Metabolic utilization of dietary carbohydrates in lean and fat lines of rainbow trout (Oncorhynchus mykiss)

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Metabolic utilization of dietary carbohydrates in lean and fat lines of rainbow trout (*Oncorhynchus mykiss*)

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(Editor's choice article, July 2012 issue; featured in Inside JEB as "Trout can cope on some carbs"; shortlisted in top 8 outstanding student research articles 2011-12).

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Communications

Kamalam, B.S., Medale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S., Panserat, S. Effect of genetic selection and dietary carbohydrates on hepatic intermediary metabolism of rainbow trout. *Aquaculture Europe,* 18-21 October 2011, Rhodes, Greece. **(Oral presentation)**

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LIST OF ABBREVIATIONS

0C	diet without carbohydrates
20C	diet with carbohydrates
6PFK	6-phosphofructo-1-kinase (EC 2.7.1.11)
ACC	acetyl coA carboxylase (EC 6.4.1.2)
ACLY	adenosine triphosphate citrate lyase (EC 2.3.3.8)
ADC	apparent digestibility coefficient
Akt	protein kinase B
AMPK	5’ adenosine monophosphate activated protein kinase
ANOVA	analysis of variance
ApoA1	apolipoprotein A1
ApoA4	apolipoprotein A4
ApoB	apolipoprotein B
B(0)AT	sodium dependent neutral amino acid transporter
cDNA	complementary deoxyribonucleic acid
CPT	carnitine palmitoyl transferase (EC 2.3.1.21)
D6D	Δ6 fatty acyl desaturase (EC 1.14.19.3)
D9D	Δ9 fatty acyl desaturase (EC 1.14.19.1)
DHA	docosahexaenoic acid
EF1α	elongation factor 1 α
Elovl2	elongation of very long chain fatty acids like-2 (EC 2.3.1.199)
Elovl5	elongation of very long chain fatty acids like-5 (EC 2.3.1.199)
EPA	eicosapentaenoic acid
F	fat line
FAA	free amino acids
FAS	fatty acid synthase (EC 2.3.1.85)
FAT/CD36	fatty acid translocase
FBPase	fructose 1,6-bisphosphatase (EC 3.1.3.11)
FFA	free fatty acids
G6Pase	glucose 6-phosphatase (EC 3.1.3.9)
G6PD	glucose 6-phosphate dehydrogenase (EC 1.1.1.49)
GK	glucokinase (hexokinase IV; EC 2.7.1.2)
GLUT2	glucose facilitative transporter type 2

GLUT4	glucose facilitative transporter type 4
HK1	hexokinase 1 (EC 2.7.1.1)
HOAD	hydroxy acyl coA dehydrogenase (EC 1.1.1.35)
HSI	hepato-somatic index
IU	international units of enzyme activity
L	lean line
LC-PUFA	long chain polyunsaturated fatty acid
LPL	lipoprotein lipase (EC 3.1.1.34)
mRNA	messenger ribonucleic acid
MTP	microsomal triglyceride transfer protein
MUFA	monounsaturated fatty acids
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
PEPCK	phosphoenolpyruvate carboxykinase (EC 4.1.1.32)
PepT1	peptide transporter 1
PK	pyruvate kinase (EC 2.7.1.40)
PPAR	Peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acids
SFA	saturated fatty acids
SGLT1	sodium dependent glucose co-transporter type 1
SREBP1c	sterol regulatory element binding protein 1c
TAG	triacylglycerol
TOR	target of rapamycin
VLDLR	very low density lipoprotein receptor
VOC-	vegetable oil based diet without carbohydrate
VOC+	vegetable oil based diet with carbohydrate
VSI	viscero-somatic index

