding with diets containing different proportions of marine and plant ingredients.

Feeding alevins the plant-based diets resulted in lower fish body weight, irrespective of maternal nutritional history. No differences in whole body lipids were found between treatments, and tissue fatty acid profile strongly reflected that of the respective broodstock or first feeding diets.

We showed that the maternal diet history did not significantly affect expression of any gene before the first feeding. Interestingly, we found an effect of maternal nutritional history on gene expression in alevins after 3 weeks of feeding. The major differences in the transcriptome of alevins from VEG-fed females compared to those from COM-fed females were: *(i)* down-regulation of genes involved in muscle growth/contraction and *(ii)* up-regulation of genes involved in carbohydrate and energy metabolism related to the delay in growth/ development observed with plant-based diets. Our findings also showed an effect of the first-feeding diets, irrespective of maternal nutritional history. Specifically, the introduction of plant ingredients resulted in up-regulation of genes involved in AA/protein and cholesterol metabolism and in differences in the expression of genes related to carbohydrate metabolism. Information gained through this study opens up avenues for further reduction of marine ingredients in trout diets including the whole rearing cycle.

Keywords: fish; nutrition; plant-products; fish oil; fishmeal; fatty acid; gene expression; microarray; early stages.

Introduction

Aquaculture is currently supplying increasing proportions of fish for global human consumption, resulting in an increasing demand for feeds for farmed fish. The use of fish meal (FM) and fish oil (FO) in fish nutrition, in particular for carnivorous species such as salmonids, has been common practice for years. This is due to the fact that FM and FO constitute excellent sources of essential amino acids and fatty acids, particularly highly unsatured fatty acids [1; 2; 3]. However, the current stagnation of FM and FO production from wild fisheries might limit the growth of aquaculture unless effective alternative ingredients are found.

Terrestrial plant-based products are thus nowadays increasingly used as substitutes for marine resources in feeds for farmed fish [3; 4]. The studies conducted with diets containing little or no FM and high levels of plant protein sources have shown lower growth performance in rainbow trout, possibly linked to reduced feed intake [5]. With regard to dietary FO replacement, several studies carried out in salmonids [6; 7] have shown that complete replacement of FO in the diet by vegetable oils does not affect growth or feed efficiency when the n-3 polyunsatured fatty acid (n-3 PUFA) requirements are met by lipids contained in FM. Indeed, one of the major consequences of the replacement of marine ingredients by plant products is the drastic modification of the fatty acids (FA) content of the diets, because none of the plant-based products contain n-3 LC-PUFAs, such as eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA), known to play a key role in fish reproduction and development [8; 9]. A few studies have also been conducted on the concomitant replacement of FM and FO. These studies showed lower growth performance in fish fed the plant-based diet, an effect mainly linked to FM replacement [10; 11].

The metabolic consequences of FM and FO replacement by alternative protein or fatty acid sources are numerous and mediated by several interacting pathways. Nutrigenomic tools (*i.e.* transcriptomics) are increasingly used to investigate molecular events taking place in a genome receiving nutritional signals and responding to them through characteristic metabolic processes in the organism [12]. Nutrigenomics studies in farmed fish have addressed the replacement of different percentages of FM and/or FO with plant ingredients in diets [13; 14; 15], and the effects of such replacement are well characterized in the hepatic transcriptome of salmonids [10; 16; 17; 18] and

marine species such as sea bass [14]. For example, the replacement of fish oil by vegetable oils was found to be mainly associated with modification of genes involved in cholesterol and fatty acid biosynthesis [16; 19], while the substitution of fish meal by plant proteins was found to be associated with a decreased capacity for protein biosynthesis and variation in nitrogen metabolism in rainbow trout [17]. The replacement of both fish meal and fish oil by plant-based ingredients in the diet of rainbow trout was associated with changes in nucleic acid and glucose metabolism, in addition to the aforementioned changes in lipid and protein metabolism[10]. Other studies have investigated the intestinal gene expression profile in response to different levels of dietary replacement of marine ingredients by plant products in several fish species, such as Atlantic salmon (*Salmo salar*)[20; 21; 22] , gilthead sea bream (*Sparus aurata L.*)[23] and Atlantic cod (*Gadus morhua*)[24]. However, most of these studies were carried out on growing fish and there is still a gap in the understanding of the effects of plant-based diets on the rest of the life cycle (broodstock and early stages). In addition to the already recognised importance of broodstock nutrition on progeny survival and development, nutrients contained in the yolk sac, transmitted by broodstock to developing progeny, are also known to influence the characteristic gene expression of offspring by modifying or interacting with transcription factor or DNA structure [25]. The effects of the maternal dietary history on reproduction and metabolic capacities of the progeny are still poorly documented, especially when broodstock are fed a totally plant-based diet without any FM and FO, and thus devoid of n-3 LC-PUFA, over the whole life cycle. In an earlier trial, we showed that broodstock produced viable offspring even when reared exclusively with a plant-based diet [26]. We also showed that trout are capable of synthesizing n-3 LC-PUFAs from the dietary precursor (α -linolenic acid, 18:3 n-3) and of incorporating them into ova, which in fish represent the main sources of nutrients utilized by the embryo [27] and later by the developing alevin.

The early life stages of fish represent a transitional ontogenetic period of simultaneous growth and organ/tissue differentiation, during which fish undergo the transition from endogenous to exogenous feeding, *i.e.* from yolk consumption to ingestion of external food [28]. Moreover, previous studies carried out on developing larvae [29; 30] showed that gene expression, and the subsequent activation of the related metabolic pathways, is differentially regulated with advancing ontogenesis. Thus, regulation of gene expression during this phase is considered to be

a key mechanism underlying the management of the biological process required for harmonious development over this phase of life, during which nutritional input is of great importance.

In order to characterise the effects of broodstock nutritional history as well as those of first feeding diets with different proportions of FM and FO and plant ingredients, the whole body transcriptome of rainbow trout alevins was characterised at two different developmental stages: (i) before first feeding (end of endogenous feeding period) to assess the effects of maternal nutritional history and (ii) after 3 weeks of feeding (exogenous feeding alevins) to assess both the effects of maternal nutritional background and those of first feeding diets.

Experimental methods

Diets

Broodstock

The broodstock diets were the same as those previously described in Lazzarotto et al.[26]. Briefly, broodstock were fed either a commercial (COM) diet composed of FM, FO and plant-based ingredients (45% FM and 50% FO replaced by plant ingredients), or an experimental plant-based (VEG) diet, completely free of marine FM and FO, which were replaced by plant protein sources (22% corn gluten, 26% soybean meal, 33% wheat gluten, 7% durum wheat, 8% white lupin and 4% dehulled peas) and vegetable oils (50% rapeseed oil, 30% linseed oil, and 20% palm oil), respectively.

Alevins

Three different first-feeding experimental diets with different dietary levels of FM and FO replacement were formulated and manufactured (INRA-NuMéa, Saint-Pée-sur-Nivelle, France) : a marine (M) diet, based on marine resources (no replacement), a commercial-like (C) diet, containing both marine and plant-based ingredients (replacement of 46% FM and 69% of FO), and a completely plant-based diet (V), with total replacement of marine FM and FO by plant-based proteins and vegetable oils. The ingredients and composition of the three diets are provided in Table 1. In order to obtain total replacement of fish products, only plant-based proteins and vegetable oils (7% rapeseed oil, 7% linseed oil and 4% palm oil) were used in the

V-diet, whereas the M and C-diets contained FO (12% and 8%, respectively). Consequently, the V-diet contained no n-3 LC-PUFAs, whereas it contained a high level of 18:3 n-3, mainly derived from linseed oil, compared to the other two experimental diets (Table 2).

Table 1. Ingredients and composition of first-feeding diets

Diets	**Diet-M**	**Diet- C**	**Diet-V**
Ingredients (%)			
Fish meal *	**65.0**	**30.0**	**0.0**
Corn gluten	0.0	13.0	24.0
Soybean meal 48	0.0	6.0	2.0
Wheat gluten	0.0	10.0	22.0
Soy protein concentrate	0.0	10.0	20.0
White lupin	0.0	0.4	2.5
Peas	0.0	4.0	0.0
Rapeseed meal 00	0.0	6.2	2.3
Extruded whole wheat	21.1	1.3	0.0
Fish oil †	**11.7**	**8.1**	**0.0**
Rapeseed oil	0.0	8.1	6.7
Linseed oil	0.0	0.0	6.7
Palm oil	0.0	0.0	3.6
Min.-Vit. Premix	2.0	2.0	2.0
Soy lecithin	0.0	0.0	2.0
L-lysine	0.0	0.3	1.5
L-Methionine	0.0	0.01	0.3
CaHPO4.2H20 (18%P)	0.0	0.0	2.9
Attractant Mix	0.0	0.0	1.5
Composition (% DM)			
Dry Matter (DM, %)	94.3	95.3	95.5
Crude protein	48.9	53.3	52.9
Crude fat	21.5	22.1	21.8
Energy (kJ/g DM)	23.0	24.2	24.1
Cholesterol	0.70	0.55	0.36

* Origin co-fishery products - all species

† Origin co-fishery products – sardines

Diet-M, marine FM-FO-based diet

Diet-C, commercial-like FM-FO & plant-based diet

Diet-V, experimental 100% plant-based diet

Table 2. Fatty acid composition (% total fatty acids) of experimental diets

	Diet-M	Diet-C	Diet-V
Fatty acid			
Saturated	**30.8**	**20.9**	**18.5**
MUFA	**33.2**	**41.9**	**38.3**
18:2 n-6 (LA)	3.2	12.5	21.5
20:2 n-6	0.2	0.1	0.04
20:3 n-6	0.1	0.02	0.0
20:4 n-6 (ARA)	0.7	0.4	0.0
22:2 n-6	0.0	0.0	0.02
Σ n-6	**4.3**	**13.1**	**21.5**
18:3 n-3 (ALA)	1.1	4.8	21.3
18:4 n-3	2.1	1.2	0.0
20:3 n-3	0.1	0.0	0.0
20:4 n-3	0.6	0.3	0.0
20:5 n-3 (EPA)	11.1	6.7	0.0
22:5 n-3	1.1	0.7	0.0
22:6 n-3 (DHA)	6.7	4.2	0.0
Σ n-3	**23.3**	**18.1**	**21.3**

MUFA, Monounsaturated fatty acid; LA, linoleic acid; ARA, arachidonic acid;

ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid;

Diet-M, marine FM-FO-based diet;

Diet-C, commercial-like FM-FO & plant-based diet;

Diet-V, experimental 100% plant-based diet

Animals and experimental plan

The experiment was carried out in strict accordance with EU legal frameworks relating to the protection of animals used for scientific purposes (Directive 2010/63/EU) and according to the *National Guidelines for Animal Care of the French Ministry of Research* (Decree N°. 2001-464, May 29, 2001). It was approved by the Ethics Committee of INRA (INRA 2002-36, April 14, 2002) and the scientist in charge of the experimentation received training and personal authorisation (N°B64 10 003). Female rainbow trout were produced at the INRA facilities (PEIMA, Sizun, France - permit number B29-277-02). During the trial, they were reared under natural photoperiod and temperature conditions. At the beginning of the trial, female fish were randomly divided into two groups, fed from first feeding and through a 3-year life cycle, with either the broodstock commercial (COM) diet or the broodstock plant-based (VEG) diet (Lazzarotto, Corraze et al. 2015). At spawning ova produced by 10 female trout/group (3-year-old females) of similar body weight from each dietary treatment were fertilized with a pool of sperm from males fed a commercial diet. Eggs were transferred to our experimental hatchery (INRA, Lees Athas, France – permit number A64-104-1) where the water temperature is around 7°C all year long. Just before first feeding (62 days post-fecundation) body weights and survival rates of alevins were recorded and whole body samples of fry were collected.

The remaining alevins from both cohorts were subsequently split into three groups of fish. Each group (4 replicates) received one of the three experimental diets from first feeding, *i.e.* diet-M, diet-C or diet-V. After 3 weeks of feeding, survival rates and body weights of alevins were recorded and whole body alevins samples were collected for subsequent analysis. All the samples were frozen in liquid nitrogen and stored at -80°C until analysis.

Lipid and fatty acid analysis

Total lipids of whole body alevins collected before first feeding (pool=15 alevins/maternal group) and after 3 weeks of feeding (pool=15 alevins/dietary group), were extracted and quantified gravimetrically according to Folch et al. [(31)]. Neutral (NL) and polar (PL) lipid fractions were separated on silica cartridges (Sep-Pak, Waters, Ireland) [(32)] and fatty acid methyl esters (FAME) were prepared according to Shantha & Ackman [(33)]. FAMEs were then analysed by gas chromatography as previously described in detail [(26)].

RNA extraction

Total RNA was extracted from individual whole body swim-up fry (n=8/maternal group) and alevins (n=8/dietary group) using the TRIzol® reagent method (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. The quantity and quality of extracted RNA were analysed using a spectrophotometer (ND-1000, NanoDrop) and a Bioanalyzer (Agilent Technologies, Kista, Sweden), respectively.

cRNA synthesis, labelling and purification

Cy3-labelled experimental cRNA samples (n=8/treatment) were generated using the Agilent "One-Color Microarray-based Gene Expression Analysis" (Low Input Quick Amp Labeling-LIQA) kit, according to manufacturer's instructions. The method uses T7 RNA Polymerase Blend, which simultaneously amplifies target material and incorporates Cyanine 3-CTP (Cy3). For each sample 150ng of total RNA were used to generate fluorescent cRNA. Agilent Spike-In was included in each reaction. After the denaturation step (10 min in circulating bath at 65°C) and cRNA synthesis step (2hr at 40°C), the reactions were incubated at 70°C for 15 min to inactivate the AffinityScript enzyme. To perform the labelling reaction, cRNA samples were each mixed with 6µL of Transcription Master Mix cocktail, containing Cy3-dye, and then incubated at 40°C for two hours. Purification was performed using Quiagen RNeasy mini spin columns, eluting in 30uL of RNase-free water.

Microarray hybridization and scanning

Cy3-labelled cRNA sample yields (> 0.825µg cRNA) and specific activity (> 6pmol of Cy3/µg of cRNA) were verified using a NanoDrop ND-1000: 600ng of Cy3-cRNA were fragmented and hybridized on a sub-array, following the LIQA kit instructions. The transcriptomic analysis was conducted using a custom-commercial 8x60K oilgoarray (Agilent Technologies, Massy, France; Gene Expression Omnibus (GEO) accession no. GPL15840). The hybridization reactions were allowed to continue for 17 hours in a rotating hybridization oven (65°C), prior to washing according to the manufacturer's instructions. Slides were scanned with an Agilent Scanner (Agilent DNA Microarray Scanner, Agilent technologies, Massy, France) using the standard parameters for a gene expression 8x60K oligoarray (3µm – 20 bits). Data were then obtained

with the Agilent Feature Extraction software (10.7.1.1), according to the appropriate GE protocol (GE1_107_Sep09). The data is deposited in NCBI's GEO (GSE74271).

Quantitative RT-PCR

Six individual samples (single whole body swim-up fry or alevin) per experimental condition were used as biological replicates. Total RNA (1µg) was reverse-transcribed to cDNA with SuperScript III RNase H reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using oligo dT Primers. Real-time PCR was performed in the iCycler iQ TM (BIO-RAD, Hercules, CA, USA). Quantitative PCR (q-PCR) analyses for gene expression were performed using the Roche Lightcycler 480 system (Roche Diagnostics, Neuilly-sur-Seine, France). The assays were performed using 2 µl of diluted cDNA mixed with 3 µl of Light cycler 480 SYBR® Green I Master mix in a total volume of 6 µl, using forward and reverse primers at a final concentration of 400 nM. Primer design was performed using the Primer 3 software. Specific primer pairs were designed with an overlapping intron, when possible, using known trout sequences in nucleotide databases (GeneBank and INRA-Sigenae). Database accession numbers and the sequences of forward and reverse primers used to test each gene are provided as supplementary files (Supplementary Table 1 a-b).

Thermal cycling was initiated with the incubation at 95°C (10 min) for hot-start iTaq TM DNA polymerase activation. Forty-five cycles of PCR were performed, each consisting of a heating step at 95°C (15 sec) for denaturing, a second step at 60°C (10 sec) for annealing and a third extension step at 72°C (15 sec). Following the final cycle of the PCR, melting curves were systematically monitored (with a gradient of 0.5°C/10 s from 55°C to 94°C) to ensure that only one target fragment was amplified. Samples without reverse transcriptase and samples without RNA were run for each reaction as negative controls. mRNA levels of all target genes were normalized with the housekeeping gene α-elongation factor 1 (*EF1-α*), previously used as a reference gene in salmonids [(34)]. The expression levels were calculated according to the threshold cycle ($\Delta\Delta C_T$) method [(35)].

Statistical analysis and data mining

Data of weight, survival, lipid content and fatty acids of whole body alevins (collected before 1st feeding and after 3 weeks of feeding) are presented as means ± standard deviation (SD). Data were analysed statistically using the R-software (version 2.14.0) and the Rcmdr package. The normality and homogeneity of variance of the variables were tested with Shapiro-Wilk's test and Levene's test, respectively. Data for alevins collected before first feeding were analysed by an independent sample t-test to assess the effects of the different broodstock nutritional histories, when both conditions were satisfied. The variables with non-parametric distribution were either normalized with an arcsin transformation or, if the criteria were still not met (some fatty acids), compared using a non-parametric paired Wilcoxon test. Data for alevins collected after 3 weeks of feeding were analysed using a two-way ANOVA (*p-value <0.05*) to assess the effects of the *nutritional broodstock history* and the *first feeding diets*. The variables with non-parametric distribution were normalized with an arcsin transformation. Data from microarray analysis were normalized and analysed statistically using GeneSpring software (12.6, Agilent). Data were scale-normalized using the median value of each array to identify differentially expressed genes between conditions. An unpaired t-test was performed to determine the effects of the *nutritional broodstock history* on the transcriptome of alevins collected before first feeding, (Benjamini Hochberg FDR correction, *p-value* cut- off 0.05). For analysis of whole body alevins collected after 3 weeks of feeding, differentially expressed genes were obtained by two-way ANOVA, with the different *broodstock nutritional histories* and *first feeding diets* as independent variables (Benjamini-Hochberg correction, *p-value* cut-off *0.05*). For all genes found to be differentially expressed, Gene Ontology (GO) annotations (biological process, cellular component, molecular functions) were obtained using the Expression Analysis Systematic Explorer (EASE) software version 2.0 [(36)]. Significant enrichment of GO was tested by using EASE software and the Benjamini correction (score < 0.05). Gene expression data obtained by RT-qPCR were tested for normality and homogeneity of variances with Shapiro-Wilk's test and Levene's test, respectively. When variances were not normally distributed a logarithmic transformation was performed. To assess the effects of the *nutritional broodstock history* and the *first feeding diets*, gene expression was analysed by two-way ANOVA (*p-value <0.05*). *Post hoc* comparisons were made using Tukey's range test and differences were considered statistically significant at *p<0.05*. Correlation of the mRNA measurement by microarray with that by reverse transcription PCR

(RT-PCR) for two of the tested genes, chosen as examples, is provided as supplementary material (Supplementary Figure 1).

Results

Growth performance

Survival rates and weight of alevins before first feeding and after three weeks of feeding are given in Table 3. No statistically significant differences in survival were found in alevins from the different experimental groups, either before first feeding or after the 3-week feeding challenge.

Before first feeding, alevins developing from VEG-fed females had significantly lower body weights (-13%, *p-value <0.001*) compared to those from COM-fed females. The initial slight difference in weight resulting from the maternal nutritional history (VEG *vs* COM) was maintained after 3 weeks of feeding, irrespective of the diets fed to the alevins. After the 3-week feeding trial, alevins responded to the three dietary treatments (M, C or V) irrespective of maternal nutritional history, with lower growth when fed the V-diet (V *vs* M: -27%; V *vs* C: -15%).

Alevin whole body lipid composition

Data on alevins collected before first feeding were presented in detail in Lazzarotto et al.[26] and are summarized in Additional file 1. Briefly, before first feeding there were no significant differences in lipid content between alevins originating from COM-fed (5.9% of fresh weight) and VEG-fed (5.6% of fresh weight) females. The whole body lipid content of fry mainly comprised neutral lipids (NL: 70%; polar lipids, PL: 30%) in progeny from both broodstock groups (COM and VEG).

In alevins collected after the 3-week feeding challenge (Table 4), we observed an effect of both maternal nutritional history and first feeding diets on the whole body lipid content, whereas no interaction between the two factors was found. Lipid content was significantly higher in progeny from the VEG-fed females that received the M-diet for three weeks (5% of fresh weight), whereas the progeny from the COM-fed females that received the V-diet had the lowest whole

body lipid content (4% of fresh weight). No significant differences were found between the other treatment groups.

The respective proportions of NL and PL were similar in all experimental groups (70% NL and 30% PL) and were therefore not affected by dietary treatments.

Table 3. Survival rates and weights of alevins collected before first feeding and after 3 weeks of feeding

	Progeny-COM						**Progeny-VEG**						***Significance***		
Before first feeding[1]			Mean	SD					Mean	SD			B		
Survival (% hatched)			84.0	7.0					78.0	13.0			*ns*		
Weight (mg)			135.0	1.0					118.0	1.0			*		
	M		**C**		**V**		**M**		**C**		**V**		***Significance***		
After 3wks feeding[2]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	B	D	BxD
Survival (% fed alevins)	99.8	0.2	99.7	0.3	100.0	0.0	99.4	0.5	99.0	0.3	99.5	0.6	*ns*	*ns*	*ns*
Weight (mg)	268.0[a]	7.0	229.0[b]	4.0	190.0[c]	4.0	246.0[a]	4.0	210.0[b]	4.0	179.0[c]	1.0	*	**	*ns*

B, broodstock nutritional history effect; D, 1st feeding diet effect; BxD, interaction. Statistical differences were determined by independent sample t-test[1] or by two-way ANOVA[2] followed by Tukey's HSD comparison test, when appropriate.*, *$p<0.05$;* **, *$p<0.01$*; ***, *$p<0.001$*; *ns*: not significant

FA composition

Alevins before first feeding

Data on FA profiles of whole body alevins collected before first feeding were presented in detail in Lazzarotto et al. [26] and were used in the present study (Additional file 1) as a starting point for comparison with data from 3-week fed alevins. We found that alevins of females fed the VEG-diet had higher n-6 PUFA, 18:2 n-6, ARA and 18:3 n-3 levels before the first feeding than those from COM-fed females. In contrast, higher percentages of n-3 PUFA, EPA and DHA were found in progeny from COM-fed females, with the exception of the PL fraction, where no significant differences were found in DHA content between groups. Lower amounts of EPA + DHA were found in alevins from VEG-fed females (1.4 mg alevin^{-1}) than in alevins from COM-fed females (2.6 mg alevin^{-1}) (Table 5).

Alevins after 3 weeks of feeding

After 3 weeks of feeding, the fatty acid composition of whole body alevin samples reflected those of the respective experimental first-feeding diets M, C or V (Table 4).

Polar lipid fraction

All FA classes (except SAT) were significantly affected by both broodstock nutritional history and the dietary treatment.

Lower percentages of SAT were found in fish fed the C and V- diets, compared to M-fed fish. Levels of MUFA were higher in fish fed the C and V-diets, with higher values in fish from VEG-fed females. The percentage of total n-6 PUFA (reflecting mainly 18:2 n-6) was higher when FM and FO were replaced by plant-ingredients (C and V-diet), with higher levels in progeny from females fed the VEG-diet. On the other hand, levels of n-3 PUFA were significantly higher in progeny from COM-fed females, EPA and DHA levels being the lowest with the V-diet.

Neutral lipid fraction

Lower levels of SAT were found in alevins fed the C and V diets, the lowest levels being found in progeny from broodstock fed the VEG-diet. Higher percentages of MUFA were found in alevins fed the C-diet in both broodstock groups (mainly due to the higher 18:1 content).

Alevins originating from females fed the VEG-diet exhibited higher (or equal) levels of n-6 PUFA than those from the COM-fed broodstock, with higher levels with the V-diet compared to the other groups. Alevins receiving the C-diet had values intermediate between the M and V-fed alevins (V>C>M). These differences were related to the greater quantities of linoleic acid in alevins fed the V-diet. Higher proportions of ARA were found in alevins fed the V-diet and values in progeny of VEG-fed females were higher.

Lower n-3 PUFA levels were found in progeny from VEG-fed females compared to progeny from COM-fed females. Alevins fed the V-diet had lower percentages of n-3 PUFA compared to alevins fed the C or M-diet. Higher (or equal) proportions of 18:3 n-3 were found in alevins originating from females fed the VEG-diet compared to those from COM-fed females. Percentages of 18:3 n-3 were higher in alevins fed the V-diet, compared to those fed the C or M-diet, irrespective of broodstock nutritional history. On the other hand, lower percentages of EPA and DHA were found in alevins originating from females fed the VEG-diet. Alevins fed the V-diet had lower EPA and DHA values than alevins fed the other experimental diets (C or M).

Amounts of EPA + DHA

The difference in quantity of EPA+DHA (mg alevin^{-1}) originating from the maternal nutritional history (COM *vs* VEG) still remained after three weeks of feeding, with lower levels recovered in progeny from VEG-fed females, irrespective of the first feeding diets (Table 5). After 3 weeks of feeding, lower levels of EPA+DHA were found in progeny fed the V-Diet, irrespective of the broodstock nutritional history (COM, V *vs* M: -62% and V *vs* C: -40%; VEG, V *vs* M: -65% and V *vs* C: -44%).

Table 4.Total lipid content (percentage of fresh weight) and fatty acid composition (percentage of total fatty acid) of polar and neutral lipid fractions of whole body alevins collected after 3 weeks of feeding.

	Progeny-VEG						*Progeny-COM*						*Significance*		
	Diet-M		Diet-C		Diet-V		Diet-M		Diet-C		Diet-V				
Whole body lipids	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*B*	*D*	*BxD*
Total lipids	5.0	0.3	4.7	0.4	4.5	0.4	4.6	0.2	4.5	0.3	4.0	0.4	*	*	*ns*
PL	70.5	1.6	70.5	1.4	71.3	2.2	70.4	1.7	71.2	0.7	68.2	1.4	*ns*	*ns*	*ns*
NL	29.5	1.6	29.5	1.4	28.7	1.4	29.6	1.7	28.8	0.7	31.8	1.4	*ns*	*ns*	*ns*
PL fraction															
Saturated	32.5	1.2^{a}	30.0	1.0^{bc}	29.3	0.6^{bc}	31.5	1.4^{ab}	28.4	0.8^{c}	30.6	1.1^{ac}	*ns*	***	*
MUFA	18.5	0.6	20.9	0.5	19.0	0.5	17.5	0.4	20.3	0.2	18.4	0.4	***	***	*ns*
18:2 n-6	2.5	0.2	4.9	0.2	7.4	0.4	2.2	0.0	4.7	0.2	7.1	0.1	**	***	*ns*
ARA	3.3	0.2	3.8	0.1	4.6	0.2	2.4	0.1	2.5	0.2	2.6	0.1	**	***	*ns*
∑ n-6 PUFAs	7.3	0.3^{e}	10.6	0.2^{c}	14.6^{a}	0.4	5.3	0.2^{f}	8.3	0.2^{d}	11.3	0.1^{b}	***	***	***
18:3 n-3	0.8	0.1	1.5	0.1	3.3	0.2	0.4	0.0	1.2	0.0	3.1	0.1	***	***	*ns*
EPA	9.0	0.4	7.2	0.2	5.5	0.1	9.6	0.5	8.1	0.4	6.3	0.2	***	***	*ns*
DHA	23.9	2.5	22.6	1.7	21.3	0.3	28.6	2.3	27.8	1.9	23.3	1.4	***	**	*ns*
∑ n-3 PUFAs	36.5	2.7	33.9	1.8	33.6	0.4	41.2	2.3	39.5	2.1	35.8	1.6	***	**	*ns*
NL fraction															
Saturated	23.3	0.1	17.6	0.2	17.6	2.6	24.9	1.5	19.1	0.8	18.2	0.5	*	***	*ns*
MUFA	37.8	0.3^{c}	44.0	0.3^{a}	38.3	0.9^{c}	34.3	0.7^{d}	42.3	0.3^{b}	34.1	0.4^{d}	***	***	***
18:2 n-6	7.6	0.4^{c}	14.5	0.1^{b}	18.2	0.4^{a}	6.9	0.4^{c}	14.8	0.2^{b}	18.8	0.2^{a}	***	***	***
ARA	1.2	0.1	1.0	0.1	1.0	0.2	1.0	0.1	0.7	0.0	0.7	0.0	***	***	*ns*
∑ n-6 PUFAs	10.7	0.5	17.6	0.1	22.0	0.8	9.1	0.4	16.7	0.2	21.4	0.3	***	***	*ns*
18:3 n-3	3.0^{d}	0.2	5.4	0.1^{b}	10.8	0.4^{a}	1.7	0.1^{e}	4.3	0.1^{c}	10.4	0.2^{a}	***	***	**
EPA	8.6	0.6	4.4	0.2	1.6	0.3	10.0	0.5	5.5	0.5	3.3	0.1	***	***	*ns*
DHA	8.3	0.6	6.1	0.4	3.4	1.0	10.9	2.0	7.0	1.2	6.4	0.4	***	***	*ns*
∑ n-3 PUFAs	25.7	0.8	20.1	0.6	21.3	1.9	28.5	2.8	20.6	1.8	25.7	0.5	**	***	*ns*

B, broodstock nutritional history effect; D, 1st feeding diet effect; BxD, interaction. PL, Polar Lipids; NL, Neutral lipids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Statistical differences were determined by two-way ANOVA followed by Tukey's HSD comparison test (superscript letters), when appropriate. *, *p<0.05*; **, *p<0.01*; ***, *p<0.001*; *ns*, not significant

Table 5. EPA and DHA content (mg alevin^{-1}) in whole body alevins collected before first feeding and after 3 weeks of feeding.

	EPA+DHA		
	before 1st feeding		3 weeks' feeding
		diets	
Progeny-COM	2.6	Diet-M	3.2
		Diet-C	2.0
		Diet-V	1.2
Progeny-VEG	1.4	Diet-M	2.6
		Diet-C	1.6
		Diet-V	0.9

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Transcriptomics

Microarray results

Transcriptome of alevins collected before first feeding

While 3185 genes exhibited fold changes (FC) >1.5, 624 (FC >2), and 114 (FC >3) between progeny originating for COM and VEG-fed females (Table 6a), none of the changes was statistically significant (*p-value >0.05, FDR >5%*).

Transcriptome of alevins collected after 3 weeks of feeding

Two-way ANOVA analysis of the transcriptome profile of whole body alevins collected 3 weeks after first feeding revealed that 71 genes were significantly differentially expressed in response to the broodstock nutritional background, and 249 gene features in response to the first-feeding diets. No significant interaction between the nutritional background of female broodstock and first-feeding diets was detected at the level of gene expression (Table 6b). The GO enrichment analysis highlighted changes in expression of genes involved in different GO categories (Figure 1a-b). In the following discussion we will focus on the main overrepresented processes, which are principally involved in metabolism-related biological processes.

Effects of broodstock nutritional history

As regards analysis of the effects of broodstock nutritional history (VEG *vs* COM) on gene expression in alevins, 54 of the 71 differentially expressed probes had an assigned gene annotation. The GO enrichment analysis highlighted changes in metabolism-related biological processes (EASE score <0.05). In particular, 11 genes involved in carbohydrate metabolism and energy pathways (20% of annotated genes) were found to be down-regulated in the transcriptome of whole-body alevins originating from females fed the VEG-diet, compared to alevins from COM-fed broodstock. The GO enrichment also indicated differential expression of 12 genes related to muscle growth and contraction (22% of annotated genes). For these genes, microarray analysis also revealed overall down-regulation in the transcriptome of whole bodies of progeny of females fed the VEG-diet, compared to those of COM-fed females (Table 7).

Effects of first feeding diets

Of the 249 probes corresponding to genes differentially expressed in response to the first feeding diets, 133 had an assigned gene annotation. GO enrichment for the biological process was performed to interpret this list of genes further. The GO enrichment analysis revealed overrepresentation of biological processes related to amino acid/protein metabolism (16 genes, 17% of annotated genes), lipid/cholesterol metabolism (13 genes, 14% of annotated genes), carbohydrate and energy metabolism (11genes, 12% of annotated genes), transport and catabolism (9 genes, 10% of annotated genes) and muscle contraction (7 genes, 8% of annotated genes). The other GO processes affected by the first feeding diets (oxidation-reduction process, transcription/translation and trans-sulfuration pathways) and their respective percentages are shown in Figure 1b. The microarray analysis showed up-regulation of the genes involved in both amino acid/protein metabolism and lipid and cholesterol metabolism with the introduction of plant-based ingredients in the diets (Table 8). By studying the expression of genes involved into carbohydrate and energy metabolism, we observed down-regulation of glucokinase (GCK) with the C-diet, and this effect became more evident when fish were fed the V-diet. In contrast, up-regulation of hexokinase (HK2) was found with the C-diet, which became more pronounced with the V-diet. Down-regulation of genes involved in muscle contraction was also observed in the transcriptome of fish fed the C and V-diets, compared to those fed the M-Diet. Genes involved in transport and catabolism were up-regulated in fish fed the plant-based C and V diets. A complete list of the pathways which have been found to be affected by first feeding diet is provided as supplementary material (Supplementary Table 2).

Table 6a. Whole body transcriptome of alevins collected before first feeding: Fold Changes and number of differentially expressed genes between groups (VEG-fed *vs* COM-fed).

Fold Change	Number of genes	*Significance*
FC > 1.5	3185	*ns*
FC > 2.0	624	*ns*
FC > 3.0	114	*ns*

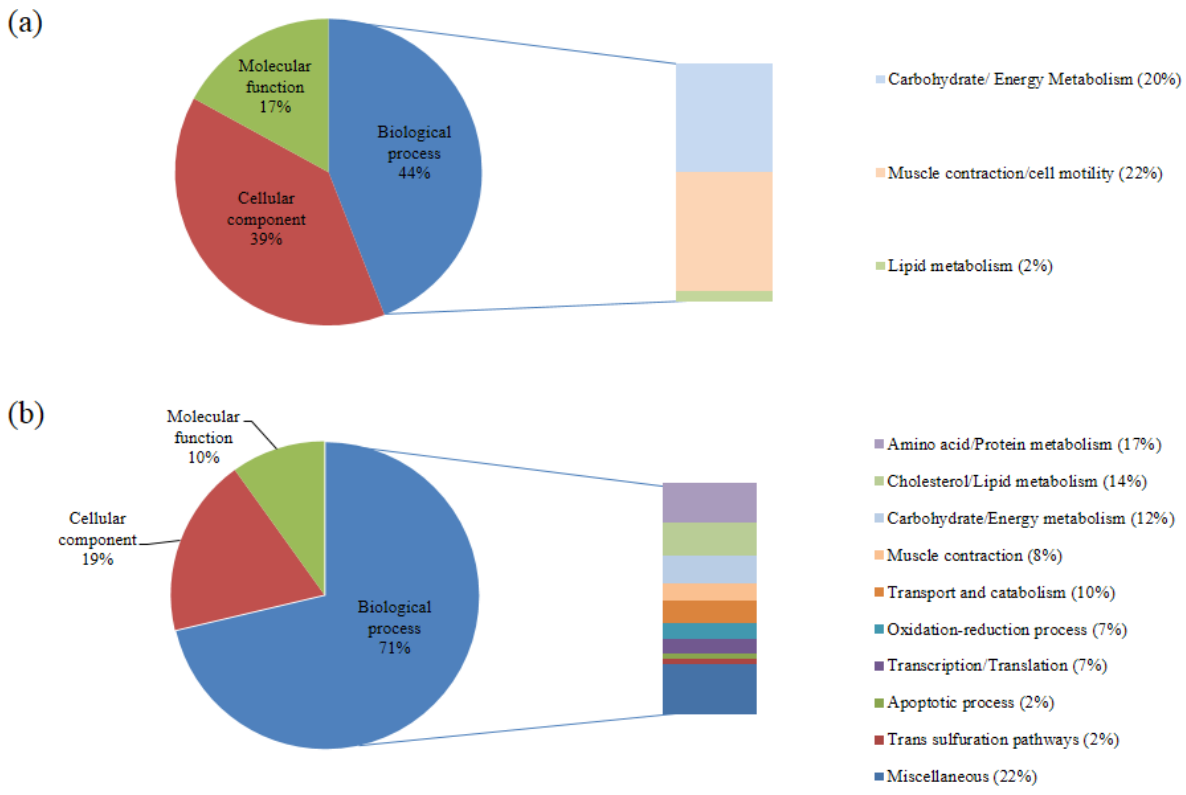
FC, Fold changes; *ns*, not significant

Table 6b. Whole body transcriptome of alevins collected after 3 weeks of feeding.

Factor	Number of genes	*P-value*
Broodstock nutritional history	71	*<0.05*
First feeding diet	249	*<0.05*
B x D	0	-

B, Broodstock nutritional history; D, First feeding diet; B x D, interaction.
Data were obtained by two-way ANOVA (p<0.05, Benjamini-Hochberg correction, *p-value* cut-off 0.05).

Figure 1 a-b. Whole-body alevins transcriptome: proportions of different GO-categories represented by differentially expressed genes obtained by a two-way ANOVA (FDR 0.05). a: broodstock nutritional history effect; b: first feeding diet effect.



RT-quantitative PCR (qPCR)

Effects of broodstock nutritional history on gene expression

Of the genes found to be differentially expressed by microarray approach, four of the genes involved in muscle growth and contraction were analysed by RT-qPCR (ACTA1, CKM, MYBPC1, MYBPC2), and are presented in Supplementary Figure 1a. The analysis revealed down-regulation of these genes in progeny originating from females fed the VEG-diet, confirming the microarray results. In addition, RT-qPCR showed an effect of the first feeding diets ($p<0.01$) for ACTA1 and an interaction between the broodstock nutritional history and the first feeding diets for CKM ($p<0.05$) that were not evident on microarray analysis.

Of the genes involved in carbohydrate metabolism and energy pathways (Supplementary Figure 1b), PGK1 was up-regulated in progeny from VEG-fed females, not confirming microarray analysis. Expression levels of five other genes (PYGM, PYGL, 6PFKM, SDHA and GPD1) were not significantly changed when measured by RT-qPCR.

Effects of first feeding diets on gene expression

A number of genes involved in amino acid and protein metabolism (IARS, LARS, EPRS and DARS) were assayed by RT-qPCR, confirming the up-regulation with the V-diet observed with the microarray analysis (Supplementary Figure 2a). With regard to cholesterol metabolism, two genes involved in cholesterol synthesis were analysed by RT-qPCR (HMGCR and HMGCS1), and the results are presented in Supplementary Figure 2b. Up-regulation of these genes was observed with the introduction of plant-based ingredients in the diet (C-diet and V-diet). Among the genes involved in carbohydrate metabolism that showed changed expression in the array analysis, three were also analysed by RT-qPCR (GCK, HK2 and LDHA). GCK and LDHA were down-regulated with the V-Diet, whereas up-regulation of HK2 expression was observed, confirming the microarray results (Supplementary Figure 2c).

Table 7. Impact of broodstock nutritional history on whole body transcriptome of alevins collected after three weeks of feeding.

			Fold change (FC)			
Probe Name	**Gene Symbol**	**Description**	***VEG-M/COM-M***	***VEG-C/COM-C***	***VEG-V/COM-V***	***p-value***
Muscle contraction/cell motility						
CUST_8882_PI425536763	**ACTA1**	**actin, alpha 1, skeletal muscle**	**-1.3**	**-1.2**	**-1.2**	***0.012***
CUST_7196_PI425536763	ACTN2	actinin, alpha 2	-1.3	-1.1	-1.4	*0.022*
CUST_8889_PI425536763	ACTB	actin, beta	-1.2	-1.2	-1.2	*0.029*
CUST_21547_PI425536763	CKB	creatine kinase, brain	-1.2	-1.2	-1.2	*0.039*
TC126460	**CKM**	**creatine kinase, muscle**	**-1.3**	**-1.2**	**-1.2**	***0.003***
CUST_21418_PI425536763	**MYBPC1**	**myosin binding protein C, slow type**	**-1.3**	**-1.3**	**-1.3**	***0.019***
CUST_20928_PI425536763	**MYBPC2**	**myosin binding protein C, fast type**	**-1.4**	**-1.3**	**-1.3**	***0.010***
CUST_21270_PI425536763	MYH2	myosin heavy chain	-1.1	-1.3	-1.5	*0.041*
TC98395	MYOM1	myomesin 1	-1.3	-1.2	-1.6	*0.037*
TC128672	MYOM2	myomesin 2	-1.3	-1.2	-1.3	*0.049*
CUST_21086_PI425536763	NEB	nebulin	-1.5	-1.4	-1.4	*0.025*
CUST_2237_PI425536763	TXN	thioredoxin	-1.3	-1.2	-1.2	*0.029*
Carbohydrate metabolism/Energy pathways						
CUST_8962_PI425536763	FH	fumarate hydratase	-1.3	-1.2	-1.2	*0.029*
CUST_21445_PI425536763	GPI	glucose phosphate isomerise	-1.4	-1.5	-1.5	*0.019*
TC96901	**GPD1**	**glycerol-3-phosphate dehydrogenase 1 (soluble)**	**-1.3**	**-1.5**	**-1.5**	***0.008***
CUST_3410_PI425536763	MDH1	malate dehydrogenase 1	-1.2	-1.3	-1.2	*0.041*
TC100795	**6PFKM**	**6-phosphofructokinase, muscle**	**-1.5**	**-1.6**	**-1.7**	***0.014***
CUST_8938_PI425536763	**PGK1**	**phosphoglycerate kinase 1**	**-1.3**	**-1.3**	**-1.2**	***0.034***
CUST_8841_PI425536763	PGM1	phosphoglucomutase 1	-1.4	-1.3	-1.4	*0.049*
TC109193	PHKA1	phosphorylase kinase alpha 1	-1.6	-1.7	-1.7	*0.026*
CUST_9021_PI425536763	**PYGL**	**phosphorylase, glycogen, liver**	**-1.2**	**-1.3**	**-1.4**	***0.032***
CUST_8835_PI425536763	**PYGM**	**phosphorylase, glycogen, muscle**	**-1.4**	**-1.4**	**-1.6**	***0.025***
CUST_22399_PI425536763	**SDHA**	**succinate dehydrogenase complex, subunit A, flavoprotein (Fp)**	**-1.3**	**-1.4**	**-1.5**	***0.006***

Genes tested by RT-qPCR are in bold. Fold changes refer to progeny developing from VEG-fed females compared to progeny from COM-fed females.

Table 8. Impact of experimental first feeding diets on whole body transcriptome of alevins after three weeks of feeding (main Biological Processes impacted).

			Fold change (FC)				
Probe Name	**Gene Symbol**	**Description**	***COM-C/COM-M***	***COM-V/COM-M***	***VEG-C/VEG-M***	***VEG-V/VEG-M***	***p-value***
Biological Process							
Amino acids/protein metabolism							
TC105786	AARS	alanyl-tRNA synthetase	+ 1.1	+ 1.4	+ 1.0	+ 1.3	*0.046*
CUST_8078_PI425536763	**EPRS**	**glutamyl-prolyl-tRNA synthetase**	**+ 1.0**	**+ 1.4**	**+ 1.1**	**+ 1.4**	***0.002***
CUST_5873_PI425536763	**DARS**	**aspartyl-tRNA synthetase**	**+ 1.1**	**+ 1.5**	**+ 1.0**	**+ 1.2**	***0.001***
CUST_11065_PI425536763	HARS	histidyl-tRNA synthetase	+ 1.1	+ 1.6	+ 1.0	+ 1.9	*0.001*
CUST_9823_PI425536763	**IARS**	**isoleucyl-tRNA synthetase**	**+ 1.6**	**+ 1.6**	**+ 1.3**	**+ 3.1**	***0.037***
TC99236	**LARS**	**leucyl-tRNA synthetase**	**+ 1.1**	**+ 1.3**	**+ 1.1**	**+ 1.2**	***0.017***
CUST_2969_PI425536763	NARS	asparaginyl-tRNA synthetase	+ 1.2	+ 1.7	+ 1.1	+ 1.3	*0.001*
TC113600	QARS	glutaminyl-tRNA synthetase	+ 1.2	+ 1.5	+ 1.1	+ 1.3	*0.007*
CUST_1945_PI425536763	SARS	seryl-tRNA synthetase	+ 1.1	+ 1.6	+ 1.0	+ 1.5	*0.001*
CUST_27479_PI425536763	TARS	threonyl-tRNA synthetase	+ 1.2	+ 1.6	+ 1.1	+ 1.3	*0.025*
CUST_6009_PI425536763	WARS	tryptophanyl-tRNA synthetase	+ 1.2	+ 1.6	+ 1.1	+ 1.2	*0.016*
TC108527	PHGDH	phosphoglycerate dehydrogenase	+ 1.3	+ 4.3	- 1.1	+ 2.2	*<0.001*
CUST_5305_PI425536763	EEF1E1	eukaryotic translation elongation factor 1 epsilon 1	+ 1.1	+ 1.5	+ 1.1	+ 1.3	*0.007*
TC97482	EIF2B1	eukaryotic translation initiation factor 2B, subunit 1 alpha	+ 1.1	+ 1.6	+ 1.2	+ 1.2	*0.005*
CUST_14068_PI425536763	EIF2B3	eukaryotic translation initiation factor 2B, subunit 3 gamma	+ 1.0	+ 1.6	+ 1.1	+ 1.4	*0.015*
TC95289	EIF2S2	eukaryotic translation initiation factor 2, subunit 2 beta	- 1.1	+ 2.8	+ 1.0	+ 1.9	*<0.001*

Table 8 (Continued)

Probe Name	Gene Symbol	Description	*Fold change (FC)*				
			COM-C/COM-M	***COM-V/COM-M***	***VEG-C/VEG-M***	***VEG-V/VEG-M***	***p-value***
Cholesterol/ Lipid metabolism							
CUST_16218_PI425536763	**HMGCR**	**3-hydroxy-3-methylglutaryl-CoA reductase**	**+ 1.5**	**+ 1.7**	**+ 1.4**	**+ 1.4**	***0.035***
TC114256	**HMGCS1**	**3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)**	**+ 1.7**	**+ 2.1**	**+ 1.2**	**+ 1.3**	***0.004***
CUST_8711_PI425536763	SQLE	squalene epoxidase	+ 1.6	+ 1.7	+ 1.5	+ 1.6	*0.009*
TC121390	CYB5R2	cytochrome b5 reductase 2	+ 1.4	+ 1.5	+ 1.3	+ 1.4	*0.011*
CUST_2668_PI425536763	CYP2F1	cytochrome P450, family 2, subfamily F, polypeptide 1	- 1.0	- 2.3	- 1.5	- 2.3	*0.003*
TC121294	CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1	+ 1.5	+ 1.9	+ 1.5	+ 1.6	*0.001*
TC112425	CYP46A1	cytochrome P450, family 46, subfamily A, polypeptide 1	+ 1.0	- 1.3	+ 1.3	- 1.5	*0.005*
TC107840	IDI1	isopentenyl-diphosphate delta isomerase 1	+ 1.6	+ 1.7	+ 1.2	+ 1.4	*0.006*
TC130899	INSIG1	insulin induced gene 1	+ 1.3	+ 1.7	+ 1.0	+ 1.3	*0.003*
CUST_12877_PI425536763	INSIG2	insulin induced gene 2	+ 1.5	+ 1.7	+ 1.2	+ 1.2	*0.029*
CUST_5335_PI425536763	MVD	mevalonate (diphospho) decarboxylase	+ 1.6	+ 1.9	+ 1.1	+ 1.3	*0.026*
CUST_28240_PI425536763	LSS	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	+ 1.6	+ 1.9	+ 1.3	+ 1.0	*0.007*
CUST_16670_PI425536763	HSD17B7	hydroxysteroid (17-beta) dehydrogenase 7	+ 1.3	+ 1.7	+ 1.2	+ 1.4	*0.011*

Table 8 (Continued)

Probe Name	Gene Symbol	Description	*Fold change (FC)*				
			COM-C/COM-M	***COM-V/COM-M***	***VEG-C/VEG-M***	***VEG-V/VEG-M***	*p-value*
Carbohydrate/ Energy metabolism							
CUST_8779_PI425536763	ENO1	enolase 1, (alpha)	- 1.0	- 1.5	- 1.3	- 1.7	*0.004*
CUST_21534_PI425536763	ENO2	enolase 2 (gamma, neuronal)	+ 1.0	+ 1.5	+ 1.5	+ 1.4	*0.012*
CUST_21688_PI425536763	ENO3	enolase 3, beta muscle	- 1.0	- 1.5	- 1.3	- 1.7	*0.046*
CUST_6475_PI425536763	**GCK**	**glucokinase (hexokinase 4)**	**- 1.5**	**- 2.1**	**- 1.4**	**- 1.6**	***0.031***
CUST_2361_PI425536763	**HK2**	**hexokinase 2**	**+ 1.1**	**+ 1.3**	**+ 1.2**	**+ 1.2**	***0.017***
CUST_21617_PI425536763	**LDHA**	**lactate dehydrogenase A**	**- 1.1**	**- 1.7**	**- 1.3**	**- 1.9**	***0.043***
CUST_21434_PI425536763	LDHB	lactate dehydrogenase B	- 1.0	- 1.9	- 1.3	- 1.7	*0.030*
TC95453	G6PC	glucose-6-phosphatase, catalytic subunit	- 1.4	- 2.7	- 1.2	- 1.7	*0.012*
CUST_11963_PI425536763	GSK3A	glycogen synthase kinase 3 alpha	+ 1.1	+ 1.3	+ 1.3	+ 1.4	*0.012*
CUST_8266_PI425536763	ATP5C1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	+ 1.1	- 1.2	+ 1.1	- 1.3	*0.011*
CUST_5461_PI425536763	ATP5J	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit F6	- 1.1	- 1.2	- 1.2	- 1.3	*0.047*
Muscle contraction							
CUST_8882_PI425536763	ACTA1	actin, alpha 1, skeletal muscle	- 1.1	- 1.4	-1.0	- 1.3	*0.017*
CUST_7817_PI425536763	ACTN2	actinin, alpha 2	- 1.1	- 1.3	- 1.2	- 1.3	*0.025*
CUST_18923_PI425536763	GAMT	guanidinoacetate N-methyltransferase	+ 1.1	- 1.7	+ 1.0	- 2.0	*0.027*
TC102031	TCAP	titin-cap	- 1.2	+ 1.9	+ 1.0	+ 1.7	*0.017*
TC96295	TNNI2	troponin I type 2 (skeletal, fast)	- 1.2	- 1.6	- 1.1	- 1.5	*0.027*
CUST_20992_PI425536763	TNNT2	troponin T type 2 (cardiac)	- 1.2	- 1.5	- 1.3	- 1.7	*0.002*

Table 8 (Continued)

			Fold change (FC)				
Probe Name	**Gene Symbol**	**Description**	***COM-C/COM-M***	***COM-V/COM-M***	***VEG-C/VEG-M***	***VEG-V/VEG-M***	*p-value*
CUST_8764_PI425536763	TNNT3	troponin T type 3 (skeletal, fast)	- 1.1	- 1.5	- 1.3	- 1.6	*0.001*
Transport and Catabolism							
CUST_23987_PI425536763	SLC3A2	solute carrier family 3 (amino acid transporter heavy chain), member 2	+ 1.0	+ 1.9	+ 1.3	+ 2.6	*<0.001*
TC120357	SLC15A1	solute carrier family 15 (oligopeptide transporter), member 1		- 2.4	- 1.6	- 2.5	*< 0.001*
CUST_27328_PI425536763	SLC1A4		+ 1.0	+ 4.0	+ 1.2	+ 3.2	*< 0.001*
TC119001	NXT2	nuclear transport factor 2-like export factor 2	+ 1.3	+ 1.9	+ 1.1	+ 1.2	*0.005*
TC98320	NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10,	+ 1.0	+ 1.4	+ 1.0	+ 1.8	*0.045*
CUST_8732_PI425536763	CAPN3	calpain 3	+ 1.1	- 1.2	+ 1.1	- 1.3	*0.048*
TC128968	CASP8	caspase 8, apoptosis-related cysteine peptidase	+ 1.0	+ 1.5	+ 1.5	+ 1.8	*0.036*
TC103006	CHIA	chitinase, acidic	- 1.3	- 1.9	- 1.3	- 1.7	*0.035*
TC118835	ACR	Acrosin	- 1.1	+ 1.9	+ 1.1	+ 1.5	*<0.001*

Genes tested by RT-qPCR are in bold. Fold changes referred to progeny fed the C or V diet, compared to fish fed th M diet.

Discussion

This study is to our knowledge the first investigation into the effects of a totally plant-based diet (no FM or FO) on the whole body transcriptome of rainbow trout alevins. It is also one of the first studies investigating the consequences of long-term feeding broodstock (3 years) a totally plant-based diet on the ability of progeny to respond to different first feeding diets with a replacement of marine ingredients rate of up to 100%. The relatively low values of FC found in this study (although statistically significant) suggest that the modifications induced by the diets, and therefore the metabolic consequences of the dietary replacement, are not so drastic. It is also important to bear in mind that one of the limitations of transcriptomic analysis in early stages might be linked to the use of RNA extracted from whole body fish, because such sample types include a mixture of different organs. The use of this kind of sample thus does not provide information about the regulation of expression in a specific organ and/or tissue.

Plant based diets do not have detrimental effects on survival but affect growth of alevins

We recently demonstrated that feeding broodstock the VEG-diet throughout a three-year life cycle had no detrimental effects on survival but resulted in lower body weight of fry before first feeding compared to those originating from COM-fed females [(26)]. Survival levels after the 3-week feeding challenge did not differ between alevins fed any of the three experimental diets (M, C or V), irrespective of the broodstock nutritional history (COM or VEG). We found that a 50% replacement rate (C-diet) resulted in lower body weights and the effect was more pronounced with total replacement (M > C > V), irrespective of the maternal nutritional history. The concomitant replacement of marine ingredients by plant-protein sources and vegetable oils is known to be responsible for a reduction in feed intake and feed efficiency [(37; 38; 39; 40)], resulting in reduced growth performance [(41)]. This effect is believed to be mainly related to the replacement of FM but not to FO substitution in rainbow trout [(17; 42)], European seabass [(43)] or gilthead sea bream [(44)].

Maternal nutritional history has no visible effect on whole-body transcriptome of alevins before first feeding

Early embryonic development in teleosts is governed until the start of zygotic transcription by maternally supplied mRNA that is incorporated into the oocyte during oogenesis [(45)]. Maternal

mRNA is critical to embryonic development since it implements basic biosynthetic processes, directs first mitotic divisions, and defines initial cell fate and embryonic patterning [(46)]. Given the previous findings on the effects of a plant-based diet on the transcriptome of adult fish and the importance of broodstock nutrition for the development of progeny, our hypothesis was that broodstock nutritional history can affect the progeny transcriptome. However, our results did not demonstrate any significant regulation in the whole body transcriptomic profile of alevins before the first feeding, despite differences in body weight and FA profile. These results suggest that no trans-generational effects linked to maternal nutritional background are present or visible at a molecular level at this specific developmental stage. One possible explanation could be that the transcriptional differences are governed by specific tissues such as the liver that are present in smaller proportions in whole individuals at this specific stage of development. The liver represents only a small proportion (around 1%) of alevin whole body components. Such a small proportion might have prevented detection of the transcriptional differences at the level of the whole individual. Nevertheless, we used the same type of sample to analyse the transcriptome of alevins after 3 weeks of feeding and we found a number of genes linked to intermediary metabolism that were differentially expressed i.e. according to the broodstock origin. Thus, the hypothesis of a "whole body diluted effect" related to the sample type probably cannot fully explain the absence of significant maternal effect.

Maternal nutritional history and first-feeding diets affect the whole-body transcriptome of alevins after three weeks of feeding

Muscle growth/contraction, as well as metabolism-related biological processes, constitute the largest group among the GO terms associated with the genes found differentially expressed in response to both broodstock nutritional history and first feeding diets of exogenous feeding alevins. In the following discussion we therefore focus on specific actors involved in metabolism from a nutrigenomic point of view in relation to different levels of FM/FO dietary replacement. However, since no interaction was found between the two factors, the effects of broodstock nutritional history and first feeding diets are discussed separately.

Effects of broodstock nutritional history

In contrast to what was observed in alevins collected before first feeding, we found a significant effect of the maternal dietary background on the transcriptomic profile of alevins after 3 weeks of exogenous feeding. One of the possible reasons to explain these results can be found in the switch of alevins from endogenous (vitellus) to exogenous feeding (external feeding). Indeed, the initiation of exogenous feeding is known to alter gene expression, through the activation of different metabolic pathways [(29)].

A set of genes related to different aspects of muscle development and contraction were found to be down-regulated in progeny from females fed the VEG-diet, compared to those from COM-fed broodstock. In particular, we observed down-regulation of creatine kinases (CKM-CKB) and myomesins (MYOM1 and MYOM2), which are involved in the structure of the contractile muscles, as well as down-regulation of alpha actin (ACTA1), which is the major constituent of the contractile apparatus. Down-regulation of myosin (*myosin heavy chain,* MYH2) and slow and fast type myosin-binding proteins-C (MYBPC1 and MYBPC2, respectively) was also observed in alevins from VEG-fed females. In fish, as in other vertebrates, skeletal muscle formation (myogenesis) involves the specific control of several myogenic regulatory factors which control processes such as specification, activation and differentiation of myogenic cells[(47)]. Once myogenic cells are activated, they proliferate and differentiate; finally, in the later stage of differentiation the expression of different genes that encode structural muscle protein, such as myosin light chain, actin and myosin heavy chain is up-regulated, marking sarcomeric assembly [(48)]. The down-regulation of the major muscular actors observed in the present study in progeny originating from VEG-fed females could therefore be mainly related to the delayed growth, and specifically muscle mass growth and development, rather than to the metabolism. Moreover, after three weeks of feeding the differences in body weight observed between groups in response to broodstock nutritional background became more evident. This increased difference could thus have helped in making the transcriptional changes detectable.

Furthermore, an overall decrease in expression of genes related to carbohydrate and energy metabolism was found. For example, a specific form of muscular phosphofructokinase (6PFKM), an actor of glycolysis, the main pathway providing energy for swimming activity in fish white muscle, was less strongly expressed in fish fed diets containing plant ingredients. Another gene

encoding creatine kinase (CK) was also associated with the glycolysis-related gene expression pattern. Previous studies on larva development of European sea bass [30] showed that these genes were increasingly expressed throughout larva growth, linked to development of skeletal muscle [49]. These findings suggest that the delayed growth recorded in fish from VEG-fed females in our study may be linked to delayed muscle differentiation.

Considering the expression of genes involved in carbohydrate metabolism and energy pathways, the results obtained by microarray analysis were not confirmed by RT-qPCR. This might be due to the fact that the primers designed for RT-qPCR do not necessarily match exactly the probes on the array, as it has been previously observed in a study on Atlantic salmon liver [18]. Indeed, due to the whole genome duplication that occurred in salmonids [50], transcriptomic and gene expression studies are often more challenging due to the presence of duplicated and highly similar genes whose transcripts might be differentially regulated.

Effects of first feeding diets

The dietary replacement of both marine proteins and oil sources by plant ingredients has been shown to result in changes in protein metabolism [10; 21]. Interestingly, we found up-regulation of 11 aminoacyl-tRNA synthetases, which catalyse the ligation of specific amino acids to their cognate tRNA and thereby assemble the building blocks of RNA translation and protein synthesis [51], with the plant-based diets. The results thus showed concomitantly higher expression of three initiation factors and a translation elongation factor in fish fed the V and C-diet. Taken together, these results seem to suggest that the replacement of FM and FO dietary sources by plant-based ingredients led to higher levels of protein synthesis. Previous studies in fish have shown that protein synthesis rates differ between tissues [52; 53]. In our study, we focused on the early stages, a period of major changes in development, during which fish go through differential rates of relative growth of organs, called allometry [54], in order to meet the specific needs of this critical developing stage and to ensure that the most essential organs for primary functions are developed first, followed by the development of organs with lower priority for survival [55]. According to these assumptions, and considering the delay in (muscle) growth found in fish fed the plant-based diets, we can hypothesize that the differences in gene expression between groups were mostly linked to the delay in development of the plant-fed groups. However, since a number of processes have key roles in protein and AA

metabolism, the biological significance of the changes in gene expression observed is limited and we prefer to treat this hypothesis with caution.

As for the broodstock nutritional history-related effects, down-regulation of genes involved in muscle contraction was also found in response to the first feeding diets in progeny receiving diets containing increasing levels of plant ingredients. These findings seem to confirm our previous hypothesis, reflecting the delay in growth and muscle development induced by plant-based diets.

Another metabolic pathway significantly affected by dietary FM and FO replacement was that of sterol metabolism. Our results suggest a general up-regulation of expression levels of genes involved in cholesterol metabolism in fish fed the diets containing increased levels of plant ingredients, namely the C-diet and the V-diet. Among the genes we found differentially expressed, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), a transmembrane glycoprotein involved in the rate-limiting step of sterol biosynthesis, was increased, as reported in European sea bass fed a diet where fish oil was replaced by vegetable oils [(14)]. In previous studies with Atlantic salmon [(19)] and rainbow trout [(10)], the authors found up-regulation of genes involved in cholesterol biosynthesis. Plant ingredients are in fact rich in phytosterols that can interfere with cholesterol metabolism, while diets based on marine FM and FO contain greater amounts of cholesterol [(56)]. The positive effects on genes of cholesterol biosynthesis pathways found in our study confirmed that trout fed the plant-based diets were capable of responding to the reduced dietary cholesterol levels in the diets as early as 3 weeks from first feeding. Indeed, the cholesterol content in our experimental diets was lower in the V-diet (0.34%) and the C-diet (0.52%) than in the M-diet (0.66%).

Our findings also suggest differential regulation of genes involved in different steps of glucose metabolism with the introduction of plant ingredients in the diet. In alevins fed the V-diet we observed up-regulation of HK2, a gene involved in the first step of the glycolysis pathway [(57)], and down regulation of GCK, which is involved in maintaining the hepatic glucose balance. Focusing on the latter, a previous study with rainbow trout, gilthead seabream and common carp [(58)] showed that nutritional induction of GCK gene expression and activity was associated with a high dietary carbohydrate (starch) intake. In our study, the down-regulation of GCK may have been linked to the lower level of dietary starch in the C- and V-diets (11% C-diet, 8.5% V-diet *vs* 20% in the M-diet). The low level of expression could also explain the absence of induction of genes involved in lipogenesis, this process being induced when glucose is in excess. Alpha-enolase (ENO1), which

participates in the conversion of glucose to pyruvate, a key intermediate at the intersection of multiple metabolic pathways including lipogenesis, was slightly down-regulated in fish fed the C- and V-diets, as previously observed in salmon fed rapeseed oil compared to those fed FO [16; 18].

Effects of broodstock nutritional history and first feeding diets on FA profile of alevins

In a previous study we showed that feeding broodstock a totally plant-based diet (VEG) throughout the life cycle affects the fatty profile of progeny (before first feeding) in both PL and NL fractions [26]. In the present study, the analysis of whole body FA composition of alevins showed higher percentages of 18:2 n-6 and 18:3 n-3 in those originating from broodstock fed the VEG-diet and in response to the V-diet. These results reflected the higher dietary content of these FA and were consistent with findings in many studies on feeding fish vegetable oils [24; 59; 60; 61]. Moreover, non-negligible amounts of n-3 LC-PUFAs (EPA and DHA) were found in both PL and NL fractions, although the dietary intake was nil with the plant-based diet. These results suggest active bioconversion from dietary precursor 18:3 n-3, and subsequent activation of the LC-PUFA biosynthesis pathway. Previous studies analysing fish transcriptome responses after dietary substitution of FO with vegetable oils have shown that lipid metabolism is highly affected [18; 19; 23; 62; 63] , regardless of the vegetable oil used. For instance in these studies, genes involved in LC-PUFA biosynthesis were overrepresented among the differentially expressed genes in Atlantic salmon post-smolts [19] and in juvenile rainbow trout [10]. The biosynthesis of n-3 LC-PUFAs in vertebrates involves consecutive desaturation and elongation reactions which convert the 18:3n-3 (α-linolenic acid) to longer-chain more unsatured FA of the same series, including EPA and DHA [2]. Two types of enzyme are responsible for this conversion, namely fatty acid desaturases and elongases. The former introduce a double bond in the fatty acyl chain from the carboxyl group, and elongases account for the condensation of activated fatty acids with malonyl-CoA in the FA elongation pathway. The analysis of our transcriptomic data on alevins, did not show any significant changes in the expression of genes involved in this pathway. A possible explanation of this result may be that we used RNA extracted from whole body alevins, including a mixture of different organs. Indeed, the use of this kind of sample does not allow unambiguous interpretation of the diet-induced regulation of gene expression, because regulation of genes in the liver and intestine, the main tissues in which the bioconversion of LC-PUFA occurs, can be masked by the mean expression pattern throughout the other organs/tissues of whole fish, especially the muscle.

Moreover, when comparing the amounts of EPA + DHA (mg alevin^{-1}) in whole body alevins at our starting point (before first feeding) and at the end of the trial (after 3 weeks of feeding), we observed a decrease in their relative quantities in alevins fed the V-Diet, irrespective of the broodstock nutritional history. Indeed, during the 3-week feeding trial V-fed alevins from both COM and VEG-fed females used around 54% and 36% of the amounts of EPA+DHA they had at the beginning of the trial, respectively. These results suggest that the reserves in terms of n-3 LC-PUFA provided by the mother through the egg (vitellus) are enough to satisfy the needs of alevins during early development, and therefore they do not need to activate the bioconversion pathway at this stage.

The present study confirmed that increasing replacement of fishmeal and fish oil by plant ingredients (up to total replacement) in the rainbow trout diet allowed fish to survive and grow, but with slight differences in terms of weight. The replacement of marine sources by plant-based ingredients in both broodstock and first feeding diets resulted in significant effects on the transcriptome of whole body alevins after 3 weeks of feeding. However, the relatively low values of FC found in this study (although statistically significant) suggest that the modifications induced by the diets, and therefore the metabolic consequences of the dietary replacement, are not too drastic. An organ-dedicated approach would be more informative and precise to improve understanding of the effects of external input, and specifically the replacement of FM and FO by plant ingredients.

Overall, these results improve the understanding of mechanisms and pathways activated by concomitant FM and FO replacement in diets for rainbow trout. These results also provide a framework for additional research on the consequences of maternal nutrition with reduced levels of fish meal and fish oil on the physiological and metabolic responses of progeny to different replacement rates in the first feeding diets. The results open up avenues to further reduction of the reliance of aquaculture on marine fishery resources by using plant-based diets over the full life cycle of fish, including broodstock and the early stages. Indeed, the limited negative consequences despite the suppression of FM and FO suggest that larger proportions of FM and FO can be replaced by plant ingredients in diets for trout broodstock and alevin, than what is currently practiced.

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Conflict of interest

None.

Authorship

Formulated research questions and designed the study: FM GC. Performed the experiments: VL LL FM GC. Analysed the data: VL GC DM FM. Wrote the paper: VL GC FM.

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Additional file 1

Lipid content (% wet weight) and proportion of the main FA (% total fatty acid) of polar (PL) and neutral (NL) lipid fractions of whole body alevins collected before first feeding.

	Progeny-COM		*Progeny-VEG*		
	Mean	SD	Mean	SD	*p-value*
Whole body lipids					
Total lipids	5.9	0.6	5.6	0.5	*Ns*
PL fraction	31.0	1.2	31.0	1.2	*Ns*
NL fraction	69.0	1.2	69.0	1.2	*Ns*
PL fraction					
Saturated	31.1	2.5	28.3	1.5	*Ns*
MUFA	13.0	0.4	15.1	0.6	*<0.05*
n-6 PUFAs	8.2	0.4	13.7	0.6	*<0.05*
18:2 n-6	3.2	0.1	4.3	0.2	*<0.05*
ARA	3.3	0.2	6.4	0.6	*<0.05*
n-3 PUFAs	44.4	2.7	38.6	2.6	*<0.05*
18:3 n-3	0.5	0.0	1.3	0.4	*<0.05*
EPA	9.0	0.7	7.7	0.5	*<0.05*
DHA	32.5	2.4	28.5	2.8	*Ns*
NL fraction					
Saturated	18.7	1.2	14.7	0.7	*<0.05*
MUFA	24.1	0.7	36.4	0.7	*<0.05*
n-6 PUFAs	15.5	0.2	20.3	0.3	*<0.05*
18:2 n-6	11.4	0.3	13.0	0.2	*<0.05*
ARA	1.7	0.1	2.4	0.2	*<0.05*
n-3 PUFAs	37.1	1.9	25.9	1.0	*<0.05*
18:3 n-3	2.3	0.2	6.6	0.1	*<0.05*
EPA	9.9	0.6	3.8	0.2	*<0.05*
DHA	18.3	1.1	9.6	0.7	*<0.05*

(Adapted from Lazzarotto et al., 2015)

ns: not significant. PL, Polar Lipids; NL, Neutral Lipids; MUFA, monousatured fatty acids; PUFA, polyunsatured fatty acids; ARA, arachidonic acid; EPA, eicosapentaenoic acid;DHA, docosahexaenoic acid. Statistical differences were determined by independent samples T-Test.

Supplementary material

Supplementary Table 1a-b. RT-qPCR-Primers

Supplementary Table 1a
Primer sequences for real-time q-PCR assays for transcripts expressed differentially in response to broodstock nutritional history (COM – VEG)

Gene	*Primer 5'-3' (FW)*	*Primer 5'-3' (RV)*	*Annealing temperature, C°*
Muscle growth/contraction			
ACTA1	AAAACAGGCCAGGGACAACA	CCTGGTATTGCTGCCCGTAT	60
CKM	TGCGTTGGTCTGAAAAGGATTGA	TCTCCTCAAACTTGGGGTGTGT	60
MYBPC1	CCAGCATCCAGAACCATCCT	TACACTGGGGAAGGTCGACA	60
MYBPC2	GTGAGTGTCCGTTTGTTGCC	CTGCCAAGTGAGACTGACGT	60
Carbohydrate/energy metabolism			
PYGM	TGCAATGTGTGTCGGTGTTG	AAGTTCCCTGGAGACCACGA	60
PYGL	AACCGACACCTCCACTTCACC	CCTGCATCTTCCTCCATCTC	60
6PFKM	GAGGGCGAAGATGAAGCTTG	GGGACCTCGAGATGAACGTA	60
SDHA	TGGTGTTTGGACGTGCCTGC	AACACAGCGGCGTGGTTCTG	60
GPD1	CTTCGCCCGGATATTCTGCA	GACCCTGGAGCTTCTGCCCA	60
PGK1	TTCGGCACAGCACACAGAGC	AAAGGGCCTGGCTGGTTTCTCC	60
Reference gene			
EF1α	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	59

ACTA1, actin-alpha 1; CKM, creatin kinase muscle; MYBPC1, myosin binding protein-C slow type; MYBPC2, myosin binding protein-C fast type; PYGM, phosphorylase glycogen muscle; PYGL, phosphorylase glycogen liver; 6PFKM, 6-phosphofructokinase muscle; SDHA, succinate dehydrogenase complex, subunit A, flavoprotein; GPD1, glycerol-3-phosphate dehydrogenase 1; PGK1, phosphoglycerate kinase 1; EF1α, α-elongation factor-1.

Supplementary Table 1b
Primer sequences for real-time q-PCR assays for transcripts expressed differentially in response to first feeding diets (M-C-V)

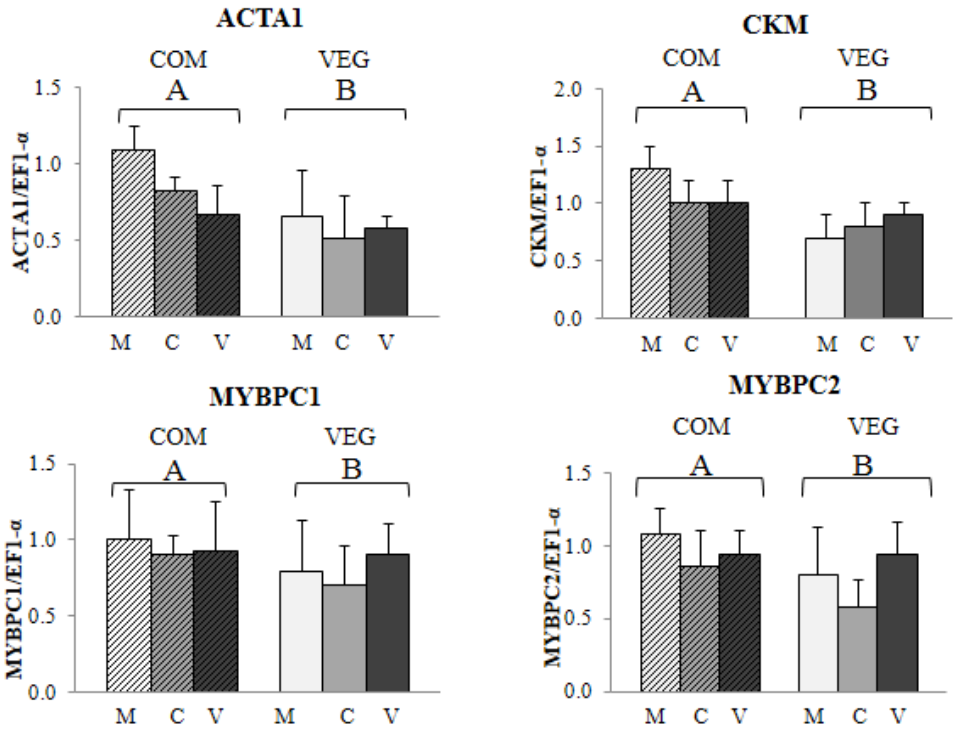
Gene	*Primer 5'-3' (FW)*	*Primer 5'-3' (RV)*	*Annealing temperature, C°*
Carbohydrate metabolism			
GCK	GCACGGCTGAGATGCTCTTTG	GCCTTGAACCCTTTGGTCCAG	60
HK2	CGCCGTGGTCGATAAGAT	TGATGAGAGCCGCCCCCTTT	60
LDHA	ATGCGTGCTGGGCAACAGTG	GCTGATAAATTAACCCTCCGC	60
Lipid/cholesterol metabolism			
HMGCR	GAACGGTGAATGTGCTGTGT	GACCATTTGGGAGCTTGTGT	60
HMGCS1	AGTGGCAAAGAGAGGGTGTG	TTCTGGTTGGAGACGAGGAG	60
Amino acids/protein metabolism			
DARS	GACCTGGCGGACATTGTGAA	GAGAGGGCCATTCACCACAA	60
EPRS	GTCGTCTGATGCCCTCTTGA	TGAAGCAGGGTCAGTGTGTG	60
IARS	ACATCGTGACTCGCTTCGCC	CTACAACCGTCAGATACGCGG	60
LARS	CGGCAGTGACATGAATGCAG	CCACTGGCCACAATGCTTTC	60
Reference gene			
EF1α	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	59

GCK, glucokinase; HK2, hexokinase-2; LDHA, lactate dehydrogenase A; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; DARS, aspartyl-tRNA synthetase; EPRS, glutamyl-prolyl-tRNA synthetase; IARS, isoleucyl-tRNA synthetase; LARS, leucyl-tRNA synthetase; EF1α, α-elongation factor-1.

Supplementary Figure 1. Gene expression of selected genes differentially expressed in response to broodstock nutritional history (COM vs VEG) and involved in (a) muscle growth/contraction, (b) carbohydrate/energy metabolism. Expression values are normalized by elongation factor-1 alpha (EF1-α) expressed transcripts. Data are presented as mean ± S.D. (n=6 individuals/treatment) and were analyzed using two-way ANOVA ($p<0.05$). Values not sharing a common lowercase letter are significantly different from each other ($p<0.05$).

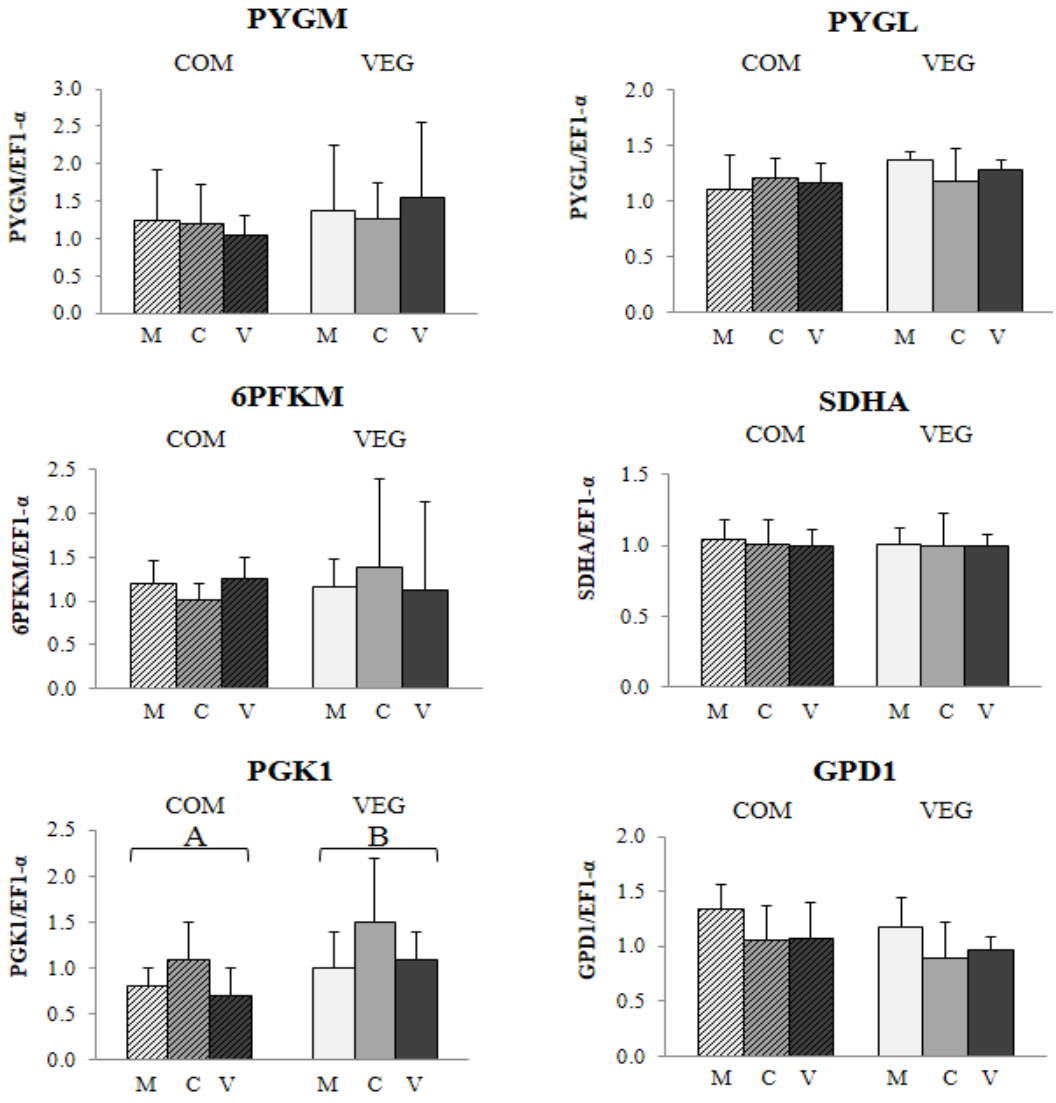
Supplementary Figure 1a.

Muscle growth/contraction. Actin-alpha skeletal muscle (ACTA1), muscle creatine kinase (CKM), myosin binding protein-C slow type (MYBPC1) and fast type (MYBPC2) mRNA levels were measured using RT-qPCR.



Supplementary Figure 1b.

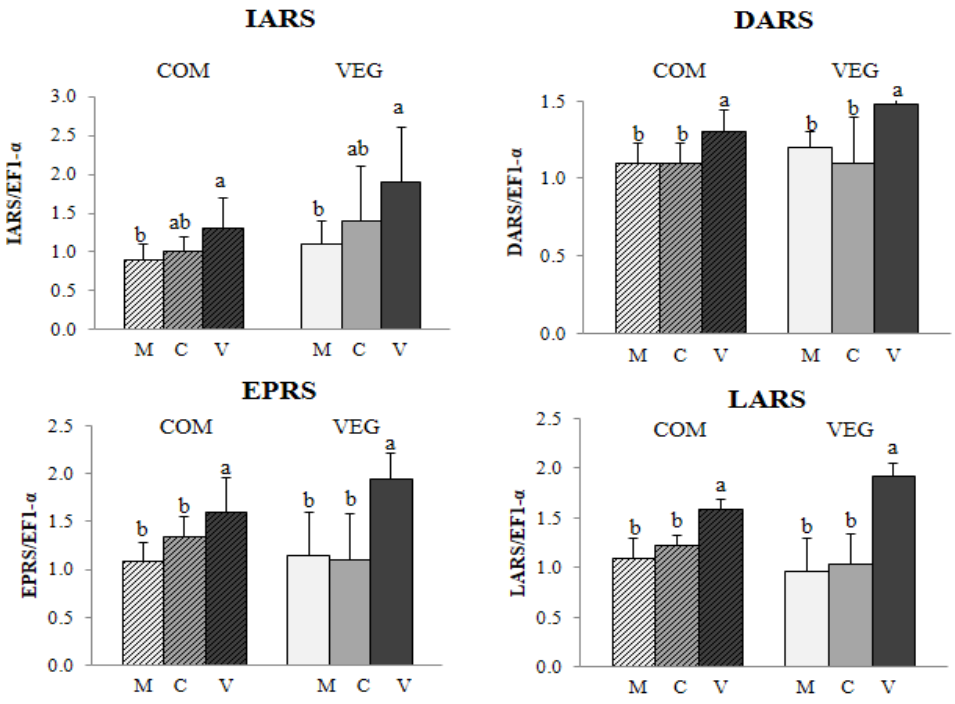
Carbohydrate/energy metabolism. Glycogen phosphorylase- muscle (PYGM) and liver (PYGL), phosphofructokinase-muscle (6PFKM), succinate dehydrogenase complex-A (SDHA), glycerol-3-phosphate dehydrogenase2 (GPD1) and phosphoglycerate-kinase 1 (PGK1) mRNA levels were measured using RT-qPCR.



Supplementary Figure 2. Gene expression of selected genes differentially expressed in response to alevin diets (M-C-V) and involved in (a) amino acid/protein metabolism, (b) lipid/cholesterol metabolism and (c) carbohydrate/energy metabolism. Expression values are normalized by elongation factor-1 alpha (EF1-α)-expressed transcripts. Data are presented as mean ± S.D. (n=6 individuals/treatment) and were analyzed using two-way ANOVA ($p<0.05$). Values not sharing a common lowercase letter are significantly different from each other ($p<0.05$).

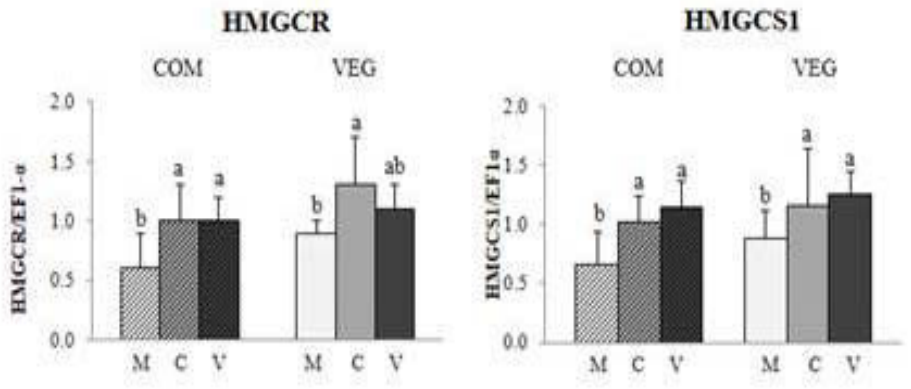
Supplementary Figure 2a.

AA/protein metabolism. Isoleucyl-tRNA synthetase (IARS), aspartyl-tRNA synthetase (DARS), glutamyl-prolyl-tRNA synthetase (EPRS) and leucyl-tRNA synthetase (LARS) mRNA levels were measured using real-time quantitative PCR.



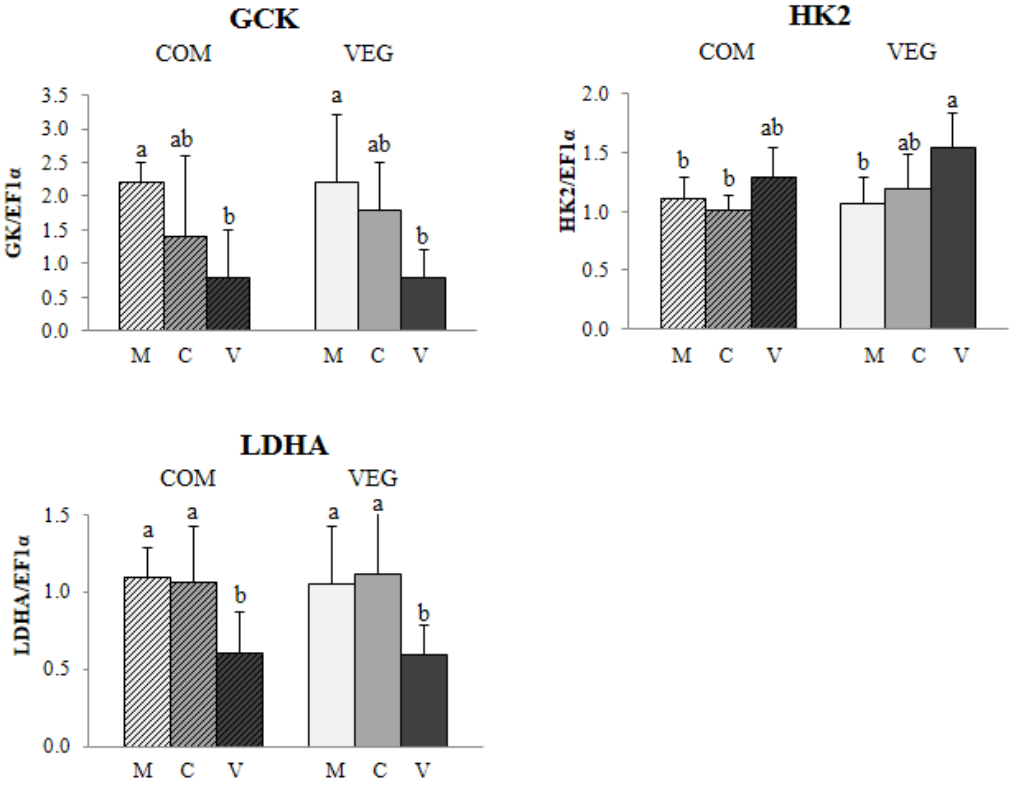
Supplementary Figure 2b.

Lipid/cholesterol metabolism. 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS1) mRNA levels were measured using RT-qPCR.



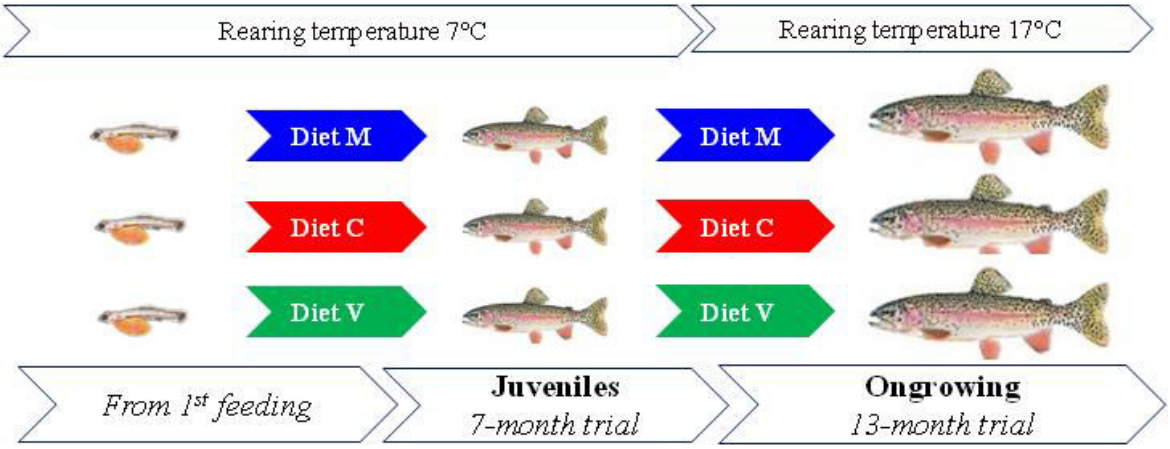
Supplementary Figure 2c.

Carbohydrate/energy metabolism. Glucokinase (GCK), hexokinase-2 (HK2) and lactate dehydrogenase-A (LDHA) mRNA levels were measured using RT-qPCR.



Publication #3

Figure 3.3 Experimental design of Publication 3.



Presentation of the article

Objective

The effects of long-term feeding trout plant-based diets since first feeding, were studied in juveniles (7-month feeding trial, 10 g fish) and ongrowing fish (13-month feeding trial, 250-350 g fish). In particular, we wanted to establish, if potential diet-induced changes observed on intestinal and hepatic transcriptomic profiles of juveniles would be maintained over time. We chose to focus on intestine and liver, because of the crucial involvement of the digestive tract in nutrient absorption, and the role of the liver as a hub of regulation of intermediary metabolism.

Experiment & analysis

Fish were fed from first feeding, one of the three experimental diets, with different inclusion levels of plant ingredients: diet-M (marine ingredients), diet-C (mix marine & plant ingredients) or diet-V (plant ingredients only, without any FM and FO). During the first period of the trial, fish were reared at 7 °C during 7 months. At the end of this first period, fish were transferred to another fish facility, where the water temperature (17°C) favoured fish maximum growth performance for 6 additional months. During the study, feeds formula were slightly changed in terms of protein and fat content in order to adapt to the different developmental stages. Fish were periodically bulk weighed. At the end of each period (7 months and 13 months feeding trial), samples of blood, intestine (mid gut), liver, and whole body fish were collected 48 hr after the last meal. Growth performance (body weight), whole body FA composition, plasma metabolites and gene expression in intestine and liver were studied.

Results and Conclusions

No significant differences were found between groups in terms of body weight at the end of the first period. However, after 13 months of feeding, body weight of the V-fed fish was significantly lower than that of fish of the other two groups. This result can be due to the fact that M and C-fed fish were able to express their maximum growth potential when reared at 17°, while during the first period at 7 °C they were restricted in their growth potential by the low water temperature. The inclusion of plant ingredients in the diet induced changes in the intestinal gene expression of juveniles, with down-regulation of genes involved in protein and FA catabolism as well as

carbohydrate metabolism, compared to the M-group. These changes were not maintained in ongrowing fish, probably as a consequence of the differences in water rearing temperatures, or because of the different developmental stages or a combination of these two factors. In the liver, enhanced expression of genes involved in cholesterol biosynthesis was found in fish fed the C and V-diet and this effect was maintained in ongrowing fish. This effect could be seen as an attempt of rainbow trout to compensate for the lower dietary cholesterol intake, as reflected by the lower plasma cholesterol levels in V and C fed fish at the physiological level. The enhanced expression of genes involved in energy pathways and electron transport identified by the transcriptomic approach in the liver of juveniles fed the V-diet, was not confirmed by RT-qPCR and were not observed in ongrowing fish. In the hepatic transcriptome of juveniles fed the plant-based diets, one elongase involved in n-3 LC-PUFA biosynthesis pathway (Elovl2) was found to be up-regulated. The hepatic up-regulation of Elovl2 with plant-based diet was confirmed by RT-qPCR in juveniles, and was also found in ongrowing fish. When tested by RT-qPCR, Elovl2 was also highly expressed in intestine of plant-fed juveniles and ongrowing fish. The expression Δ-6 desaturase, another gene involved in n-3 LC-PUFA biosynthesis pathway, was also found increased in both liver and intestine of C and V-fed juveniles and ongrowing fish, when assessed by RT-qPCR. Together, these observations suggest an active bioconversion from dietary precursor. This was confirmed by the higher amounts (g $fish^{-1}$) of EPA+DHA found in whole body of ongrowing fish compared to juveniles. However, this biosynthesis was not enough to compensate for the dietary lack of these n-3 LC-PUFAs, as reflected by the lower percentages of EPA and DHA found in whole body fish fed the V-diet.

Overall, this study showed that feeding trout diets with combined and total replacement of FM and FO by plant ingredients from first feeding did not have major effects on metabolism at the level of gene expression. This is reflected by the relatively low number of metabolism-related genes found to be differentially expressed in intestine and liver of juveniles in responses to dietary treatments.

In spite of the fact that no major deficiencies, for example for AA, exist in our experiment diets, small adjustments of the feed-formula are still needed to further optimize growth performance. In addition, this study pointed out the importance of conducing long term trials and considering all rearing parameters before drawing conclusions, especially when drastic changes in feed formulation are studied.

Long-term dietary replacement of fish meal and fish oil in diets for rainbow trout: effects on hepatic and intestinal gene expression

Viviana Lazzarotto, Françoise Médale[§], Laurence Larroquet and Geneviève Corraze

[1]INRA - UMR 1419 "Nutrition Métabolisme Aquaculture", Aquapôle, 64310 Saint Pée-sur-Nivelle, France

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Abstract

The intestinal and hepatic gene expression of juveniles and ongrowing trout was studied to investigate the effects of long-term feeding fish with diet containing different levels of marine and plant ingredients (*i.e.* V-diet: 100% plant-based, C-diet: mix of marine & plant ingredients, M: 100% marine-based). Molecular effects were analysed using microarrays and targeted RT-qPCR in juveniles after 7months of feeding (7°C). To investigate temporal robustness of dietary gene expression changes, the expression of differentially regulated genes identified in juveniles were assessed by RT-qPCR in ongrowing fish after 6 additional month of feeding (17°C). Survival and body weight were monitored during the entire duration of the trial. Plasma metabolites, whole body lipids and fatty acid profiles were analysed. After 7 months at 7°C, all juveniles reached the same body weight (10g), while V-fed ongrowing fish exhibited lower body weight. The V-diet led to higher body lipid content, to a fatty acid profile that mirrored that of the diet, and to a decreased plasma cholesterol, without affecting plasma glucose levels, at both sampling periods. In intestine of juveniles fed plant-based diets (but not in ongrowing fish), down-regulation of genes involved in protein catabolism, carbohydrate metabolism and transport was detected. Genes involved in lipid and cholesterol metabolism were up-regulated in livers of fish fed plant-based diets at both sampling periods. Overall, this study showed that feeding trout from first-feeding a totally plant-based diet did not strongly affects metabolism gene expression in juveniles reared at 7°C, as reflected by the relatively low number of genes found to be differentially expressed between groups in the intestine and liver.

Keywords: fish; nutrition; plant proteins; vegetable oils; fatty acid; microarray; transcriptome

Introduction

Aquaculture production has expanded almost 12-fold [(1)] during the last three decades in order to compensate for the decline in fish capture and to provide growing population with a consistent supply of high-quality and sustainable seafood. With the ever increasing demand for aquafeeds and the reduction in availability of the traditionally used fish meal (FM) and fish oil (FO), alternative sources of protein and lipids are now required. Significant research efforts have been made in recent decades to replace FM and FO by plant ingredients. However, the use of plant products is recognized to have some disadvantages, particularly related to differences in amino acid, cholesterol and fatty acid (FA) composition compared to marine resources, but also related to the presence of anti-nutritional factors in vegetables [(2)]. In view of these limitations and the dietary requirements of different species, efforts have been made to develop diets with low marine ingredient content and much progress has been made in the substitution of both FM and FO in feeds for aquaculture [(3)]. Nevertheless, certain deleterious effects of the changes in nutritional input still remain. For example, in a study involving Atlantic salmon (Salmo salar) juveniles [(4)] the authors shown reduced levels of growth performance in fish fed a diet with high levels of replacement of FM (80% replaced) and FO (70% replaced) with plant ingredients. Decreased growth was also observed in juvenile rainbow trout (Oncorhynchus mykiss) fed a totally plant-based diet (0%FM and 0%FO), compared to fish fed diets containing marine ingredients [(5)]. These authors suggested that the lower growth observed was mainly related to the substitution of FM, rather than FO replacement. The replacement of marine ingredients, and in particular FO substitution by plant ingredients, is also known to drastically modify FA composition of the diet. Indeed, none of the vegetable oils contain n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFAs), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), while they are rich in 18:0,18:1, 18:2 n-6 and 18:3 n-3. Reflecting FA composition of the diet, the FA composition of fish tissue also changes, as shown in previous studies in which FM and FO were largely [(4;7)] or totally [(4; 6; 7)] replaced by plant ingredients in diets.

In addition to traditional measures of the effects of substitution of marine ingredients with plant sources, such as growth performance and tissue FA composition, recent advances in functional genomics (*i.e.* gene expression) have opened up new avenues for understanding the basic molecular pathways involved in the response of fish to new diets or feeds [(8; 9; 10)]. In particular, determining

patterns of gene expression through study of tissue transcriptomes (mRNA expression) can provide extensive information about how dietary ingredients are perceived by fish and thus provide a molecular snapshot of the physiological response of specific tissue [5; 11]. Several studies in recent years, have addressed the effects of replacement of different proportions of FM and/or FO with plant ingredients on gene expression in tissues of different fish species, such as Atlantic salmon [4; 8; 12], rainbow trout [5; 13] and European sea bass (*Dicentrarchus labrax*) [6]. However, among the studies focusing on the liver, very few have investigated the transcriptional effects of total and concomitant replacement of marine ingredients by plant sources. For example, in a study involving rainbow trout juveniles, the authors found that the concomitant replacement of FM and FO by plant ingredients induced changes in hepatic expression of genes involved in nucleic acid and glucose metabolism, as well as in the expression of genes involved in lipid and protein metabolism [5]. In another study with European sea bass, the authors found up-regulation of several genes involved in the LC-PUFA and cholesterol biosynthetic pathways in the livers of fish fed a totally plant-based diet compared to those fed a marine diet, suggesting stimulation of the lipogenic pathways [6]. While slightly less extensive than for the liver, a growing number of reports are now available on the effects of plant-based diets on intestinal gene expression. Recent studies have investigated the intestinal gene expression profile in response to different levels of dietary replacement of marine ingredients by plant products in several fish species, such as Atlantic salmon [12; 14; 15], Atlantic cod (*Gadus morhua*) [16] and gilthead sea bream (*Sparus aurata*) [17]. In a previous study investigating the effects of dietary FO replacement by vegetable oil on the intestinal transcriptome of Atlantic salmon, the authors observed that lipid and energy metabolisms, were the functional category most affected by diet [14]. On the other hand, in a study with Atlantic cod juveniles fed diets containing increasing proportions of vegetable oils (33% up to 100%) replacing FO, the authors did not find major diet-induced metabolic changes in the intestine, while genes potentially able to alter cellular proliferation and death or change the structural property of intestinal muscle were found to be up-regulated [16]. In a previous study considering FM replacement in diets for Atlantic halibut (*Hippoglossus hippoglossus*) juveniles fed for three weeks with a diet with partial (30%) replacement of FM by plant-ingredients, the authors found up-regulation in the intestinal expression of genes involved in immune responses and in xenobiotic detoxification and down regulation of genes involved in lipid transport, protein synthesis and cell growth [18]. In another study investigating the effects in Atlantic salmon of FM substitution by plant protein (50% FM replaced),

the authors found changes in the intestinal expression of genes involved in protein and energy metabolism, as well as in genes involved in cell proliferation and apoptosis [12].

However, the few investigations on the impact of total and concomitant substitution of FM and FO with plant products on the metabolic response of fish tissue have been mainly on relatively short- or middle-term.

The main aim of the study presented here was therefore to investigate whether the diet-induced transcriptional changes observed in fish at certain points in their development are maintained in the longer term. In particular, we want to establish whether the changes we observed in the intestinal and hepatic gene expression of juveniles after 7 months of feeding plant-based diets might be considered representative of the rest of the life cycle and thus if these changes would be maintained when fish are reared at optimum growth water temperature and for a longer rearing period.

Materials and methods

Diets and feeding trial

The experimental plan of the present study can be divided into two sequential periods, as shown in Figure 1.

The diets used in the first period (long term feeding trial) were the same as those previously described in Lazzarotto et al., (2015). Briefly, fish were fed either a marine M1-diet (based on FM and FO), or a commercial-like C1-diet (composed of marine and plant-based ingredients, with 46% FM and 69% FO replaced by plant ingredients), or a totally plant-based V1-diet (0% FM and 0% FO, 100% plant protein and vegetable oils).

The diets used during the second part of the experiment (very long-term feeding trial) contained the same ingredients but in slightly different proportions in order to adapt the formulation to different developmental stages and fish size. The composition was slightly different to that of diets during the first period.

The ingredients and composition of the experimental diets are given in Table 1 and the proportions of the main FA of the diets in Table 2.

The experiment was carried out in strict accordance with EU legal frameworks relating to the protection of animals used for scientific purposes (Directive 2010/63/EU) and according to the

National Guidelines for Animal Care of the French Ministry of Research (Decree N° 2001-464, May 29, 2001). It was approved by the Ethics Committee of INRA (INRA 2002-36, April 14, 2002) and the scientist in charge of the experimentation received training and personal authorisation (N°B64 10 003).

The first period of the feeding trial took place at the INRA fish facilities of Lees-Athas (Permit N° A64 104 1), where the water temperature is around 7°C all year long and under natural photoperiod conditions. At the beginning of the trial, approximately 3700 rainbow trout fry, mean weight 135 ± 1 mg, were split into three groups (4 replicates per group). Fish were fed one of the three experimental diets M1, C1 or V1 from first feeding for 7 months.

For the second period of the trial, the remaining fish were transferred to the INRA experimental facilities in Donzacq (Permit N° A40 2281) and reared at a constant water temperature of 17°C and under natural photoperiod conditions. Fish were fed for 6 months (*i.e.* until the end of the trial) the M2-diet, the C2-diet or the V2-diet (3 tanks per dietary treatment).

The fish in each group were bulk-weighed throughout the feeding trial every 3 weeks in order to check the evolution of body weight as the experiment progressed. After 7 months and 13 months of feeding, 16 and 12 fish per dietary treatment, respectively, where sedated by immersion in 2% benzocaine solution. Blood was then removed from the caudal vein, collected in heparinised syringes 48h after the last meal and centrifuged (3000g, 5 min). The plasma recovered was immediately frozen and kept at -20°C until further analysis. Fish were then euthanised by immersion in a 6% benzocaine solution (anesthetic overdose) and collected for whole body composition analysis. At the end of first and the second trial periods, livers and mid gut were also sampled from eight and six individuals per dietary treatment, respectively, immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Table 1. Ingredients and composition of the experimental diets.

	alevins – juveniles			***ongrowing fish***		
Diets	**M_1**	**C_1**	**V_1**	**M_2**	**C_2**	**V_2**
Ingredients (%)						
Fish meal *	**65.2**	**30.0**	**0.0**	**54.3**	**30.0**	**0.0**
Corn gluten	0.0	13.2	24.0	0.0	10.2	18.0
Soybean meal 48	0.0	6.1	2.0	0.0	6.3	4.3
Wheat gluten	0.0	10.0	22.0	0.0	5.0	12.1
Soy protein concentrate	0.0	10.2	20.0	0.0	3.5	18.1
White lupin	0.0	0.4	2.5	0.0	6.5	5.0
Peas	0.0	4.1	0.0	0.0	6.9	2.4
Rapseed meal 00	0.0	6.2	2.3	0.0	6.3	9. 8
Extruded whole wheat	21.1	1.3	0.0	30.1	7.2	2.8
Fish oil **	**11.7**	**8.1**	**0.0**	**13.6**	**8.0**	**0.0**
Rapeseed oil	0.0	8.1	6.7	0.0	8.0	7.3
Linseed oil	0.0	0.0	6.7	0.0	0.0	7.3
Palm oil	0.0	0.0	3.6	0.0	0.0	3.0
Min.-Vit. Premix	2.0	2.0	2.0	2.0	2.0	2.0
Soy lecithin	0.0	0.0	2.0	0.0	0.0	2.0
L-lysine	0.0	0.3	1.5	0.0	0.1	1.5
L-Methionine	0.0	0.01	0.3	0.0	0.0	0.3
CaHPO4.2H20 (18%P)	0.0	0.0	2.9	0.0	0.0	2.6
Attractant Mix	0.0	0.0	1.5	0.0	0.0	1.5
Composition (% DM)						
Dry Matter (DM, %)	94.3	95.3	95.5	93.8	95.2	95.0
Crude protein	48.9	53.3	52.9	44.4	46.3	47.2
Crude fat	21.5	22.1	21.8	22.0	24.2	24.5
Starch	20.5	11.5	8.2	20.0	11.5	8.0
Energy (kJ/g DM)	23.0	24.2	24.1	23.9	24.3	25.1
Cholesterol	0.70	0.55	0.36	0.68	0.51	0.41

*Origin co-fishery products - all species

** Origin co-fishery products – sardines

M: marine FM-FO-based diet

C: commercial-like FM-FO & plant-based diet

V: experimental 100% plant-based diet

Table 2. Proportions of the main fatty acids (% of total FA) in experimental diets.

	alevins – juveniles			***ongrowing fish***		
	M_1	**C_1**	**V_1**	**M_2**	**C_2**	**V_2**
Fatty acid						
Saturated	30.8	20.9	18.5	26.6	18.2	17.6
MUFA	33.2	41.9	38.3	28.9	42.9	37.9
18:2 n-6	3.2	12.5	21.5	2.9	12.0	21.4
20:4 n-6	0.7	0.4	0.0	0.8	0.4	0.0
PUFA n-6	4.3	13.1	21.5	4.3	12.8	21.4
18:3 n-3	1.1	4.8	21.3	0.8	4.5	22.7
18:4 n-3	2.1	1.2	0.0	2.3	1.3	0.0
20:5 n-3	11.1	6.7	0.0	14.7	8.3	0.0
22:5 n-3	1.1	0.7	0.0	1.7	1.1	0.0
22:6 n-3	6.7	4.2	0.0	9.9	5.5	0.0
PUFA n-3	23.3	18.1	21.3	30.8	21.4	22.7

M: marine FM-FO-based diet

C: commercial-like FM-FO & plant-based diet

V: experimental 100% plant-based diet

MUFA: monounsaturated fatty acid;

PUFA: polyunsaturated fatty acid

Plasma metabolite analysis

Plasma glucose (Glucose RTU, BioMérieux, Marcy-l' Etoile, France) and cholesterol (CHOL100, Sobioda) levels were determined using commercial kits adapted to a micro-plate format, according to the recommendations of the manufacturer.

Lipid and fatty acid analysis

Whole body total lipids were extracted and quantified gravimetrically according to Folch et al. [(19)]. Fatty acid methyl esters (FAME) were prepared according to Shantha & Ackman [(20)]. FAMEs were then analysed by gas chromatography as previously described in detail [(21)].

Individual FAs were expressed as percentage of total FAME identified and also as quantity of EPA + DHA (g fish-1).

RNA isolation

Total RNA was extracted from individual mid gut and livers of juveniles (sampled at the end of the first period of the trial, n=8 per dietary group) and ongrowing fish (sampled at the end of the second period of the trial, n= 6 per dietary group). Extraction was performed using the TRIzol® reagent method (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. The quantity and quality of extracted RNA were analysed using a spectrophotometer (ND-1000, Nanodrop) and a Bioanalyzer (Agilent Technologie, Kista, Sweden), respectively.

Microarray hybridisation and analysis

The microarray analysis was performed on samples of RNA from individual liver and intestine (mid gut) of juveniles (8 individual samples /diet), using a custom-commercial 8X60K oligoarray (Agilent Technologies, Massy, France; Gene Expression Omnibus (GEO) Accession No. GPL15840).

Cy3-labelled experimental cRNA samples were generated using the Agilent "One-Color Microarray-based Gene Expression Analysis" (Low Input Quick Amp Labeling-LIQA) kit, as previously described in detail (Lazzarotto et al., 2016). Cy3-labelled cRNA sample yield (>0.825µg cRNA) and specific activity (>6pmol of Cy3/µg of cRNA) were verified using a NanoDrop ND-1000. Forty-eight samples (two tissues x three dietary treatments x eight replicates) were processed.

For each reaction, 600ng of Cy3-cRNA were fragmented and hybridized on a sub-array, following the LIQA kit instructions. The hybridization reactions were allowed to occur for 17h in a rotating hybridization oven (65°C) prior to washing according to the manufacturer's instructions. Samples were randomized, preventing samples from the same dietary treatment being overrepresented in a particular batch in order to avoid unintentional biases. Slides were scanned with an Agilent Scanner (Agilent DNA Microarray Scanner, Agilent technologies, Massy, France) using the standard parameters for an 8x60K gene expression oligoarray (3µm – 20 bits). Data were then obtained with the Agilent Feature Extraction software (10.7.1.1) according to the appropriate GE protocol (GE1_107_Sep09).

Real Time q-PCR (RT-qPCR)

For each experimental condition, six samples of intestine (mid gut) and liver from individual juveniles and ongrowing fish were used as biological replicates for RT-qPCR analysis.

In addition to validating differentially expressed genes obtained by the microarray analysis, we also analysed the expression of additional candidate genes related to lipid metabolism in the livers (Elovl5 and hmgcr) and intestines (Δ-6desaturase, Elovl2 and Elovl5) of juveniles.

The same genes were also studied in the intestines and livers of ongrowing fish (6 samples per tissue per experimental condition). Primer design was performed using Primer 3 software. Specific primer pairs were designed with an overlapping intron when possible, using known trout sequences in nucleotide databases (GeneBank and INRA-Sigenae). Database Accession Numbers and the sequences of forward and reverse primers used for each gene are provided in Table 3.

For the RT-qPCR, total RNA (1µg) was reverse-transcribed to cDNA with the SuperScript III RNase H reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) using oligo dT Primers. Real-time PCR was performed in the iCycler iQ TM (BIO-RAD, Hercules, CA, USA). Quantitative PCR analyses for gene expression were performed on 10µl of the RT reaction mixture using the iQ TM SYBR ® Green Supermix (BIO-RAD, Hercules, CA, USA). The total volume of the PCR reaction was 25µl containing 200nM of each primer. Thermal cycling was initiated with incubation at 95°C (90 sec) for hot-start iTaq TM DNA polymerase activation.

Table 3. Primer sequences of genes selected for analysis by RT-q PCR.

Gene	***Primer 5'-3' (FW)***	***Primer 5'-3' (RV)***	***Anealing temperature, C°***
CTSZ	GGAGCCCTTCATCAACCACA	TTGTTGGTCCACTGCCTGTT	60
CTSS	TTTGCCTCATTGCGTGTTCC	GTCTTTCATCAGCTGGCCCT	60
FAAH	TCCCTGTCTCCACGGTAACA	AACAGCCTCTCCACCTCTCT	60
CYP51A1	CCCGTTGTCAGCTTTACCA	GCATTGAGATCTTCGTTCTTGC	60
HMGCR	GAACGGTGAATGTGCTGTGT	GACCATTTGGGAGCTTGTGT	60
DHCR7	GTAACCCACCAGACCCAAGA	CCTCTCCTATGCAGCCAAAC	60
MDH2	TTGACATTGCCCACACACCT	AGATCATCACGGGTCATGCC	60
COX5B	AGATCACTGCCACGACACTATG	CTTTCCTTTCTTCAGTGCCTGC	60
COX7A2L	CCCTTGATGTGGACTGGCAA	GAGGCTTCACACCGAGTACA	60
Elovl2	TGTGGTTTCCCCGTTGGATGCC	ACAGAGTGGCCATTTGGGCG	59
Elovl5	GAACAGCTTCATCCATGTCC	TGACTGCACATATCGTCTGG	59
Δ6-desaturase	AGGGTGCCTCTGCTAACTGG	TGGTGTTGGTGATGGTAGGG	59
Reference genes			
EF1α	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	59
18S	CGGAGGTTCGAAGACGATCA	TCGCTAGTTGGCATCGTTTAT	56
β-actin	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59

CTSZ, cathepsine Z; CTSS, cathepsine S; FAAH, fatty acid amide hydrolase; CYP51A1, cytochrome P450, family 51, subfamily A, polypeptide 1; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; DHCR7, 7-dehydrocholesterol reductase; MDH2, malate dehydrogenase 2, NAD (mitochondrial); COX5B, cytochrome c oxidase, subunit Vb; COX7A2L, cytochrome c oxidase, subunit VIIa polypeptide 2 like; Elovl2, polyunsaturated fatty acid elongase 2; Elovl5, polyunsaturated fatty acid elongase 5; Δ6-desaturase, delta-6-desaturase; EF1α, eukaryotic translation initiation factor 1 alpha 1; 18S, 18S ribosomal RNA; β-actin, beta actin.

Thirty-five steps of PCR were performed, each consisting of a heating step at 95°C (20s) for denaturing, and at 59°C 30s for annealing and extension. Melting curves were systematically monitored following the final PCR cycle (with a gradient of 0.5°C/10 s from 55°C to 94°C) to ensure that only one fragment was amplified. Samples without reverse transcriptase and samples without RNA were run for each reaction as negative controls. Expression of three references genes, i.e. elongation factor-1α (EF1-α), β-actin and 18S, was quantified for both tissues types in samples from juveniles and from ongrowing fish. mRNA levels of all target genes studied in the liver were initially normalized with the housekeeping gene EF1-α, previously used as a reference gene in salmonids [22], and the expression levels were calculated according to threshold cycle (ΔΔCT). However, due to the fact that none of the three reference genes tested for the intestine was stable between the experimental groups, mRNA levels of target genes studied were normalized following the method proposed Matz et al.[23], our dataset being applicable to this method. Moreover, to validate our analytical choice, we also tested the data of liver gene expression and we compared them with the data obtained by the "classical" method (housekeeping-gene normalization). Since the results obtained with these two approaches were the same, we assumed that our choice was appropriate. In order to be able to compare gene expression levels in the two types of tissue studied, we decided to normalize data on mRNA levels of all target genes studied (in both the liver and the mid gut) following the method proposed by Matz et al. The results were analysed using the MCMC.qpcr R-package that implements generalized linear mixed model analysis of RT-qPCR data, based on the lognormal-Poisson model.

Statistical analysis and data mining

Data of biometric parameters, lipid content and fatty acids are presented as mean ± standard deviation (SD). Data were analysed statistically using the R software (version 2.14.0) and the Rcmdr package. The normality and the homogeneity of variance of the variables were tested with Shapiro-Wilk's and Levene's tests, respectively. When both conditions were satisfied, a one-way ANOVA (p-value <0.05) was performed to assess the effects of the diets. The variables with non-parametric distribution were normalized with an arcsin transformation. If the criteria were still not met (some fatty acids), a non-parametric test was used for the analysis.

Data from microarray analysis were normalized and analysed statistically using GeneSpring software (12.6, Agilent). Data were scale-normalized using the median value of each array to

identify genes differentially expressed between conditions. Differentially expressed genes were obtained by 1-way ANOVA (diet, $p<0.05$). For all genes found to be differentially expressed, GO ontologies were obtained using the Expression Analysis Systematic Explorer (EASE) software, version 2.0 (24). Significant GO enrichment was tested using EASE software, with Benjamini-Hochberg correction (score <0.05). Data from RT-qPCR were analysed by one-way ANOVA (diet, *p-value* <0.05) followed by a Tukey's post hoc test (*p-value*<0.05).

Results

Survival and growth

Data on survival and body weights of juveniles and ongrowing fish are given in Figure 2a and Figure 2b, respectively. At the end of the first period of the trial, significantly lower survival rates were observed in the V-fed group compared to groups fed the C and M-Diet (65% *vs* 95%), mainly due to the high mortality recorded in the V-fed group during the first three months. During the second period of the trial, no significant differences were found in survival rates between groups (96-98% throughout the period).

Despite the lower growth recorded for the V-fed fish during the first 3 months (Figure 2b), at the end of the first period of the trial (7 months) fish reached the same mean body weight (10 ±1 g). However, at the end of the second period of the trial (13 months) significantly lower body weights were recorded for fish fed the V-diet compared to the M-fed and the C-fed groups. No significant differences in body weight were found between the C- and M-fed groups (Figure 2b).

Figure 2a. Survival during the first (on the left) and the second period (on the right) of the feeding trial. Data are expressed as mean of tank. Fish survival is expressed as % of survivors in rapport to the initial number of fish in each tank, at each experimental period. *, statistically significant differences between V vs C and M-fed fish (one-way ANOVA, $p<0.05$).

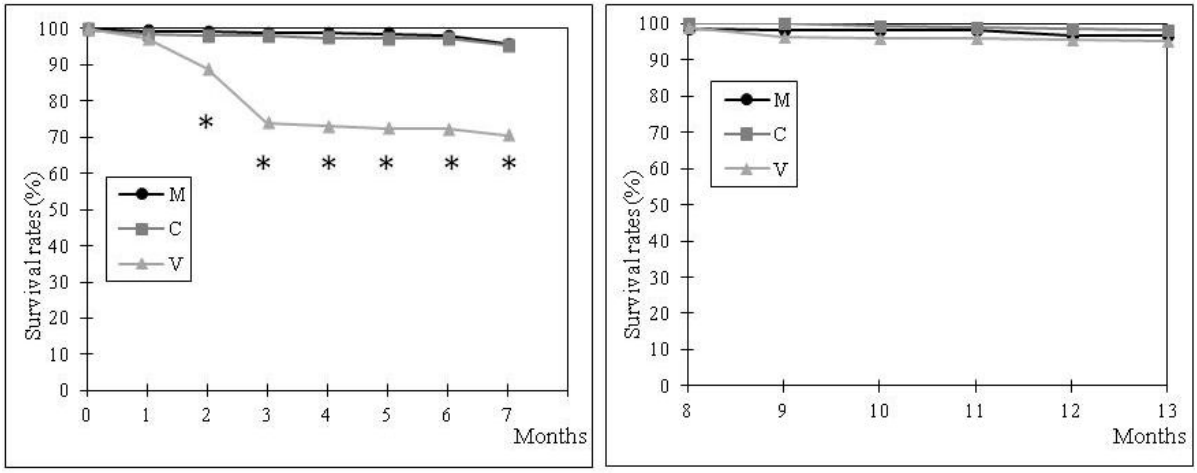
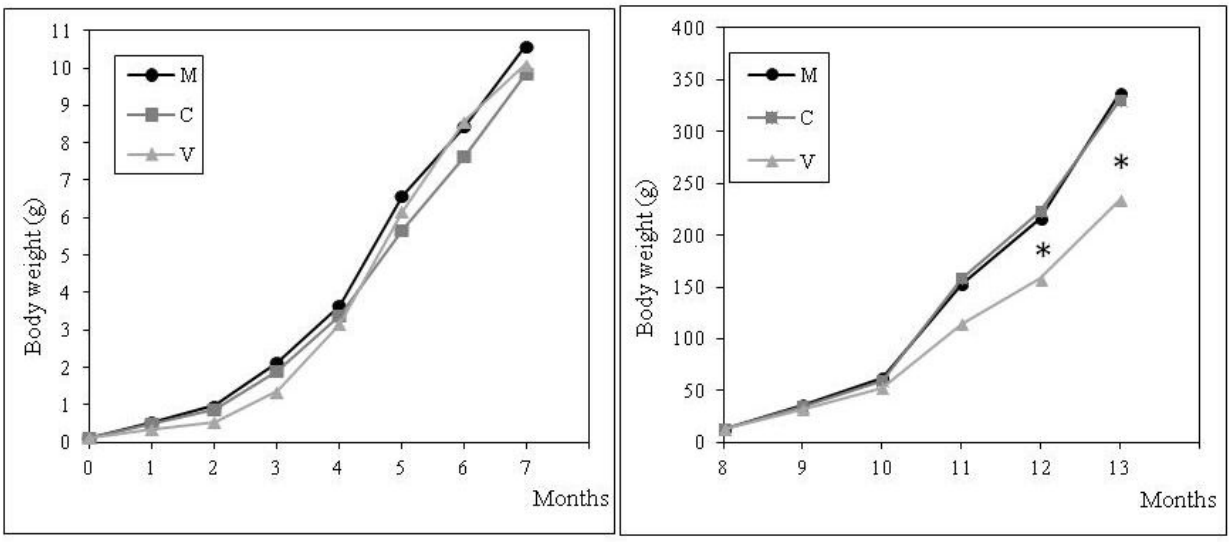


Figure 2b. Body weight of rainbow trout during the first (on the left) and the second period (on the right) of the feeding trial. Data are expressed as mean, fish were monthly bulk weighed during the whole duration of the feeding trial. *, statistically significant differences between V vs C and M-fed fish (one-way ANOVA, p<0.05).



Plasma metabolites

Plasma metabolites were measured 48h after feeding at the end of each period of the trial (7 months and 13 months, Figure 3). Plasma glucose levels were not significantly different between dietary treatments, either in juveniles or in ongrowing fish. However, lower glucose levels were found in ongrowing fish than in juveniles, irrespective of the dietary treatment. Significantly lower plasma cholesterol levels were found in both juveniles and ongrowing fish fed the plant- based diets compared to the M-fed group. Plasma levels of cholesterol were slightly higher in ongrowing fish than in juveniles, irrespective of the diet.

Whole body lipids and FA profile

Significantly higher whole body lipids (Table 4) were found in juveniles fed the V_1-diet (+23%), at the end of the first period of the trial, compared to those fed the M_1 or the C_1-Diet, whereas no differences were recorded in ongrowing fish (around 15% body lipids) (Table 4).

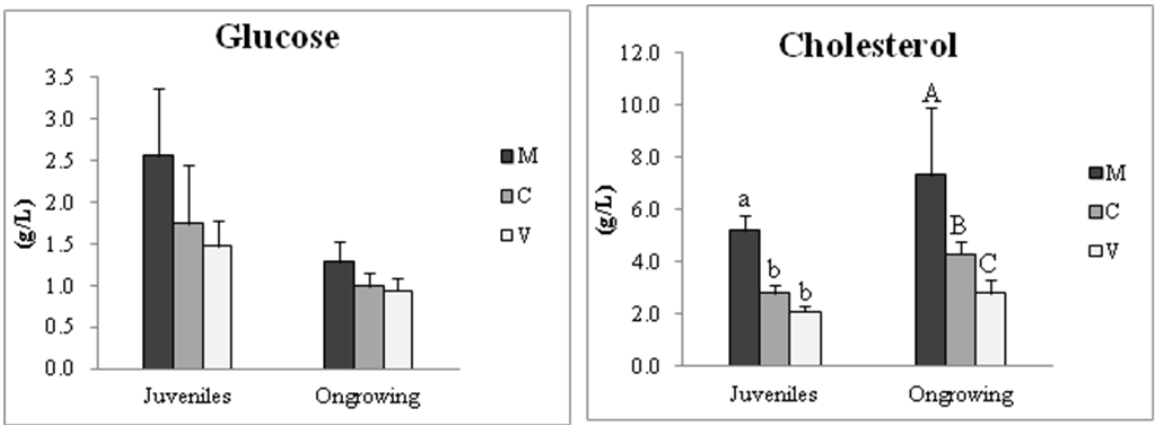
The whole body fatty acid composition of juveniles and ongrowing fish reflected that of the respective diets (Table 4). Lower percentages of SAT were found in fish fed the plant based diets (C_{1-2} or V_{1-2}), compared to M_{1-2}-fed fish. Levels of MUFA were higher in fish fed the C_{1-2} and V_{1-2}-diet compared to those fed the M_{1-2}-diet. Trout fed the V_{1-2}-diet exhibited the highest levels of n-6 PUFA, mainly linked to the high percentages of 18:2 n-6. The ARA levels were higher in fish fed the M_{1-2}-diet, compared to those fed the C_{1-2} and the V_{1-2}-diet. 18:3 n-3 levels were higher in fish fed diets containing plant ingredients (V_{1-2}>C_{1-2}>M_{1-2}), whereas lower percentages n-3 LC-PUFA (1% EPA and 2% DHA) were found in fish fed the V_{1-2}-diet than in fish fed the C_{1-2} or M_{1-2}-diet. Amounts of of EPA and DHA in terms of g $fish^{-1}$ (Table 5) are lower in body lipids with the inclusion of plant ingredients in the diet (M_{1-2}>C_{1-2}>V_{1-2}). Amounts of EPA + DHA were higher in ongrowing fish than in juveniles, irrespective of the administrated diet.

Table 4. Total lipid whole body content (% of fresh weight) and proportions (% of total FA) of the main fatty acids in juveniles and ongrowing fish.

Diets	**M**		**C**		**V**		
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	*p-value*
Juveniles							
Lipids (%)	9.40[a]	0.38	9.94[a]	1.52	13.20[b]	0.88	*<0.01*
Saturated	24.7[a]	0.4	18.2[b]	0.5	16.5[c]	1.1	*<0.001*
MUFA	34.1[c]	0.3	42.8[a]	0.1	39.1[b]	0.6	*<0.001*
18:2 n-6	3.5[c]	0.2	12.6[b]	0.2	18.7[a]	0.4	*<0.001*
20:4 n-6	0.8	0.02	0.5	0.04	0.5	0.05	*<0.001*
PUFA n-6	4.9[c]	0.2	14.3[b]	0.2	22.4[a]	0.5	*<0.001*
18:3 n-3	1.0[c]	0.0	3.8[b]	0.1	11.3[a]	0.3	*<0.001*
20:5 n-3	8.3[a]	0.2	4.2[b]	0.1	1.0[c]	0.1	*<0.001*
22:6 n-3	14.5[a]	0.4	9.4[b]	0.2	2.0[c]	0.2	*<0.001*
PUFA n-3	29.1[a]	0.8	20.7[b]	0.2	20.7[b]	1.1	*<0.001*
Ongrowing							
Lipids (%)	15.1	1.1	16.2	0.8	15.2	0.9	*Ns*
Saturated	30.7[a]	0.5	22.5[b]	0.5	18.0[c]	0.1	*<0.001*
MUFA	31.3[c]	0.7	43.1[a]	0.3	38.2[b]	0.3	*<0.001*
18:2 n-6	3.0[c]	0.1	11.8[b]	0.3	19.3[a]	0.2	*<0.001*
20:4 n-6	0.7[a]	0.0	0.4[c]	0.0	0.5[b]	0.0	*<0.001*
PUFA n-6	4.5[c]	0.1	13.2[b]	0.3	20.8[a]	0.2	*<0.001*
18:3 n-3	0.8[c]	0.0	3.5[b]	0.1	13.2[a]	0.2	*<0.001*
20:5 n-3	8.9[a]	0.4	4.0[b]	0.1	0.8[c]	0.0	*<0.001*
22:6 n-3	10.4[a]	0.6	6.5[b]	0.2	1.9[c]	0.3	*<0.001*
PUFA n-3	23.5[a]	1.2	12.9[b]	0.3	3.7[c]	0.4	*<0.001*

MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; ns, not significant. Statistical differences were determined by one-way ANOVA followed by Tukey's HSD comparison test. Values that do not share a common letter are significantly different ($p<0.05$). Mean n=3.

Figure 3. Plasma metabolites in juveniles and ongrowing fish (g/L).



Data are means ± SD. Differences between diets were analyzed by one-way ANOVA, followed by Tukey's test. Values that do not share a common letter are significantly different ($p<0.05$), with lower and upper case letters indicating differences between dietary groups within juveniles and ongrowing fish, respectively.

Table 5. Amounts of EPA + DHA (g fish^{-1}) at different developmental stages in response to the experimental diet

	EPA + DHA	
Diets	*Juveniles*	*Ongrowing fish*
Diet-M	0.2	8.9
Diet-C	0.1	5.4
Diet-V	0.04	0.9

Microarray analysis in juveniles

Intestinal transcriptome

Analysis by one-way ANOVA of the intestinal transcriptome of juvenile rainbow trout showed that 143 genes were significantly differentially expressed in response to the dietary treatments. Of these, 45 had an assigned gene annotation (Figure 4a and Supplementary Table 1). The GO enrichment analysis mainly highlighted changes in metabolism-related biological processes (62% of enriched genes, EASE score <0.05, Table 6). Specifically, eight genes involved in protein degradation (17 % of annotated genes) were found to be down-regulated in fish fed the plant based diets, the down-regulation being more pronounced in the C-fed group. The same down-regulation trend was observed for seven genes involved in carbohydrate metabolism (16% of annotated genes). The GO enrichment also indicated down-regulation of three genes involved in the apoptotic process and immune response (7% of annotated genes), as well as two genes involved in fatty acid catabolism (4%) in fish fed the plant-based diets, compared to those fed the M-diet. One gene involved in transcription and activation processes (4% of annotated genes) was up-regulated in fish fed the C diet, compared to fish fed the other two experimental diets. Global down-regulation with the C-diet was highlighted by the GO analysis of genes belonging to the GO molecular function category (25% of enriched genes) and the GO cellular component (13% of enriched genes) compared to the M- and V-fed fish.

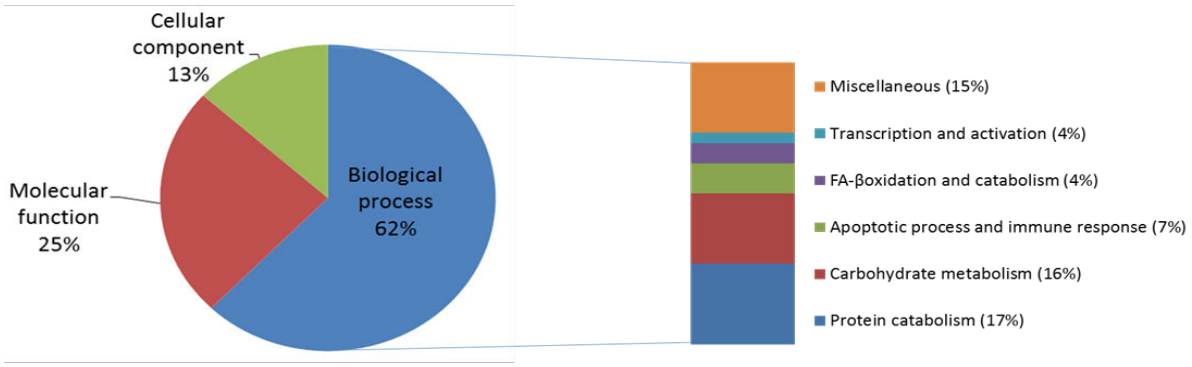
Hepatic transcriptome

The one-way ANOVA revealed that 53 genes were differentially expressed in the liver in response to the dietary treatment. Of these, only 22 had an assigned gene annotation (Figure 4b and supplementary table 2). The GO enrichment analysis highlighted changes in metabolism-related biological processes (43% of enriched genes, EASE score <0.05, Table 7). Of these, the pathways most affected by the dietary treatments were lipid/cholesterol metabolism (4 genes, 17% of annotated genes), energy pathways (4 genes, 17 % of annotated genes) and electron transport (2 genes, 10 % of annotated genes). Among the genes involved in lipid metabolism, we observed up-regulation of those involved in LC-PUFA biosynthesis as well as those involved in cholesterol biosynthesis with the V-diet compared to the other treatment groups. Genes involved in energy pathways were also up-regulated in fish fed the V-diet. Up-regulation with plant based diets was

also found for the two genes involved in electron transport. Differential regulation in response to the diet was also observed for genes belonging to the GO molecular function category (48% of enriched genes) with, in particular, global up-regulation of genes involved in macromolecule biosynthesis with the V-diet compared to the other two experimental groups (V>M>C). Diet also affected the expression of two genes belonging to the GO category of cellular components (9% of enriched genes).

Figure 4 a-b. Intestinal and hepatic transcriptome: proportions of different GO-categories represented by differentially expressed genes obtained by a one-way ANOVA (factor: *diet*, FDR 0.05).

a. Intestinal transcriptome



b. Hepatic transcriptome

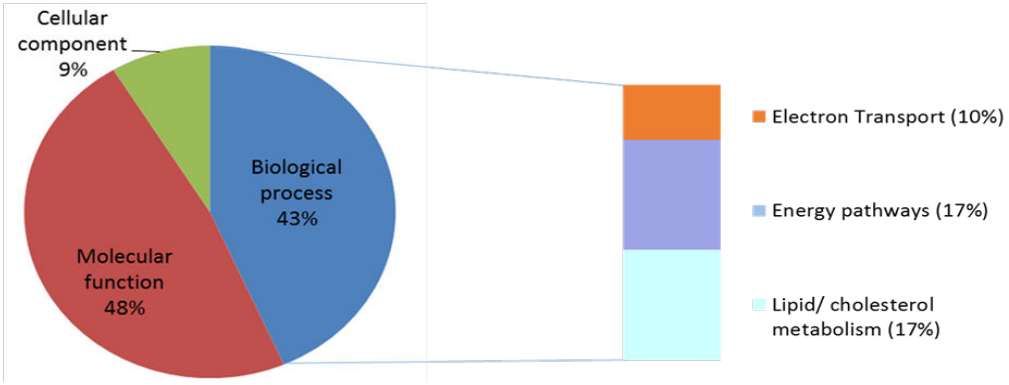


Table 6. Impact of dietary treatments on the intestinal transcriptome of juveniles related to the expression of genes involved in GO biological processes. Genes tested by RT-q PCR are in bold.

			Fold Change (FC)			***Significance***
Probe Name	**Gene Symbol**	**Description**	***C vs M***	***V vs M***	***V vs C***	***p-value***
Protein catabolism						
TC99247	CTSH	cathepsin H	- 5.1	- 2.6	+ 2.0	*0.035*
CUST_68_PI425708691	CTSL2	cathepsin L2	- 11. 1	- 4.0	+ 2.8	*0.041*
CUST_8157_PI425536763	**CTSS**	**cathepsin S**	**- 11.0**	**- 3.8**	**+ 2.9**	***0.041***
CUST_24029_PI425536763	**CTSZ**	**cathepsin Z**	**- 5.7**	**- 2.9**	**+ 2.0**	***0.013***
TC106655	DPP7	dipeptidyl-peptidase 7	- 2.2	- 2.1	+ 1.0	*0.036*
CUST_20321_PI425536763	FOLH1	folate hydrolase	- 3.9	- 1.8	+ 2.2	*0.041*
TC110997	LGMN	legumain	- 9.0	- 4.5	+ 2.0	*0.041*
CUST_25677_PI425536763	ENPEP	glutamyl aminopeptidase (aminopeptidase A)	- 13.8	- 1.9	+ 7.2	*0.041*
Carbohydrate metabolism						
CUST_21158_PI425536763	MAN2B1	mannosidase, alpha, class 2B, member 1	- 7.1	- 5.4	+ 1.3	*0.012*
CUST_17398_PI425536763	FUCA1	fucosidase, alpha-L- 1, tissue	- 4.4	- 2.8	+ 1.5	*0.035*
CUST_12758_PI425536763	FUCA2	fucosidase, alpha-L- 2, plasma	- 8.3	- 3.8	+ 2.2	*0.025*
TC114862	GLB1	galactosidase, beta 1	- 11.4	- 3.5	+ 3.3	*0.016*
TC104967	NAGA	N-acetyl galactosaminidase, alpha	- 11.2	- 3.8	+ 3.0	*0.042*
TC108468	NEU1	neuraminidase 1	- 25.8	- 7.2	+ 3.6	*0.025*
TC123951	**PFKFB3**	**6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3**	**- 2.8**	**- 3.0**	**+ 1.1**	***0.042***
Apoptotic process/immune response						
CUST_14242_PI425536763	MPO	myeloperoxidase	-1.5	- 4.9	- 3.3	*0.035*
CUST_7188_PI425536763	BAD	BCL2-associated agonist of cell death	- 2.3	- 1.3	+ 1.8	*0.045*

Table 6. (*Continued)*

Probe Name	Gene Symbol	Description	Fold Change (FC)			*Significance*
			C vs M	***V vs M***	***V vs C***	***p-value***
Fatty acid catabolism						
TC94736	CPT1A	carnitine palmitoyltransferase 1A (liver)	+ 1.4	+ 1.0	- 1.4	*0.045*
TC121737	**FAAH**	**fatty acid amide hydrolase**	**- 18.6**	**- 3.0**	**+ 6.2**	***0.041***
Transcription and activation						
TC125816	PQBP1	polyglutamine binding protein 1	+ 1.3	- 1.1	- 1.4	*0.042*
Miscellaneous						
CUST_17716_PI425536763	MXD4	max dimerization protein 4	- 1.7	- 1.6	+ 1.1	*0.025*
CUST_1243_PI425536763	POLR2F	polymerase (RNA) II (DNA directed) polypeptide F	+ 1.4	- 1.1	- 1.5	*0.039*
TC118891	PRPSAP2	phosphoribosyl pyrophosphate synthetase-associated protein 2	+ 1.4	- 1.0	- 1.4	*0.025*
CUST_9923_PI425536763	TTC4	tetratricopeptide repeat domain 4	+ 1.8	+ 1.5	- 1.2	*0.039*
CUST_6882_PI425536763	RENBP	renin binding protein	- 4.4	- 2.5	+ 1.8	*0.036*
TC95545	ASAH1	N-acylsphingosine amidohydrolase 1	- 2.6	- 1.9	+ 1.4	*0.041*
CUST_15445_PI425536763	ASH2L	ash2 (absent, small, or homeotic)-like	- 2.2	- 1.7	+ 1.3	*0.049*

Table 7. Impact of dietary treatments on the hepatic transcriptome of juveniles related to the expression of genes involved in GO biological processes. Genes tested by RT-q PCR are in bold.

			Fold Change (FC)			***Significance***
Probe name	**Gene Symbol**	**Description**	***C vs M***	***V vs M***	***V vs C***	***p-value***
Biological process						
Lipids/Cholesterol Metabolism						
CUST_14393_PI425536763	**ELOVL2**	**polyunsaturated fatty acid elongase**	**+ 1.4**	**+ 2.6**	**+ 1.8**	***0.049***
TC130473	**CYP51A1**	**cytochrome P450, family 51, subfamily A, polypeptide 1**	**+ 2.3**	**+ 4.1**	**+ 1.8**	***0.046***
TC130143	**DHCR7**	**7-dehydrocholesterol reductase**	**+ 2.3**	**+ 3.8**	**+ 1.6**	***0.049***
CUST_9914_PI425536763	TM7SF2	transmembrane 7 superfamily member 2	+ 2.6	+ 5.7	+ 1.8	*0.030*
Energy pathways						
CUST_21841_PI425536763	ATP5B	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit	- 1.0	+ 1.5	+ 1.5	*0.030*
CUST_20841_PI425536763	ATP5C1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	+1.1	+ 1.4	+ 1.3	*0.040*
CUST_11055_PI425536763	**MDH2**	**malate dehydrogenase 2, NAD (mitochondrial)**	**+1.0**	**+ 1.7**	**+ 1.7**	***0.030***
TC114386	UQCRC1	ubiquinol-cytochrome c reductase core protein I	-1.0	+ 1.7	+ 1.7	*0.030*
Electron Transport						
TC105004	**COX5B**	**cytochrome c oxidase subunit Vb**	**-1.2**	**+1.4**	**+ 1.6**	***0.038***
TC99046	**COX7A2L**	**cytochrome c oxidase subunit VIIa polypeptide 2 like**	**-1.2**	**+1.5**	**+ 1.9**	***0.027***

RT-qPCR

The intestinal and hepatic expression of the genes tested by RT-qPCR is shown in Figure 5a and Figure 5b, respectively.

Intestinal gene expression

The expression of selected genes was measured by RT-qPCR in order to validate microarray results in juveniles. A good match between RT-qPCR and microarray results was found for the genes tested (CTSS, CTSZ, PFKFB3, FAAH and Elovl5), although differences between experimental groups assessed by RT-qPCR were not significant for CTSZ.

In terms of genes involved in protein catabolism, CTSS was down-regulated in fish fed the V-diet compared to the other experimental groups, while we observed the highest levels of expression for the C-fed fish at the end of the first rearing period. Different diet-induced effects were observed in ongrowing fish, the CTSS expression level being enhanced in both C- and V-fed fish compared to those fed the M-diet. No statistical differences between groups were observed in the expression levels of CTSZ in juveniles, whereas higher levels were found in ongrowing fish fed the plant-based diets ($p<0.05$), compared to those fed the M-diet. In terms of carbohydrate metabolism, PFKFB3 was down-regulated in juveniles with the inclusion of plant ingredients in the diet (M≥C≥V), while up -regulation was observed in C and V-fed ongrowing fish. In terms of fatty acid catabolism in juveniles, we observed significantly lower expression of FAAH in the C- fed group compared to the M-fed group and intermediate levels for the V-fed fish. Enhanced expression of FAAH was observed in ongrowing fish fed the plant based diets, compared to those fed the M-diet. Although we did not find any difference on microarray analysis, three candidate genes involved in FA bioconversion were also tested by RT-qPCR (Elovl5, Elovl2 and Δ6-desaturase). No statistically significant differences were observed between groups in the expression levels of Elovl5, in either juveniles or ongrowing fish. In terms of the expression of Elovl2, we observed the highest levels in V-fed fish, while the lowest levels were observed for the C-fed group. Up-regulation of Elovl2 was found in both C and V-fed groups of ongrowing fish compared to fish fed the M-diet. Up-regulation of Δ6-desaturase was observed in V-fed juveniles compared to the other experimental groups. Up-regulation of Δ6-desaturase was observed in ongrowing fish in both the C-and V-fed groups, compared to those fed the M-diet.

Hepatic gene expression

The RT-qPCR performed on the livers of juveniles confirmed overall the microarray results for the genes involved in fatty acid bioconversion (Elovl2) and cholesterol biosynthesis (CYPP51A1 and DHCR7). In contrast to the results observed with the microarray approach, no statistically significant differences were observed for genes involved in energy pathways (MDH2) and electron transport (COX5B and COX5A2L) either in juveniles or in ongrowing fish.

Among the genes involved in fatty acid bioconversion, Elovl2 and Δ6-desaturase were found to be up-regulated in V-fed juveniles and ongrowing fish, compared to the other experimental groups whereas no statistically significant diet-induced effect was observed in the levels of expression of Elovl5 at both stages. Up-regulation of CYP51A1, DHCR7 and HMGCR was found in juveniles fed the V-diet compared to the C and M-fed fish, and the same expression pattern was observed in ongrowing fish (V> C and M).

Figure 5a. Intestinal gene expression in juveniles and ongrowing fish. Data are mean ± S.D. (n=6 individuals/treatment). Differences between diets were analyzed by one-way ANOVA, followed by Tukey's test. Values that do not share a common letter are significantly different ($p<0.05$), with lower and upper case letters indicating differences between dietary groups within juveniles and ongrowing fish, respectively.

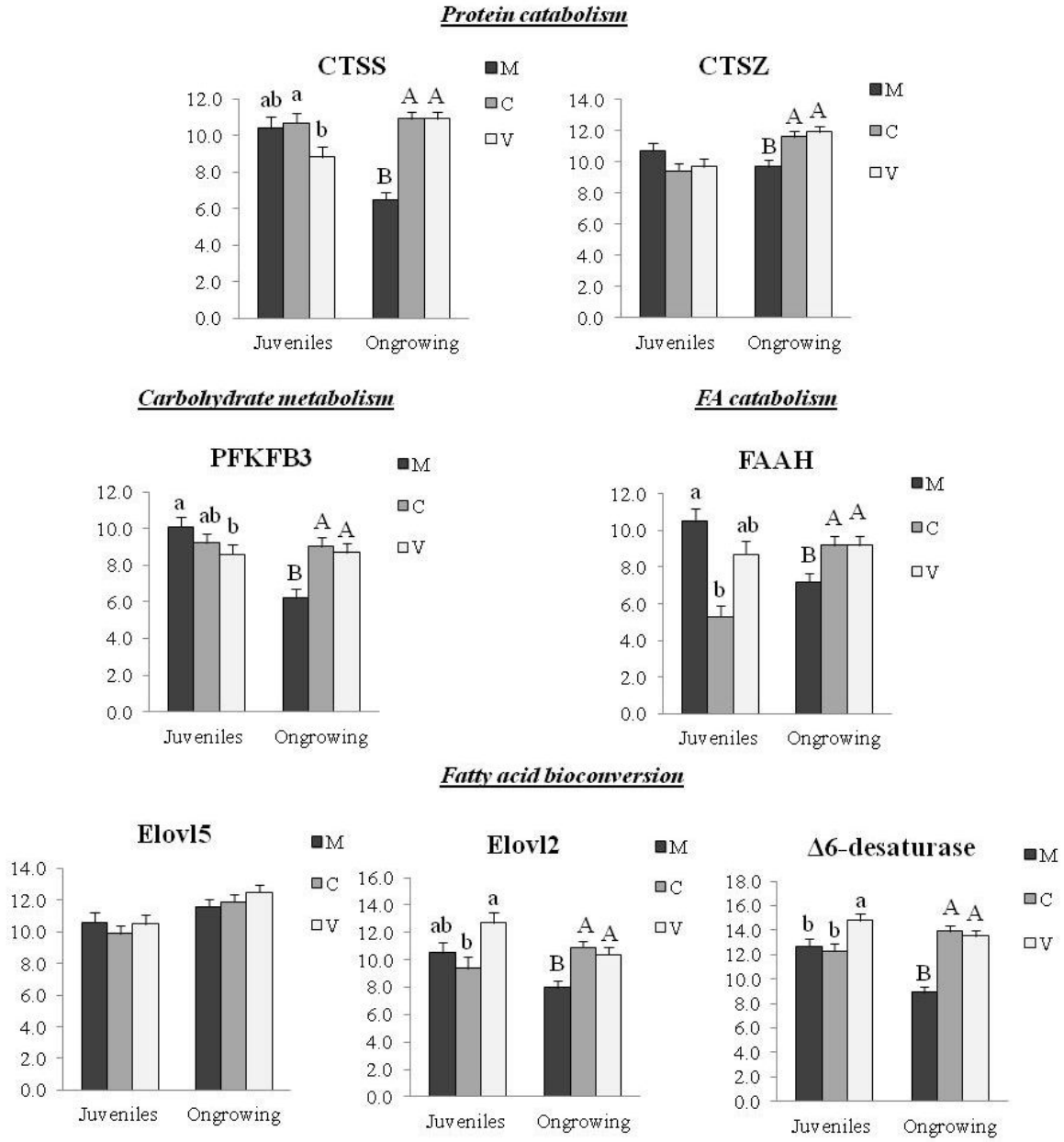
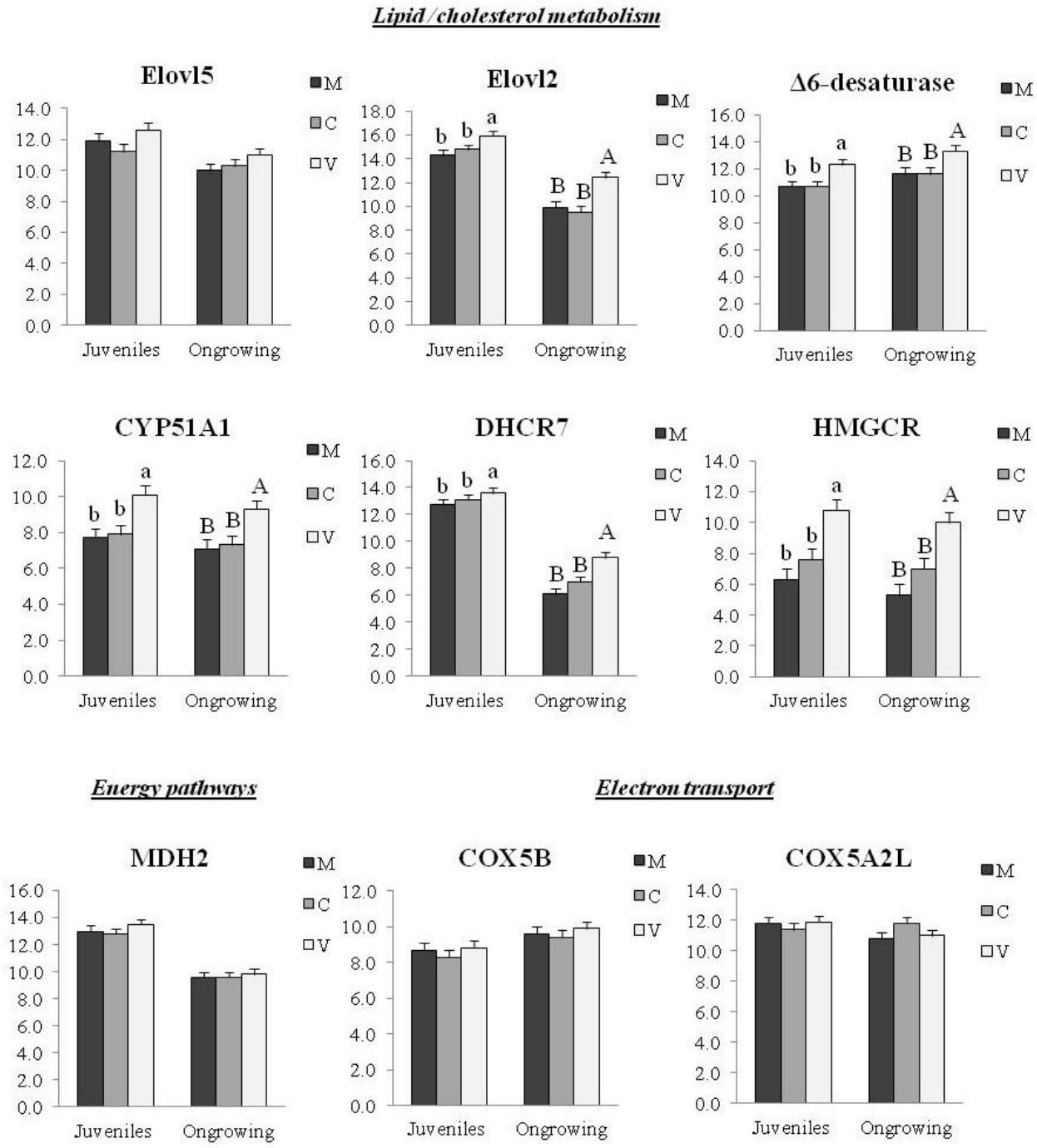


Figure 5b. Hepatic gene expression in juveniles and ongrowing fish. Data are mean ± S.D. (n=6 individuals/treatment). Differences between diets were analyzed by one-way ANOVA, followed by Tukey's test. Values that do not share a common letter are significantly different ($p<0.05$), with lower and upper case letters indicating differences between dietary groups within juveniles and ongrowing fish, respectively.



Discussion

In the context of sustainable aquaculture, replacing FM and FO in feeds for farmed fish with alternative less costly and more readily available products such as vegetable sources is becoming a common practice [2]. We recently demonstrated that feeding rainbow trout diets containing different levels of plant ingredients (up to 100% plant-based diets) from first feeding affected the whole body transcriptome of alevins [Lazzarotto et al., accepted] after a 3-week feeding trial. The main aim of the present study was to elucidate the long term effects of rainbow trout diets, including increasing levels of FM and FO, on the response of fish after 7 months and 13 months of feeding. In order to assess these effects from a molecular point of view, we used a microarray approach which allowed us to obtain a snapshot of the physiological responses of specific tissues through gene expression. On the basis of the results obtained by the microarray analysis in juveniles after 7 months of feeding, we further investigated whether the changes in gene expression had been maintained in the longer term, *i.e.* after the additional 6 months of feeding. The relatively low number of genes found to be differentially expressed in both the intestine and the liver in response to our experimental diet, in addition to the relatively low overall fold changes (FC) obtained suggested that the modifications induced were not drastic. In the following discussion we focus on the main overrepresented processes highlighted by the enrichment analysis, in particular on genes involved in metabolism-related biological processes.

Plant based diets: effects on growth and survival

Levels of fish survival were strongly affected by the V-diet only during the first three months after first feeding. After that time, the survival rates did not differ for fish fed any of the experimental diets (M, C or V) at any of the developmental points analysed, suggesting adaptation of the fish to the plant-based diet. These results are in accordance with previous studies involving rainbow trout, in which the authors have demonstrated the remarkable ability of rainbow trout to survive and adapt to a totally plant based diet, completely devoid of marine ingredients [25]. Moreover, when we looked at body weight at the end of the first rearing period (7-month feeding trial), we did not find any significant differences between groups, and these results seem to support the hypothesis of adaptation. However, it is important to note that this first period of the trial was carried out at 7°C. Several studies in salmonids have shown that a

low rearing temperature leads to a reduced feed intake and consequently to lower growth performance [26; 27]. In addition, feed intake is also known to decrease with the dietary replacement of marine ingredients (mainly FM) with plant sources [28; 29; 30; 31]. Taken all together these results suggest that at 7°C fish fed the C and M-diets were somehow feed-restricted (depressed FI) and therefore did not express their maximum growth potential , while V-fed fish did. Moreover, when considering feed efficiency in ongrowing fish at the end of the second rearing period, we observed lower values with the V-diet (1.04), compared to the M-(1.19) and C-fed fish (1.22). Although not statistically significant ($P>0.05$) this decrease could explain the lower body weight observed at the end of the second rearing period in fish fed the V-diet.

Gene expression changes in the intestine

As the first organ in contact with feed components, the intestine, has a key role in the digestion and absorption of nutrients and may be very sensitive to dietary changes. The introduction of plant ingredients in aquafeeds has been shown to induce changes in intestinal protein metabolism in fish, and specifically to induce up-regulation of genes related to both protein synthesis and degradation when FM is replaced by plant protein [12; 14]. Our transcriptomic analysis of the intestines of juvenile trout revealed differential regulation of a certain number of genes involved in protein metabolism. Specifically, we observed global down-regulation of several genes involved in protein catabolism with the plant-based diets (C and V- diet) compared to fish fed the M-diet. Among these genes, several types of cathepsins (cathepsin-H, -L2, -S and –Z) were down-regulated in the intestine. Cathepsins are lysosomal cysteine proteases, which have important metabolic roles in the maintenance of cellular homeostasis [32; 33] . These proteases are involved in a variety of processes, and they have a wide range of functions, including intracellular protein degradation and turnover [34]. In a previous study with Atlantic salmon fed a diet in which FM was replaced by plant ingredients, the authors found up-regulation of several forms of cathepsins with the plant-based diet and associated these results with a high protein turnover because, in addition to the up-regulated cathepsins involved in protein degradation, the authors also found concurrent expression of genes involved in protein synthesis. These results are in contrast to what we observed in juveniles fed the V-diet, but are completely in agreement with the enhanced expression of CTSS and CTSZ we found in ongrowing fish fed the C-and V-diets, compared to those fed the M-diet. This supports the hypothesis of a high turnover in the

intestines of fish fed plant-based diets. However, we observed differential dietary regulation of CTSS and CTSZ in juveniles and ongrowing fish. One of the reasons could be related to the fact that intestinal protein metabolism and its capacity to respond to different types of dietary input changes during trout development. In addition, two different rearing water temperatures were used during the two periods of the present trial, and temperature is known to alter metabolic and feeding rates [27]. Fish response to dietary inclusion of plant ingredients could have been masked or altered at a low temperature and/or the turnover in the intestine could have been stimulated by a higher temperature.

Another pathway found to be highly affected by dietary replacement was fatty acid catabolism. For example, FAAH (fatty acid amide hydrolase) was found to be down-regulated in juveniles fed C-diet compared to fish of the M-fed group (FC= -18.6). On the other hand, up-regulation of this gene was found in ongrowing fish fed the C- and V-diets, compared to those fed the M-diet. FAAH is a membrane-associated protein that is localized in internal membranes, such as the endoplasmic reticulum, in which it is active. In a study with mammals, FAAH was shown to be involved in maintaining intestine physiological balance by intervening in the regulation of intestinal motility [35] . Previous transcriptomic studies on salmonids have demonstrated that plant-based diets negatively affect cellular and physiological processes, altering nutrient absorption and digestion [9; 36]. The results of the present study suggest that the intestinal physiological balance in fish fed diets containing plant ingredients is also affected in terms of motility. Specifically, our results suggest an increase in motility after a long-term feeding period at a relatively high water temperature. Indeed, possible explanations of the differences between juveniles and ongrowing fish observed in the present study could be related to the different developmental stages compared or to the differences in rearing water temperature- or to a combination of these two factors. Whatever the explanation, our study provides evidence for nutritional regulation of intestinal metabolism at the gene expression level in trout. Further investigations will be needed to improve understanding of the biological and physiological roles of FAAH in the intestine of fish.

Another aspect of metabolism in the intestine which is affected by the dietary inclusion of plant ingredients is carbohydrate metabolism. Our microarray analysis highlighted an overall down-regulation of several genes involved in carbohydrate metabolism in C and V-fed fish compared

to the M-group. Most of these changes were observed in genes encoding enzymes related to sugar digestion, such as fucosidase isoforms and mannosidase (FUCA1, FUCA2 and MAN2B1). In another study investigating the transcriptional effects of a plant-based diet in the salmon intestine, the authors reported a high diet-induced effect on genes involved in sugar degradation by plant ingredients [(9)]. Because glycolysis represents the major route of glucose catabolism and previous studies in fish have consistently demonstrated a negative effect of plant-based diets on the hepatic expression of genes involved in this pathway [(37)], we validated the expression of PFKFB3 by RT-qPCR. We observed significant down-regulation of PFKFB3 in V- and C-diets, compared to the M-diet. PFKFB3 is a powerful activator of 6-phosphofructo-1-kinase, the rate-limiting enzyme of glycolysis [(38)]. In our study, the down-regulation of PFKFB3 may have been linked to the lower levels of dietary starch in the C-diet (11.5 % of DM) and V-diet (8 % of DM), compared to that of the M-diet (20.5% of DM). Interestingly, we observed a different expression profile for this gene in ongrowing fish, with enhanced expression of PFKFB3 in C- and V-fed fish. These results seem to suggest an adaptation of fish to plant-based diets during the feeding trial.

Gene expression changes in the liver

The liver is arguably the key metabolically active tissue which responds to circulating dietary nutrients absorbed through the intestine. The present study revealed an increase in expression levels of genes involved in sterol metabolism in fish fed the V-diet. Among the genes found to be differentially expressed, lanosterol 14-alpha demethylase (CYP51A1) and 7-dehydrocholesterol reductase (DHCR7) were up-regulated in fish fed the V-diet and these results were confirmed by RT-qPCR. Moreover, this up-regulation was maintained in ongrowing fish. Our results are in accordance with previous studies carried out on Atlantic Salmon [(8)] and rainbow trout [(5)] showing higher expression levels of genes involved in the cholesterol biosynthesis pathway in fish fed plant based diets. In order to corroborate our results, we also analysed the expression of another gene, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), which is a rate-limiting step in sterol biosynthesis. In both juveniles and ongrowing trout, the hepatic expression of HMGCR was significantly increased with the V-diets, confirming previous findings [(Lazzarotto et al., submitted [(6)]. A possible explanation of the up-regulation observed in the cholesterol biosynthetic pathway might be linked to the lower dietary cholesterol provision in fish fed the V-diet. Indeed, higher

amounts of cholesterol are present in marine FM and FO than in plant-based sources [39]. Moreover, plant ingredients, particularly vegetable oils, are also rich in phytosterols [40], which can interfere with cholesterol metabolism. The marked hypocholesterolemic effect observed in the present study is in accordance with results from previous studies in various species fed diets with different rates of replacement of FM [41; 42] or FO [43]. Taken together, the molecular and physiological results of the present study suggest that rainbow trout compensate for the lower dietary cholesterol input by increasing the expression of genes involved in the cholesterol biosynthesis.

Our transcriptome findings from juvenile trout livers revealed enhanced expression of genes involved in energy pathways with the introduction of plant ingredients in diets. Overall, the V-diet induced increases in the expression of genes involved in mitochondrial metabolism, especially the electron transport chain and oxidative phosphorylation (e.g. ATP synthase components ATP5B and ATP5C1). Additionally, in the transcriptomic analysis expression of malate dehydrogenase-2 (MDH2), a key mitochondrial component of the Krebs cycle, was increased in V-fed fish compared to the other groups. However, RT-qPCR validation failed to confirm this expression pattern. These results are in contrast to those reported in the livers of trout fed vegetable oils [13]. Similarly, the expression of two genes involved in electron transport, (COX5B and COX7A2L) was significantly different between experimental groups in the microarray analysis ($p=0.038$ and $p=0.027$, respectively), while no differences were confirmed in either juveniles or ongrowing fish by RT-qPCR. For all three genes the FC detected by microarray analysis were very small ($FC < 2$). The lack of differences in gene expression between groups observed by RT-qPCR analysis may reflect the small diet-induced effect. Moreover, it is important to bear in mind that a total match between the microarray and the RT-qPCR results should not be expected due to the approach taken to design RT-qPCR primers which do not necessarily exactly match the probe on the array, as previously observed in a study on Atlantic salmon liver [44]. Indeed, due to the genome duplication (4G) that occurred in salmonids[45], transcriptomic and gene expression studies are often more challenging due to the presence of duplicated and highly similar genes whose transcripts might be differentially regulated. The transcriptional effects observed in response to the introduction of plant ingredients in the diets are therefore difficult to validate. Overall, any conclusions drawn from

the present study need to be further validated in order to reach reliable conclusions regarding the biological consequences of these molecular-level changes.

Gene expression changes in both intestine and liver

Previous studies investigating the gene expression response of fish after dietary replacement of FM and/or FO by plant-ingredients have shown that, irrespective of the oil used, lipid metabolism and in particular fatty acid bioconversion are highly affected in both the liver and intestine [8; 17; 44; 46; 47]. These two tissue types are known to be central actors in FA bioconversion in fish. In the present study, gene expression related to lipid metabolism, particularly LC-PUFA biosynthesis, was found to be up-regulated in both the livers and intestines of fish fed the V-diet, compared to the other experimental groups. For example, polyunsaturated fatty acid elongase-2 (Elovl2) was up-regulated in the intestines and livers of juveniles fed the V-diet and this up-regulation was maintained in ongrowing fish. In order to corroborate our results, we also investigated the expression of two other key genes of the LC-PUFA biosynthesis pathway (Elovl5 and Δ6-desaturase). Both genes have key roles in fatty acids bioconversion in the liver and intestine [48; 49]. Δ6-desaturase was up-regulated in fish fed the V-diet in both tissue types analysed. These results are in accordance with previous studies [21; 50; 51], confirming the capacity of salmonids to synthesize LC-PUFA from dietary C18 precursors. Intestinal and hepatic expression of Elovl5 did not significantly change in response to diets in either juveniles or ongrowing fish. These results are somewhat surprising, given the importance of this elongase in LC-PUFA biosynthesis, in particular in the elongation from C18 to C22 [52]. On the other hand, it is known that both Elovl5 and Elovl2 have roles in the elongation of C18 into longer C-chains. One difference lies in the fact that Elovl5 does not have the capacity to elongate beyond C22 [53]. Given the crucial importance of DHA in fish, it can be hypothesized that the higher expression of Elovl2 in fish fed the V-diet is linked to preference given to the biosynthesis of DHA, rather than EPA. Moreover, it has been proven that EPA can also represent a substrate for DHA production [54]. This enhanced biosynthesis of DHA is confirmed by the higher percentages of DHA (2% of total FA) we found in whole body juveniles and ongrowing fish, compared to EPA (1% of total FA).

In addition, we observed an increase in the quantities of EPA+DHA (g $fish^{-1}$) at both stages of our feeding trial, from juveniles to ongrowing, supporting evidence of the capacity of rainbow trout to synthesize LC-PUFA from dietary precursor. However, as previously demonstrated [55], this biosynthesis was not sufficient to compensate for the lack of provision of dietary n-3 LC-PUFAs. This was reflected by the whole body FA profile, which mirrored the composition of the different diets. Consequently, lower proportions of EPA and DHA were found in body lipids of juveniles and ongrowing fish fed the V-diet.

This study showed that long-term feeding trout with a totally plant-based diet from first feeding onwards has slight effects on metabolism, as shown by the relatively low proportion of metabolism-related genes found to be differentially expressed in the intestine and liver transcriptome of juveniles. Our finding largely confirmed previous studies performed during a shorter feeding period, especially regarding changes in the expression of genes involved in the bioconversion of cholesterol and fatty acid. Our study therefore showed that these dietary-induced molecular and biochemical changes in lipid metabolism were maintained in the longer term. Our results also characterize new molecular actors affected by the nutritional stress induced in the fish intestine by introducing plant ingredients in diets for rainbow trout, especially genes involved in intestinal motility. The results provide a framework for further studies aimed at reducing the reliance of aquaculture on marine fishery resources by developing new alternative plant-based feeds.

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Conflict of interest

None.

Authorship

GC, FM: formulated research questions and designed the study. VL, LL, FM, GC: performed the experiments. VL, FM, GC: analysed the data. VL FM GC: wrote the paper.

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Supplementary Material.
Supplementary Table 1. Impact of dietary treatments on the intestinal transcriptome of juveniles. Genes tested by RT-q PCR are in bold.

			Fold Change (FC)			***Significance***
Probe Name	**Gene Symbol**	**Description**	***C vs M***	***V vs M***	***V vs C***	***p-value***
Protein catabolism						
TC99247	CTSH	cathepsin H	- 5.1	- 2.6	+ 2.0	*0.035*
CUST_68_PI425708691	CTSL2	cathepsin L2	- 11. 1	- 4.0	+ 2.8	*0.041*
CUST_8157_PI425536763	**CTSS**	**cathepsin S**	**- 11.0**	**- 3.8**	**+ 2.9**	***0.041***
CUST_24029_PI425536763	**CTSZ**	**cathepsin Z**	**- 5.7**	**- 2.9**	**+ 2.0**	***0.013***
TC106655	DPP7	dipeptidyl-peptidase 7	- 2.2	- 2.1	+ 1.0	*0.036*
CUST_20321_PI425536763	FOLH1	folate hydrolase	- 3.9	- 1.8	+ 2.2	*0.041*
TC110997	LGMN	legumain	- 9.0	- 4.5	+ 2.0	*0.041*
CUST_25677_PI425536763	ENPEP	glutamyl aminopeptidase (aminopeptidase A)	-13.8	- 1.9	+ 7.2	*0.041*
Carbohydrate metabolism						
CUST_21158_PI425536763	MAN2B1	mannosidase, alpha, class 2B, member 1	- 7.1	- 5.4	+ 1.3	*0.012*
CUST_17398_PI425536763	FUCA1	fucosidase, alpha-L- 1, tissue	- 4.4	- 2.8	+ 1.5	*0.035*
CUST_12758_PI425536763	FUCA2	fucosidase, alpha-L- 2, plasma	- 8.3	- 3.8	+ 2.2	*0.025*
TC114862	GLB1	galactosidase, beta 1	- 11.4	- 3.5	+ 3.3	*0.016*
TC104967	NAGA	N-acetyl galactosaminidase, alpha	- 11.2	- 3.8	+ 3.0	*0.042*
TC108468	NEU1	neuraminidase 1	- 25.8	- 7.2	+ 3.6	*0.025*
TC123951	**PFKFB3**	**6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3**	**- 2.8**	**- 3.0**	**+ 1.1**	***0.042***
Apoptotic process/immune response						
CUST_14242_PI425536763	MPO	myeloperoxidase	-1.5	- 4.9	- 3.3	*0.035*
CUST_7188_PI425536763	BAD	BCL2-associated agonist of cell death	- 2.3	- 1.3	+ 1.8	*0.045*
TC104795	MAP3K7	mitogen-activated protein kinase kinase kinase 7	- 1.1	- 1.5	+ 1.6	*0.049*

Supplementary Table 1 (*Continued*)

Probe Name	Gene Symbol	Description	Fold Change (FC)			*Significance*
			C vs M	***V vs M***	***V vs C***	***p-value***
Fatty acid catabolism						
TC94736	CPT1A	carnitine palmitoyltransferase 1A (liver)	+ 1.4	+ 1.0	- 1.4	*0.045*
TC121737	**FAAH**	**fatty acid amide hydrolase**	**- 18.6**	**- 3.0**	**+ 6.2**	***0.041***
Transcription and activation						
TC125816	PQBP1	polyglutamine binding protein 1	+ 1.3	- 1.1	- 1.4	*0.042*
Miscellaneous						
CUST_17716_PI425536763	MXD4	Max dimerization protein 4	- 1.7	- 1.6	+ 1.1	*0.025*
CUST_1243_PI425536763	POLR2F	polymerase (RNA) II (DNA directed) polypeptide F	+ 1.4	- 1.1	- 1.5	*0.039*
TC118891	PRPSAP2	phosphoribosyl pyrophosphate synthetase-associated protein 2	+ 1.4	- 1.0	- 1.4	*0.025*
CUST_9923_PI425536763	TTC4	tetratricopeptide repeat domain 4	+ 1.8	+ 1.5	- 1.2	*0.039*
CUST_6882_PI425536763	RENBP	renin binding protein	- 4.4	- 2.5	+ 1.8	*0.036*
TC95545	ASAH1	N-acylsphingosine amidohydrolase 1	- 2.6	- 1.9	+ 1.4	*0.041*
CUST_15445_PI425536763	ASH2L	ash2 (absent, small, or homeotic)-like	- 2.2	- 1.7	+ 1.3	*0.049*
Molecular function						
CUST_17473_PI425536763	ABCC12	ATP-binding cassette, sub-family C (CFTR/MRP), member 12	- 2.1	- 2.1	+ 1.0	*0.049*
TC108892	ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	- 5.0	- 2.0	+ 2.5	*0.039*
TC104435	ARHGAP12	Rho GTPase activating protein 12	- 2.1	- 1.4	+ 1.5	*0.049*
CUST_6855_PI425536763	ATP2C1	ATPase, Ca++ transporting, type 2C, member 1	- 4.0	- 3.6	+ 1.1	*0.029*

Supplementary Table 1 (*Continued*)

Probe Name	Gene Symbol	Description	Fold Change (FC)			*Significance*
			C vs M	***V vs M***	***V vs C***	***p-value***
Molecular function (continued)						
CUST_18236_PI425536763	ATP6V0A1	ATPase, H+ transporting, lysosomal V0 subunit a1	- 3.2	- 1.4	+ 2.2	*0.043*
CUST_5593_PI425536763	ATP6V0C	ATPase, H+ transporting, lysosomal 16kDa, V0 subunit c	- 1.9	- 1.7	+ 1.1	*0.039*
TC112228	CDKN1B	cyclin-dependent kinase inhibitor 1B	+ 4.8	+ 1.5	-3.2	*0.046*
TC117615	CUBN	cubilin (intrinsic factor-cobalamin receptor)	- 45.4	- 5.0	+ 9.0	*0.042*
CUST_21136_PI425536763	SLC16A1	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	+ 1.9	- 1.4	- 2.6	*0.049*
CUST_7347_PI425536763	SNX8	sorting nexin 8	- 2.6	- 1.7	+ 1.5	*0.042*
TC104334	TTN	titin	- 2.7	- 1.4	+ 1.9	*0.045*
Cellular component						
CUST_23323_PI425536763	ADCY1	adenylate cyclase 1	- 1.8	- 1.6	+ 1.1	*0.042*
TC100687	PSAP	prosaposin	- 1.8	- 1.6	+ 1.1	*0.025*
TC128912	RPS14	ribosomal protein S14	- 2.3	- 2.1	+ 1.1	*0.048*
TC107355	SNAP23	synaptosomal-associated protein 23	- 2.0	- 1.2	+ 1.7	*0.048*
CUST_5785_PI425536763	VPS41	vacuolar protein sorting 41 (yeast)	- 1.9	- 1.3	+ 1.4	*0.030*
CUST_21742_PI425536763	ST7	suppression of tumorigenicity 7	+ 1.3	- 1.2	- 1.5	*0.046*

Supplementary Table 2. Impact of dietary treatments on the hepatic transcriptome of juveniles. Genes tested by RT-q PCR are in bold.

			Fold Change (FC)			***Significance***
Probe name	**Gene Symbol**	**Description**	***C vs M***	***V vs M***	***V vs C***	***p-value***
Biological process						
Lipids/Cholesterol Metabolism						
CUST_14393_PI425536763	**Elovl2**	**polyunsaturated fatty acid elongase**	**+ 1.4**	**+ 2.6**	**+ 1.8**	***0.049***
TC130473	**CYP51A1**	**cytochrome P450, family 51, subfamily A, polypeptide 1**	**+ 2.3**	**+ 4.1**	**+ 1.8**	***0.046***
TC130143	**DHCR7**	**7-dehydrocholesterol reductase**	**+ 2.3**	**+ 3.8**	**+ 1.6**	***0.049***
CUST_9914_PI425536763	TM7SF2	transmembrane 7 superfamily member 2	+ 2.6	+ 5.7	+ 1.8	*0.030*
Energy pathways						
CUST_21841_PI425536763	ATP5B	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit	- 1.0	+ 1.5	+ 1.5	*0.030*
CUST_20841_PI425536763	ATP5C1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	+1.1	+ 1.4	+ 1.3	*0.040*
CUST_11055_PI425536763	**MDH2**	**malate dehydrogenase 2, NAD (mitochondrial)**	**+1.0**	**+ 1.7**	**+ 1.7**	***0.030***
TC114386	UQCRC1	ubiquinol-cytochrome c reductase core protein I	-1.0	+ 1.7	+ 1.7	*0.030*
Electron Transport						
TC105004	**COX5B**	**cytochrome c oxidase subunit Vb**	**-1.2**	**+1.4**	**+ 1.6**	***0.038***
TC99046	**COX7A2L**	**cytochrome c oxidase subunit VIIa polypeptide 2 like**	**-1.2**	**+1.5**	**+ 1.9**	***0.027***
Molecular function						
TC100568	ACTN3	actinin alpha 3a	- 1.4	- 3.6	- 2.5	*0.027*
TC103820	CSRP1	cysteine and glycine-rich protein 1a	+ 1.0	- 2.2	- 2,3	*0.046*
CUST_8095_PI425536763	DBI	diazepam binding inhibitor (GABA receptor modulator, acyl-CoA binding protein)	+ 1.4	+ 2.3	+ 1.6	*0.030*
CUST_10542_PI425536763	DRG1	developmentally regulated GTP binding protein 1	- 1.5	+ 1.5	+ 2.2	*0.027*
CUST_21649_PI425536763	NDUFA9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9	+ 1.1	+ 1.5	+ 1.4	*0.046*

Supplementary Table 2 (*Continued*)

			Fold Change (FC)			***Significance***
Probe name	**Gene Symbol**	**Description**	***C vs M***	***V vs M***	***V vs C***	***p-value***
TC102108	RAE1	RNA export 1 homolog (S. pombe)	- 1.2	+ 1.3	+ 1.5	*0.030*
Macromolecule biosynthesis						
TC100227	DENR	density-regulated protein	- 1.3	+ 1.3	+ 1.7	*0.049*
TC95842	EIF5	eukaryotic translation initiation factor 5	- 1.4	+ 1.4	+ 2.0	*0.033*
CUST_7899_PI425536763	MRPL3	mitochondrial ribosomal protein L3	- 1.2	+ 1.3	+ 1.5	*0.030*
CUST_3849_PI425536763	MRPS18B	mitochondrial ribosomal protein S18B	- 1.4	+ 1.2	+ 1.6	*0.030*
Cellular component						
CUST_17684_PI425536763	SEC23IP	SEC23 interacting protein	- 1.4	+ 1.5	+ 1.4	*0.036*
TC112917	NCOA4	nuclear receptor coactivator 4	+ 2.4	- 2.1	- 5.1	*0.027*

Chapter 4

General discussion

4.1 Discussion

The experiments undertaken in this PhD thesis were designed to study the effects of concomitant dietary replacement of both FM and FO by plant ingredients (up to 100% plant-based diet) during the whole life cycle of rainbow trout. Specifically, we focused on female reproduction and potential intergenerational effects, which have never been studied before. Moreover, we also studied the long-term effects of experimental diets with different proportions of FM, FO and plant ingredients (0 to 100%) distributed since the first feeding on survival, growth and different aspects of trout metabolism, including gene expression in two target tissues: intestine and liver. The idea behind this experimental design was to determine if trout fed with plant-based diets since first feeding developed adaptation mechanisms that were stable at long term and that enable them to efficiently use plant-based diets later during their life cycle and in different rearing conditions.

While a detailed discussion of the results can be found in the appropriate section of this thesis, the following section contains a comprehensive discussion outlining how these findings have answered the initial questions and hypotheses and the knowledge they provide in the field of the reduction of marine resources incorporation in rainbow trout diets. The section is organized according to the specific research questions, as described in the introduction of the thesis, and concludes with future research recommendations based on the findings presented in this thesis.

For practical reasons, the following denominations are used for specific diets in this discussion, in order to facilitate the clear distinction between broodstock diets and progeny diets distributed from first feeding:

VEG: completely-plant based broodstock diet; *COM*: commercial broodstock diet (mix of marine and plant ingredients);

V: completely plant-based diet at first feeding; *C*: commercial-like diet at first feeding (mix of marine and plant ingredients); *M*: FM/FO-based diet at first feeding.

Are female rainbow trout able to survive, grow and reproduce when reared on a totally plant-based diet from first feeding and during the whole life cycle? Which effects -if any- does this plant-based diet have on ova and offspring?

Female trout are able to survive, grow and reproduce when reared from first feeding and during a 3-year life cycle on a totally plant based diet

One major result of the present PhD thesis is the first demonstration, that rainbow trout being fed a plant-based diet totally devoid of marine resources can achieve a 3-year breeding cycle including two spawning events. This clearly indicates that female rainbow trout can be grown and reach reproductive capacity in timeframes suitable for rainbow trout aquaculture operations, without any dietary supply of FM and FO.

With regard to reproductive success, we observed some negative effects of the VEG-diet with quite high mortality at the first spawning event and lower ova weight at both spawning events. The survival rate of progeny from VEG-fed females was almost twice lower than that of progeny from COM-fed females at the first spawning event. Furthermore there was a high variability in survival rates of progeny from VEG-fed broodstock, which was not observed for progeny from COM-fed females. However, this negative impact of the plant based diet free from marine resources was not observed at the second spawning event, suggesting a potential adaptation of female broodstook to the plant-based diet. Egg quality of the first spawn in the trout life is generally considered lower compared to the subsequent spawning events, whatever the broodstock diet. Besides, for this reason, it is a common practice in trout aquaculture not to utilize the first spawn for reproduction operation. In our experiment it seems that this poor quality was still reinforced by feeding females with the VEG-diet.
Diet-induced differences were found in the weight of the ova at both, the first and second spawning event: eggs produced by VEG-fed broodstock were lighter (-17% at first spawning and -13% at the second one) compared to eggs produced by COM-fed broodstock.

Pereira et al. (1998), who studied reproductive performance of female trout fed diets with different protein sources, pointed out strongly reduced feed intake in fish fed a diet made of plant protein sources and devoid of fish meal. This reduction in feed intake impairs energy balance and may limit energy allocation to reproductive processes, thus resulting in poor reproductive

performance. On the basis of Pereira's results, we designed our experiment in order to avoid the effect of differences in feed intake. We choose to feed the VEG-females ad libitum, while the COM-fed females were given the same food ration as the VEG-fed females, in order to assess the effect of feed composition, and not that of the amount of feed intake on reproductive performance. The fact that the VEG-fed trout were able to accumulate high amount of lipids in the perivisceral adipose tissue, the primary source of energy for vitellogenesis in trout according to Manor et al. (2012), supports the hypothesis that the food-intake of VEG-fed trout was sufficient to allow them to store reserves and to enable gonad formation, albeit at a lower level, as suggested by the lower weight of ova and the lower gonadosomatic index (GSI).

One of our questions was related to the qualitative aspect of the dietary supply in terms of FA and its impact on reproductive success. While general energy intake and lipid deposition appear adequate in both COM- and VEG-diet fed fish, qualitative differences in FA supply exist because of the different composition of the dietary oils sources. It is known that the FA profile plays a particularly important role in egg quality and offspring survival (Izquierdo, Fernandez-Palacios et al. 2001). Therefore, in order to assess the reproductive success in the light of the FA profile, specifically EPA, DHA, and ARA, we analyzed the consequences of the diets on FA profiles of maternal body compartments (digestive tract, liver, ova and carcass *i.e.* the remaining part of the fish) at the time of ova collection, as well as in the eggs and swim-up fry.

Dietary FA profile affects fatty acid profile in maternal broodstock tissues, ova and offspring.

The FA profile of female broodstock tissues globally reflected that of the respective diets. Increasing levels of MUFA, 18:2 n-6, 18:3 n-3, and a concomitant decrease in n-3 LC-PUFA (EPA and DHA) were found in tissues of females fed the VEG-diet, confirming previous results from shorter-term studies (Benedito-Palos, Navarro et al. 2008, Torstensen, Espe et al. 2011, Matos, Gonçalves et al. 2012). However, in spite of a complete absence of dietary intake of EPA and DHA since first feeding, measurable concentrations of EPA and DHA were found in all maternal tissues, albeit in higher proportions in the liver and the ova. This indicates that female rainbow trout maintained on an exclusively plant-based diet across an entire life cycle were capable to synthesize EPA and DHA from a dietary precursor (18:3 n-3), and to store synthesized EPA and DHA in specific tissues. This data confirms the previously described enhanced capacity of salmonids to synthesize LC-PUFA when fed diets containing vegetable oils

i.e. when dietary supply of LC-PUFA was a low or null (Tocher 2003, Betancor, Sprague et al. 2015). However, this bioconversion was not sufficient to fully compensate for the total absence of these two FA in the VEG diet, as demonstrated by the lower levels of EPA and DHA found in VEG-fed female trout compared to the COM-fed counterparts.

Higher concentrations of EPA and DHA were measured in ova compared to maternal tissues, indicating active transfer of the n-3 LC-PUFA neo-synthesized in the liver specifically towards the ova. These results are in accordance with previous studies showing that elevated levels of EPA and DHA are present in fish eggs (Tocher and Sargent 1984, Izquierdo, Fernandez-Palacios et al. 2001), supporting their important role in eggs and subsequent offspring development. n-3 LC-PUFAs are stored as reserves for potential use during development (Tocher 2003) or are catabolized for energy production after hatching (Rønnestad, Koven et al. 1998). In addition, they play an important role as components of structural phospholipids (Sargent 1995) assuring fluidity of cell membranes.

Interestingly, we observed that the decreases in proportions of EPA and DHA resulting from the VEG-diet in comparison with the COM-diet were much less pronounced in the ova than in the maternal tissues (especially the digestive tract and carcass) even in neutral lipids. Such data suggest that VEG-fed female broodstock efficiently prioritize the storage of EPA and DHA in the ova.

Nevertheless, the EPA and DHA concentrations remained significantly lower in progeny of VEG-fed females, from both the first and second spawning event, and EPA and DHA levels remained consistent within each experimental group between spawning events. Because similar reproductive success of offspring was observed with both dietary treatments at the second spawning event, our data suggest that reproductive success is not limited by the EPA and DHA levels in ova from VEG-fed broodstock or at least, that sufficient amounts of EPA and DHA were transferred to ova to enable development of viable embryos and fry, especially at the second spawning event.

In contrast, our data provides evidence for a limiting effect of egg weight in this experiment: while eggs from VEG-fed females remain significantly smaller compared to eggs from COM-fed females, an increase in egg weight is observed in both groups at the second spawning event. This suggests that a minimum egg weight is required for successful progeny survival and

development. At the second spawning event, eggs from VEG-fed progeny attained approximately the same weight as eggs from COM-fed females at the first spawning event. This might also reflect a temporal adaptation of the female broodstock to the plant diet, resulting in larger eggs in the second spawning event.

Does maternal diet history affect alevin survival, and its response to different diets at first feeding? What are the underlying molecular mechanisms at the transcriptional level?

The first experiment demonstrated that broodstock can be fed on an exclusively plant- based diet across their life cycle, and furthermore synthesize and allocate sufficient amounts of EPA and DHA to developing eggs to allow fry survival. The second experiment extended the investigation to intergenerational effects, by quantifying potential maternal effects on early offspring ontogenesis (prior to first feeding), and exploring the concept of nutritional programming through maternal dietary history. We hypothesized, that feeding broodstock with a totally plant-based diet would endow the progeny with a higher capacity to positively respond to a plant-based diet. We investigated several parameters, which are key in aquaculture production: survival and body weight of fish, as well as FA composition, especially EPA and DHA proportions, and changes in gene expression, as indicators of pathways impacted by the dietary treatments.

Effects of broodstock nutritional history on progeny survival and body weight

Feeding broodstock a totally plant-based diet did not result in any effects in survival of offspring developing from the second spawning event, either before first feeding, or after 3 weeks of feeding. However, we did observe differences in the alevins body weight. Indeed, just prior to first feeding, the body weight of fry originating from females fed the VEG-diet displayed a significantly lower body weight (-13%), compared to those originating from COM-fed females. This difference is likely the result of smaller sized eggs produced by females fed the plant-based diet. Smaller eggs, in turn, may lead to smaller offspring, as previously demonstrated (Kamler 2005). We therefore hypothesize that this difference in terms of weight is the reflection of a certain “fragility”, essentially derived from broodstock nutritional history. Three weeks after first feeding, broodstock diet history still significantly affected alevins’ body weight, with a lower body weight of progeny from VEG-fed broodstock (VEG<COM). Additionally, first feeding diets also significantly affected body weight of alevins after the first three weeks of feeding (M >

C > V). Body weight data therefore do not support our initial hypothesis that feeding females with a plant-based diet could endow the next generation with a higher capacity to positively respond to such plant-based diet.

Effects of broodstock nutritional history on progeny whole body transcriptome

Since no studies carried out to date had investigated the mechanisms of biological responses of offspring issued from female broodstock fed diets with different amounts of plant ingredients as substitutes for FM and FM, there was a gap in knowledge regarding the potentially affected pathways. This is the reason why we applied a transcriptomic approach on whole alevins to elucidate the effects of broodstock nutritional history and first feeding diets.

Gene expression changes related to broodstock dietary history

Surprisingly, broodstock nutritional history had no visible effects on the transcriptome of whole body alevins collected just prior to first feeding. Therefore, our hypothesis of increased fragility as a result of feeding broodstock with the totally plant-based diet was not supported at the level of gene expression in alevins prior to first feeding. In contrast, significant effect of maternal nutritional history emerged in alevins after 3 weeks of feeding, whatever the first feeding diet. The fact that no significant interaction between broodstock dietary history and first feeding diets was observed at the molecular level is in agreement with the findings at the level of phenotype (body weight).

The presence of a significant maternal effect in alevins after 3 weeks of feeding, suggests life-stage-dependent effects of broodstock dietary history. One potential explanation is that maternal effects may be masked in swim-up fry before exogenous feeding because the offspring are still dependent on endogenous feeding from the vitellus, while this effect emerges once alevins transit to exogenous feeding. This period is critical for early stage-development, because gene expression is altered through the activation of specific metabolic pathways enabling the utilization of exogenous feeds by fish (Mennigen, Skiba-Cassy et al. 2013, Bicskei, Bron et al. 2014).

Advantages and limitations of using a transcriptomic approach

The transcriptomic approach is an integrative research tool of nutrigenomics studies. Nutrigenomics is a science still in its infancy, but is defined as studying dietary effects on the transcriptome. In nutrigenomics, nutrients are considered as signals through which cells interpret information about the environment (diet) and respond, according to necessity, by modifying metabolic pathways through regulation of gene and protein expression (Müller and Kersten 2003). However, as well as any other approach, transcriptomic analysis has both advantages and disadvantages, particularly when it is used to analyze entire organisms rather than differentiated and functionally distinct tissues. When analyzing entire trout fry, transcriptional effects related to a specific pathway, which may be specifically, rather than ubiquitously, active in different tissues, could be diluted and therefore potentially meaningful transcriptional effects in specific tissues can be masked. Moreover, a common difficulty when using transcriptomic tools, is that even low fold changes can mean significant alterations at a biological levels and therefore the interpretation of the results is sometimes complicated. On the other hand, nutrigenomics provides an overview of diet-induced molecular changes, in connection with physiological responses, that occur when dietary ingredients are changed (De Santis, Crampton et al. 2015). The main advantage of this approach is that it is without any *a-priori* hypothesis. It can therefore serve to identify new molecular actors and pathways, underlying responses at the whole organism level (*i.e.* survival, growth) and biochemical level (*i.e.* FA profiles). Although regulation of transcription initiation is considered to be the most important step in gene control in eukaryotes, it is known that there are also regulatory processes after the mRNA (Adeli 2011, Vogel and Marcotte 2012). We understand that the expression of biologically active proteins is controlled at multiple points (*i.e.* chromatin structure, initiation, processing and modification of mRNA transcripts, etc., translation, posttranscriptional modifications), and that the differences in terms of mRNA levels as proxies for the concentration/activities of the corresponding proteins may not always reflect the functional biological process. However, the main mechanism by which animals adapt to their nutritional environment is the regulation of gene expression by nutrients (Girard, Ferré et al. 1997).

The significant gene expression changes related to broodstock dietary history reflect delayed growth observed at the phenotypic level (body weight). Specifically, transcriptomic responses, especially the muscle specific transcripts detected in entire alevins are suggestive of delayed growth in offspring stemming from VEG-fed broodstock. We found global significant down-regulation of different major actors of muscle development and contraction, such as CKM, CKB, myomesins, as well as ACTA1 and MYH2 in progeny originating from VEG-fed broodstock. This down-regulation points to altered muscle growth as a major factor of lower body weight. In a previous study investigating gene expression patterns during larval development of European sea bass, the authors described a progressive increase in the expression of muscle-related genes across development (Darias, Zambonino-Infante et al. 2008). These data seem to confirm our hypothesis of a delayed growth, largely mediated by muscle development, which is also reflected by the lower whole body weight observed in alevins.

Effects of first feeding diets on whole body transcriptome of alevins

Among the significant changes in gene expression related to first feeding diets, a down-regulation of muscle-specific transcripts was also observed in groups fed the diets containing plant ingredients (C-V *vs* M). Together, the significantly decreased expression of muscle related transcripts in response to both, maternal dietary history (VEG), and first feeding diets (C-V), shows that inclusion of plant ingredients in either broodstock or alevin diets may lead to deregulation of muscle growth. In other words, the dietary inclusion of plant ingredients has negative consequences on alevin development, as reflected by decreased expression of muscle related genes, irrespective of whether broodstock or alevins themselves were reared on these diets. However, the fact that the same muscle related target genes (*i.e.* ACTA1, ACTN2) were significantly decreased in expression when considering each factor (broodstock or alevin diets), suggests the observed changes are possibly a reflection of the lower body weight phenotype. Indeed, mechanistically important gene expression changes may have been masked by the strong effect of muscle growth, related to both factors.

With regard to specific metabolic pathways, a general decrease in the expression of several transcripts related to carbohydrate metabolism and energy pathways was observed in alevins from VEG-fed females after 3 weeks of feeding. Genes involved in carbohydrate metabolism, which were significantly down-regulated in alevins from VEG-fed broodstock, included

glycolytic enzymes (*i.e.* 6PFKM, GPI), glycogen metabolism (*i.e.* PYGL, PYGM, PHKA1, PGM1) and Krebs cycle (*i.e.* MDH1, SDHA, FH). This suggests a significant decrease of major energy generating pathways in alevins from VEG-fed females after 3 weeks of feeding.

When considering effects of first feeding diets, subtle yet significant increases in gene expression of several t-RNA synthetases and protein translation related transcripts were observed with introduction of plant ingredients in the diet, particularly in V-fed alevins. These observations suggest that, translation processes are increased in V-fed alevins, compared to the other groups, potentially reflecting the initiation of catch-up growth after 3 weeks of feeding. This is reflected in terms of body weight, when considering its differential development between dietary groups until the juvenile stage. While V-fed fish exhibited a significantly lower body weight in the first 3 months, this difference disappeared after four months and remained stable until the end of the 7-month feeding trial.

Overall, our results suggest that while feeding broodstock a totally plant-based diet resulted in viable egg production, as previously discussed, some effects are present in progeny, which depend on either maternal dietary history or diets at first feeding. These effects indicate a developmental delay, which can be a cause or a consequence but they seem not to be highly deleterious, at least at 7 °C, as juveniles of all groups reached similar body weight of 10g at the end of the 7-month feeding trial.

Effects of broodstock nutritional history and first feeding diets on progeny fatty acid bioconversion and composition

Feeding broodstock with the VEG-diet resulted in significantly higher levels of 18:2 n-6 and 18:3 n-3, and lower EPA and DHA in alevins before and after first feeding. These observations indicate a persisting effect of maternal nutritional history on alevins FA profile until at least 3 weeks after first feeding. As well, first feeding diets significantly influenced precursors and EPA and DHA fatty acid profile. Generally, precursors (18:2 n-6 and 18:3 n-3) levels were highest in V-fed alevins, followed by C-fed and M-fed alevins (V>C>M). Conversely, EPA and DHA concentration were highest in M-fed alevins, followed by C-fed and V-fed alevins (M>C>V) irrespective of the maternal origin of the alevins. The absence of interaction between nutritional history of broodstock and the responses of the alevins to the composition of the first feeding diets

in terms of EPA and DHA deposition suggests that feeding female broodstock with a plant-based diet does not endow the progeny of a higher capacity to synthesize EPA and DHA when fed a diet free from such n-3 LC-PUFA.

Furthermore, the transcriptomic analysis of entire alevins did, somewhat surprisingly, not reveal any changes in the expression of genes implicated in the LC-PUFA biosynthesis pathway before and after 3 weeks of feeding. This lack of differential expression may be linked to the sampling time: the machinery for elongation/desaturation may become functional later on during ontogeny of rainbow trout. This hypothesis however seems less likely in the light of recent findings, obtained by Monroig et al. (2014) who addressed the question of ontogenetic expression of elongases and desaturases which is poorly documented in fish. The authors provided evidence for the expression of relevant desaturases and elongases during zebrafish development. While these data suggest that fish may be able to biosynthesize LC-PUFA in early development, one has to keep in mind that fish are a phylogenetically diverse group. Specifically, zebrafish development occurs at a faster rate that rainbow trout development. Therefore, the situation in salmonids remains uncharacterized and should be addressed by future studies.

Another possibility may be the fact that female broodstock were able to transfer sufficiently high amounts of LC-PUFAs to the ova (as discussed above) to not only allow embryonic development until hatching, but also fulfill nutritional requirements during the 3 weeks post hatching. This hypothesis is supported by the decrease in the amounts of EPA and DHA between the eggs, the 'swim-up fry before first feeding' stage and the 'alevins after 3 weeks of feeding' stage (Table 4.1).

Table 4.1 Amounts of EPA+ DHA (mg egg^{-1} or mg alevin-1) across rainbow trout ontogeny.

	Σ EPA+DHA			
	Eggs	before 1st feeding		3 weeks feeding
			Diets	
Progeny-COM	7.8	2.6	Diet-M	3.2
			Diet-C	2.0
			Diet-V	1.2
Progeny-VEG	2.3	1.4	Diet-M	2.6
			Diet-C	1.6
			Diet-V	0.9

Long- and very-long term plant-based feeding trials since first feeding in rainbow trout: are there any major diet-induced changes on survival and body weight? Do plant-based diets alter intestinal and hepatic gene expression? If any, are these effects maintained over the time?

V-diet induced effects on survival and body weight at long term

The importance of running long-term feeding trials, especially when high inclusion levels of alternative ingredients are being evaluated, had been pointed out by Torstensen et al. (2008). For the long-term feeding experiment, we focused on the progeny of the COM-fed females only. With regard to mortality, no differences were observed between dietary groups after 3 weeks of feeding. However, after this first period, we observed a high mortality in the V-fed fish, until the third month of feeding, compared to the other groups. These observations suggest that, during the first 3 weeks of rearing, the alevins are still using the reserves transferred by mother in the ovum (vitellus). Considering survival later on, between 4 and 7 months, survival rates stabilized without significant differences between dietary treatments.

With regard to body weight, V-fed alevins exhibited a significantly lower body weight, compared to the C and V-fed alevins (V<C<M) after 3 weeks of feeding. This difference disappeared after 3 months of feeding, and was no longer evident in juveniles at the end of the 7-month feeding trial.

Together, survival and body weight data suggest that V-fed fish may have undergone a selection process. Indeed, it is possible that only alevins with higher capacity for adaptation to the V-diet survived during the first 3 months of feeding. Subsequent survivors in the V-group were then able to reach the same body weight (~10 g fish) as fish fed the other diets containing marine ingredients.

Temperature-dependent effects of long-term feeding with the V-diet on fish body weight

For the very long-term feeding trial (7-13 months), fish were transferred to another experimental fish facility, where groups were maintained on diets with the same proportions of FM, FO and plant ingredients, albeit at a higher water temperature of 17 °C. This temperature allows fish to express their maximum growth potential. No difference in survival rates between dietary groups was observed during this rearing period.

Contrarily to what we observed after 7 months feeding at 7 °C, at the end of the trial at 17 °C, fish fed the V-diet had a significantly lower body weight (-30%) compared to the other experimental groups. These results partially allowed us to reject general validity our first hypothesis of nutritional programming, as the adaptation was no longer evident at this later time-point at higher water temperature.

It has been observed in several studies with rainbow trout fed plant-based diets that lower body weight is mainly related to reduced feed-intake (Pereira, Reis-Henriques et al. 1998, Geurden, Cuvier et al. 2005, Geurden, Corraze et al. 2007, Le Boucher, Vandeputte et al. 2013). This was not the case in the present experiment. One possible explanation is that lower water temperature results in lower metabolic rates, which in turn limit the dietary intake in C and M-fed trout, while V-fed trout already exhibit their maximum growth potential. At a higher temperature (17 °C), nutrients supply by the V-diet did not sustain maximum growth potential. This hypothesis is supported by the lower values of feed efficiency in the V-fed group during the trial period 7-13 months at 17 °C.

Molecular effects: lipid metabolism

Considering the long-term effects of including plant ingredients in feeds for rainbow trout, some metabolism-related gene expression changes occurred in intestine and liver of fish after 7 months of feeding. In the intestine, relatively minor changes in metabolic gene expression were observed, suggesting few consequences on intestinal metabolism with plant-based diets. In the liver, clear-cut changes were observed with regard lipid metabolism gene expression, with significant increases in expression of genes with a function in cholesterol metabolism and in FA bioconversion in V-fed fish, compared to the other dietary groups. It is interesting to notice that the enhanced expression of genes of cholesterol biosynthesis was found at each developmental stage, encompassing entire alevins after 3 weeks of feeding and hepatic tissue in 7 and 13 months old fish, independently of the rearing water temperature. This suggests that, in the presence of dietary plant ingredients as nutritional stimulus, these processes are maintained across the developmental stages investigated and likely represent a compensatory mechanism to meet the metabolic demand in cholesterol in the V-fed fish that is not meet by the dietary supply. Moreover, the fact that cholesterol related gene expression is increased even in entire alevins suggests a strong (temporal) conservation of this effect in V-fed fish.

Comparing gene expression of FA bioconversion-related genes, a significant increase in the expression of Δ6-desaturase and Elongase2 was observed in intestine and liver of trout after 7 and 13 months feeding trial, while no diet-induced effects were visible in alevins. However, is difficult to make strong conclusions about the temporal persistence of this effect, since different biological material (entire alevin or specific tissues) was used for analyses.

When considering the relevant literature, most of the studies carried out to date using transcriptomic tools reported that the FM and/or FO impact fish metabolic responses to nutrients, showing changes at the transcriptional level. The comparison between studies at the level of gene expression is difficult due to the use of different species, life stages, tissues and microarray design. Consequently, common changes in individual gene expression have been rarely reported under similar experimental conditions, in which the transcriptome is dynamic in nature. Analysis of changes through biological pathway enrichment tends to be a much better and consistent predictor of functional importance, and provided more often consistent results between different studies (Roberge, Einum et al. 2006). For example, significant pathway enrichment of lipid metabolism (*e.g.* n-3 LC-PUFA and cholesterol biosynthesis) in response to the inclusion of plant ingredients in the diets described in our study confirms previous enrichment of these pathways in (juvenile) salmonids fed plant-based diets (Leaver, Villeneuve et al. 2008, Panserat, Ducasse-Cabanot et al. 2008, Panserat, Hortopan et al. 2009, Betancor, Sprague et al. 2015).

Overall, our results on gene expression not only confirmed the regulation of cholesterol and FA bioconversion in previous studies of plant-based diets in salmonids species, but extend the validity of these findings to different ontogenetic stages and rearing temperature conditions.

Additional metabolism-related gene expression changes

Differential expression of genes related to carbohydrate and protein metabolism was observed in the intestine of fish between dietary groups after 7 and 13 months feeding treatments, indicating that gene expression in these pathways depends on ontogeny/developmental stage of fish and/or water temperature. Analysis of the liver transcriptome showed that the V-diet significantly enhanced expression of genes involved in the electron transport chain of oxidative phosphorylation and mitochondrial ribosomal proteins. Oxidative phosphorylation in mitochondria represents the final step in macronutrients catabolism. Therefore such results suggest that V-fed fish may have an increased capacity for energy generation (ATP), compared

to the other groups when reared at 7 °C. These effects were not confirmed by RT-qPRC in samples collected either after 7 month feeding trial or at longer term (end of the 13 month feeding trial). Because of the importance of energy metabolism in determining feed efficiency in fish, it would be worth investigating these pathways at both molecular and biochemical levels, which was not possible in the timeframe of my PhD.

Furthermore, some results of our approach without *a priori* hypotheses have laid the foundation for future studies of identified targets. For example, data from transcriptomic analysis of the intestine of juveniles reveald novel actors regulated by dietary treatments, such as fatty acid amide hydrolase (FAAH), which has been characterized as a regulator of intestinal motility in mammals (Capasso, Matias et al. 2005). In order to better understand the nature of the changes we observed at the molecular level, it would be interesting to confirm if these changes are also reflected at the enzymatic activity level and/or result in histological changes.

4.2 Conclusions and perspectives

Overall, the present thesis work showed, for the first time, that feeding rainbow trout plant-based diets across the whole life-cycle results in successful female egg production, and successful, albeit slightly slower, development and growth of alevins, as indicated by consequences on biometric, biochemical and molecular parameters. Results from our integrative approach linking different levels of analysis (whole animal, biochemical (FA profiles) and molecular) globally provides no evidence of a programming effect, as offspring derived from VEG-broodstock did not more efficiently utilize subsequent plant-based diets. Therefore, manipulation of female broodstock diet is not an efficient way to improve capacity of progeny to utilize diets rich in plant ingredients with low levels or without marine ingredients. However, the negative effects of feeding female broodstock of trout with a diet free from marine resources and rich in plant ingredients are not strongly deleterious, so that plant-based diets can be recommended for feeding trout broodstock in commercial operation, provided that some adjustments of feed formula are made. When considering long-term feeding effects independent of maternal dietary history, we showed stage and temperature dependent effects in V-fed trout. In other words, diet-induced effects observed at one specific developmental stage in specific rearing conditions are not representative of the effects during the whole life cycle of trout and at different water temperatures.

Based on these results, several topics for future studies emerged: (i) ontogeny of capacity of LC-PUFA biosynthesis according to dietary treatments; (ii) nutritional regulation of genes implicated in intestine motility and consequences on digestive tract physiology and the digestive function (iii) effects of the dietary plant-sources on energy metabolism and link with feed efficiency (iv) interactions between water temperature et utilization efficiency of plant-based diets at different stages of the fish life cycle.

Another direction emerging for innovative future research is the investigation of potential paternal effects on progeny from male broodstock fed diets with different levels of marine and plant ingredients. The number of studies addressing this question in fish is very limited and focused on male reproductive capacity assessed by sperm quality and offspring development.

While no data was available on potential transgenerational effects of broodstock nutrition in fish at the beginning of this 3 year PhD work, very recent literature suggests potentially different mechanisms in transgenerational effects in fish compared to mammals. A study on zebrafish suggested that some heritable epigenetic (DNA methylation) effects are potentially much more linked to the paternal contribution rather than the maternal one (Jiang, Zhang et al. 2013); further investigations in progeny of male trout fed plant-based diets are therefore warranted.

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Annexes

Annexes

Annex 1 Complete fatty acid composition (% of total FA) of broodstock diets.

	Diet-COM$_{1-2}$	Diet- VEG
Saturated		
10:0		
12:0	0.13	0.06
14:0	4.82	0.21
15:0	0.42	0.03
16:0	14.36	12.71
17:0	0.47	0.15
18:0	3.41	2.63
20:0	0.06	0.34
MUFA		
14:1	0.03	
15:1	0.05	
16:1	6.40	0.17
17:1	0.06	0.05
18:1	12.55	38.69
20:1	1.81	0.67
22:1	1.63	0.24
PUFA n-6		
14 PUFA	0.12	0.10
16:2 n-4	1.15	
16:3 n-4	1.12	0.05
16:4 n-1	1.72	
18:2 n-6	5.66	21.67
18:3 n-6	0.29	
20:2 n-6	0.18	0.05
20:3 n-6	0.18	
20:4 n-6	1.33	
22:2 n-6	0.37	0.22
22:4 n-6	0.11	
PUFA n-3		
18:3 n-3	1.50	20.13
18:4 n-3	2.49	
20:3 n-3	0.11	
20:4 n-3	0.85	
20:5 n-3	17.62	
22:4 n-3	0.34	
22:5 n-3	1.92	
22:6 n-3	11.46	

COM, commercial diet *Le Gouessant*; VEG, 100% plant-based diet
MUFA, monounsaturated fatty acid; PUFA n-3, polyunsaturated fatty acids n-3; PUFA n-6, polyunsaturated fatty acids n-6

Annex 2 Complete fatty acid composition (% of total FA) of experimental diets for alevins, juveniles and ongrowing fish.

	alevins-juveniles			*ongrowing fish*		
Saturated	**M_1**	**C_1**	**V_1**	**M_2**	**C_2**	**V_2**
12:0	0.12	0.09	0.06	0.10	0.06	0.06
14:0	8.53	4.77	0.33	6.88	3.68	0.30
15:0	0.54	0.31	0.04	0.42	0.23	0.02
16:0	18.66	12.91	14.88	16.02	11.18	13.78
17:0	0.30	0.20	0.07	0.32	0.20	0.08
18:0	2.55	2.25	2.69	2.64	2.26	2.81
20:0	0.13	0.30	0.29	0.16	0.35	0.34
22:0		0.15	0.15	0.06	0.24	0.17
MUFA						
14:1	0.08			0.06		
16:1	9.40	4.93	0.26	8.41	4.61	0.22
17:1	0.07	0.08	0.04	0.06	0.07	0.01
18:1	15.31	33.58	37.37	13.31	33.90	36.98
20:1	4.45	2.38	0.55	3.34	2.33	0.60
22:1	3.89	0.93	0.08	3.76	2.03	0.11
PUFA n-6						
14 PUFA	0.072	0.022		0.05	0.02	
16:2 n-4	1.453	0.767		1.41	0.75	
16:3 n-4	1.267	0.792	0.06	1.44	0.82	0.04
16:4 n-1	1.792	1.057		1.92	1.04	
18:2 n-6	3.168	12.491	21.50	2.87	12.05	21.42
18:3 n-6	0.205	0.105		0.23	0.11	
20:2 n-6	0.181	0.111		0.21	0.15	
20:3 n-6	0.074	0.023		0.17	0.05	
20:4 n-6	0.653	0.378		0.79	0.43	
PUFA n-3						
18:3 n-3	1.07	4.79	21.25	0.85	4.51	22.7
18:4 n-3	2.13	1.15		2.32	1.27	
20:3 n-3	0.09			0.07	0.02	
20:4 n-3	0.59	0.32		0.64	0.35	
20:5 n-3	11.15	6.66	0.03	14.7	8.30	0.03
21:5 n-3	0.40	0.28		0.61	0.35	
22:5 n-3	1.13	0.70		1.69	1.03	
22:6 n-3	6.70	4.22		9.92	5.55	

M: marine FM-FO-based diet; C: commercial-like FM-FO & plant-based diet
V: 100% plant-based diet; MUFA: monounsaturated fatty acid
PUFA n-3: polyunsaturated fatty acids n-3; PUFA n-6: polyunsaturated fatty acids n-6

Annex 3 Detailed composition of vitamin and mineral premix (Vit-Min premix, UPAE INRA) used in the experimental diets.

Vitamins (IU or mg Kg^{-1} diet)	
DL-a tocopherol acetate	60 IU
Retinil acetate	15.000 IU
DL-colecalcipherol	3000 IU
Sodium menadione bisulphate	5 mg
Thiamin	15 mg
Riboflavin	30 mg
Pyridoxine	15 mg
Vitamin B12	0.05 mg
Nicotinic acid	175 mg
Folic acid	500 mg
Inositol	1000 mg
Biotin	2.5 mg
Calcium pantothenate	50 mg
Choline chloride	2000 mg

Minerals (g or mg Kg^{-1} diet)	
Dibasic calcium phosphate (20% Ca, 18% P)	5.0 g
Calcium carbonate (40% Ca)	2.15 g
NaCl	0.4 g
KCl	0.9 g
Magnesium oxyde (60% Mg)	1.24 g
Ferric citrate	0.2 g
Zinc sulphate (36% Zn)	0.4 g
Manganese sulphate (33% Mn)	0.3 g
Copper sulphate (25% Cu)	0.3 g
Cobalt sulphate	2.0 mg
Potassium iodide (75% I)	0.4 mg
Sodium selenite (30% Se)	3.0 mg

List of publications and communications

Peer-reviewed publications

Viviana Lazzarotto, Geneviève Corraze, Amandine Leprevost, Edwige Quillet, Mathilde Dupont-Nivet and Françoise Médale. 2015. Three-year breeding cycle of rainbow trout (Oncorhynchus mykiss) fed a plant-based diet, totally free of marine resources: consequences for reproduction, fatty acid composition and progeny survival. *PlosOne, DOI: 10.1371/journal.pone.0117609*

Viviana Lazzarotto, Geneviève Corraze, Laurence Larroquet, David Mazurais and Françoise Médale. 2015. Does broodstock nutritional history affect the response of progeny to different first-feeding diets? A whole-body transcriptomic study of rainbow trout alevins. (*BJN, Accepted, In press*).

Viviana Lazzarotto, Françoise Médale, Laurence Larroquet, Geneviève Corraze. 2015. Long-term dietary replacement of fish meal and fish oil in diets for rainbow trout: effectson hepatic and intestinal gene expression. (*BJN*, *to be submitted*).

Communications

V. Lazzarotto, G. Corraze, L. Larroquet, F. Médale 2014. Three years life cycle breeding with a totally plant-based diet: reproduction performance and potential carry over generation effects in rainbow trout (*Onchorynchus mykiss*). International Symposioum of Fish Nutrtion and Feeding (IVX), May 25-30, Cairns (Australia). **Poster presentation.**

V. Lazzarotto, F. Médale, S. Kaushik, L. Larroquet, G. Corraze 2014. Long term feeding rainbow trout with plant-based diets up to total replacement of fish meal and fish oil: consequences on growth performance, whole body lipid content and fatty acid profile. International Symposioum of Fish Nutrtion and Feeding (IVX), May 25-30, Cairns (Australia). **Poster presentation.**

V. Lazzarotto, G. Corraze, L. Larroquet, F. Médale 2014. L'alimentation maternelle des truites arc-en-ciel modifie-t-elle l'aptitude des descendants à utiliser des aliments à base de végétaux? Journées Recherche Filière Piscicole, July 2-4, Paris (France). **Oral presentation.**

V. Lazzarotto, G. Corraze, L. Labbé, E. Quillet, M. Dupont-Nivet, F. Médale 2014. Does the maternal diet history affect the transcriptome of swim-up alevins of rainbow trout (*Oncorhynchus mykiss)*? EAS-Aquaculture Europe 2014, October 14-17, S. Sebastian (Spain). **Poster presentation.**

V. Lazzarotto, G. Corraze, D. Mazurais, S. Kaushik, F. Médale 2014. Effect of broodstock history and first feeding diets on transcriptome of rainbow trout (*Oncorhynchus mykiss*) alevins. EAS-Aquaculture Europe 2014, October 14-17, S. Sebastian (Spain). **Oral presentation.**

V. Lazzarotto, G. Corraze, F. Médale 2015. Does the long-term dietary replacement of fish meal and fish oil affect the hepatic and intestinal transcriptome of rainbow trout (*Oncorhynchus mykiss*) juveniles? Aquaculture 2015, August 23-26, Montpellier (France). **Oral presentation.**

Thesis summary

"Consequences of long-term feeding trout with plant-based diets on the regulation of energy and lipid metabolism: special focus on trans-generational effects and early stages"

In the last years, the increase in aquaculture production has forced a change in fish feed composition, with increasing substitution of fish meal and fish oil by more available plant sources. In this context, the present PhD work aimed at analyzing the effects of different levels of concomitant dietary replacement of fish meal and fish oil by plant ingredients during the whole life cycle of rainbow trout (from first feeding to reproduction).

This work showed for the first time that rainbow trout was able to survive, grow and produce viable offsprings, when fed a totally plant-based diet throughout the whole life cycle. Such dietary replacement resulted in drastic changes in tissues fatty acid profile of broodstock females. However, we found that trout was capable to synthetize n-3 long chain- polyunsaturated fatty acids (LC-PUFA) from dietary precursors and to transfer large amounts of these fatty acids in ova, so that an efficient reproduction can occur.

We also studied the potential carry over generation effects of feeding broodstock a totally plant-based diet on progeny and their response to different diets at early stages, using molecular approaches, including the study of the whole body transcriptome. Considering the effects on progeny, the present study confirmed the capability of trout to survive and grow on a plant-based diet, but with slight differences in terms of weight. While no effects of maternal dietary background were observed before first feeding, except slightly lower body weight (-13%), significant effects on the transcriptome of whole body alevins appeared after 3 weeks of feeding. These effects of maternal nutritional history were mainly related to muscle growth/contraction and carbohydrate and energy metabolism. Irrespective of the maternal origin of progeny, first feeding diets containing plant ingredients resulted in up-regulation of genes involved in AA/protein and cholesterol metabolism, as well as in changes in the expression of genes involved in carbohydrate metabolism.

Finally, the effects of long-term feeding trout plant-based diets were also studied in juveniles (10g) and ongrowing fish (250-350g), mainly focusing on intestine and liver gene expression. The long term trial in juveniles highlighted subtle effects on both intestinal and hepatic gene expression (transcriptome), mainly related to LC-PUFA and cholesterol biosynthetic pathways,

which were enhanced in fish fed the plant based diets. This transcriptional pattern was maintained in ongrowing fish. Genes involved in protein catabolism, carbohydrate metabolism and trafficking were also affected by plant-based diets in juveniles, but these results were not fully confirmed in ongrowing fish.

Overall, the results of the present thesis allowed extending the use of diets with high replacement rates of fish meal and fish oil to the whole life cycle of fish, including broodstock and early stages. Adjustments of the feed-formula are still needed to further optimize reproductive and growth performance.

Résumé de la thèse

"Conséquences à long-terme d'une alimentation à base de matières premières végétales sur la régulation du métabolisme énergétique et lipidique chez la truite arc-en-ciel : focus particulier sur les effets trans-générationnels et les stades précoces"

Au cours des dernières années, l'augmentation de la production aquacole a nécessité un changement dans la composition des aliments pour poissons, pour remplacer la farine et l'huile de poisson par des sources végétales plus disponibles. Dans ce contexte, le travail de ma thèse avait pour but d'analyser les effets d'une substitution totale et concomitante de la farine et l'huile de poisson par des ingrédients végétaux dans les aliments distribués pendant tout le cycle de vie de la truite arc-en-ciel, de la première alimentation jusqu'à la reproduction.

Ce travail a montré pour la première fois que la truite arc-en-ciel peut survivre, se développer et avoir une descendance viable, lorsqu'elle est nourrie tout au long de son cycle de vie avec un régime 100% végétal. Un tel remplacement alimentaire a entraîné des changements radicaux dans le profil en acides gras des tissus des femelles. Cependant, nous avons constaté que la truite était capable de synthétiser des acides gras polyinsaturés à longue chaîne n-3 (AGPI-LC n-3) à partir de précurseurs présents dans l'aliment, et de stocker dans les ovules une partie de ces acides gras néosynthétisés dans le foie , permettant ainsi une reproduction efficace.

Nous avons également étudié les possibles effets trans-générationnels d'une alimentation 100% végétale des géniteurs, sur la survie et la croissance de la descendance et sur sa réponse à différents régimes aux stades précoces en nous appuyant sur des analyses du transcriptome des alevins entiers. Aucun effet significatif de l'alimentation maternelle avec des régimes à base de végétaux n'a été observé sur les descendants avant leur première alimentation, à l'exception d'un poids corporel légèrement inférieur (-13%) à celui des descendants de femelles nourries avec un aliment contenant des farines et huiles de poisson. En revanche, des effets significatifs de l'alimentation maternelle sur le transcriptome des alevins sont apparus après 3 semaines d'alimentation. L'histoire nutritionnelle de la mère a affecté principalement des gènes impliqués dans la croissance/contraction musculaire et dans les métabolismes énergétique et glucidique. Quelle que soit l'origine maternelle, l'alimentation des alevins avec des aliments contenant des ingrédients végétaux conduit à une régulation positive des gènes impliqués dans le métabolisme

des AA/protéines et le métabolisme du cholestérol, ainsi qu'à des changements dans l'expression des gènes impliqués dans le métabolisme des glucides.

Enfin, les effets à long-terme de régimes à base de matières premières végétales chez la truite arc-en-ciel ont également été étudiés chez les juvéniles (10g) et les poissons « en croissance » (250-350g), en se focalisant principalement sur l'expression des gènes dans l'intestin et le foie. Cette étude à long-terme a mis en évidence chez les juvéniles des effets subtils sur des gènes intestinaux et hépatiques (transcriptome), avec principalement une augmentation de l'expression des gènes impliqués dans les voies de synthèse des AGPI-LC et du cholestérol chez les poissons nourris avec des régimes à base de plantes. Ces changements d'expression ont été maintenus chez les poissons « en croissance ». L'expression de gènes impliqués dans le catabolisme des protéines, le métabolisme des glucides et dans le transport intracellulaire a également été modifiée par les régimes à base de plantes chez les juvéniles, mais ces résultats ne sont pas entièrement confirmés chez les poissons « en croissance ».

L'ensemble des résultats de cette thèse fournissent des informations originales sur l'utilisation de régimes alimentaires avec des taux de remplacement élevés de farine et d'huile de poisson pendant l'ensemble du cycle de vie des poissons, y compris les reproducteurs et les stades précoces, car la majorité des études précédentes avaient concerné la phase de grossissement. Des ajustements de la formule des aliments à base de végétaux sont encore nécessaires pour continuer à optimiser les performances de reproduction et surtout la croissance des poissons d'élevage.