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Chapter 1

INTRODUCTION

AND

REVIEW OF LITERATURE

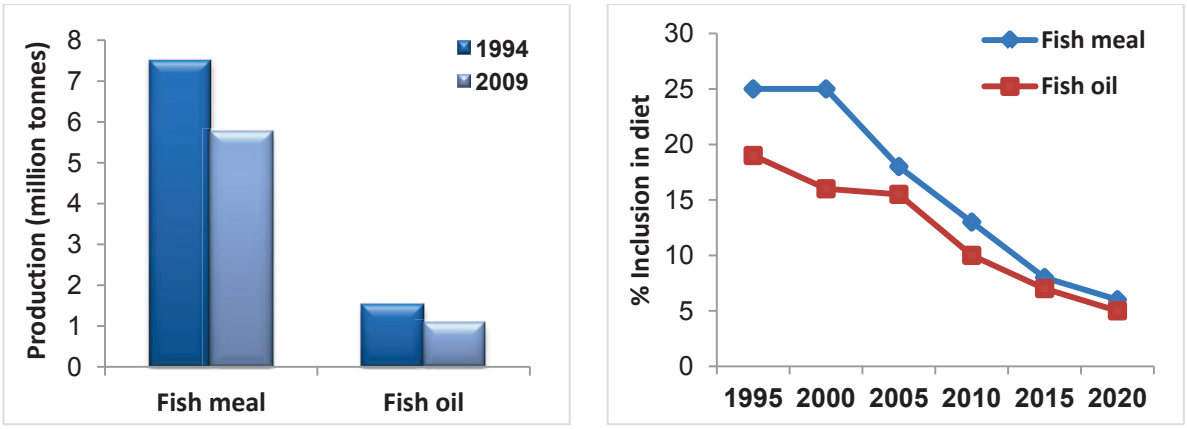
1.1. Aquaculture development and feed formulation: trends and prospects

Aquaculture has rapidly evolved from being almost negligible to fully comparable with capture production in terms of feeding the increasing global population. The contribution of farmed fish to human consumption rose to 47 percent in 2010 from a lowly 9 percent in 1980. Over these years, the world aquaculture production has expanded by almost 12 times to reach 60 million tonnes in 2010, at an average annual rate of 8.8 percent, with an estimated total value of nearly 91 billion € (FAO, 2012). On the other hand, global capture production has levelled off at around 90 million tonnes in the recent past, providing close to 68 million tonnes of capture fish for human consumption (FAO, 2012). Therefore, the additional onus to maintain the current global per capita fish consumption of 18.8 kg and to meet the growing demands of the ever increasing population is on aquaculture and so it is imperative to increase production efficiency and scale of aquaculture systems. This objective can be achieved mainly by improvements in nutritional aspects (diets) and by using genetically superior strains of fish (Naylor et al., 2000; Gjedrem et al., 2012), further supported by improving farming systems, optimisation of rearing densities, controlling maturation/gender and by reducing the concentration of nitrogen/phosphorous in effluents (Bostock et al., 2010).

Complementing the rapid growth in aquaculture, global industrial compound aquafeed production has increased almost fourfold from 7.6 million tonnes in 1995 to 29.2 million tonnes in 2008 at an average rate of 11 percent per year. It is expected to grow further to 51 million tonnes by 2015 and to 71 million tonnes by 2020 (FAO, 2012). This clearly indicates that the availability of quality feeds and feed ingredients in requisite quantities is integral to intensified production strategies. Now considering the case of fish meal and fish oil, the predominant protein and lipid source in carnivorous fish feed; production from marine capture fisheries have been decreasing in the past 15 years at annual average rates of 1.7 and 2.6 percent, respectively (Figure 1). But at the same time, aquaculture's share of consumption has more than doubled to 61% and 74%, respectively. The finite nature of marine resources, poor regulation of reduction fisheries, negatively balanced demand-supply economics and sustainability metrics such as 'fish-in fish-out' ratios has raised serious challenges to lower the aggregate level of fishmeal and fish oil inputs in feeds and to find suitable substitutes (Naylor et al., 2000; Tacon and Metian, 2008; Naylor et al., 2009; FAO, 2012).

Terrestrial plant proteins, oils and carbohydrates (cereals, including by-product meals and oils; oilseed meals and oils; pulses and protein concentrate meals) are prominent substitution

Figure 1. *Trends of global fish meal and fish oil production* (Adapted from FAO, 2012) *and their projected inclusion in rainbow trout diets* (Adapted from FEAP annual report, 2011)



This figure shows the decreasing global production of fish meal and oil in the last two decades (left) and the subsequent changes achieved and projected in the incorporation levels of these marine ingredients in commercial trout feed formulations in Europe (right).

prospects due to their abundance and competitive pricing (Gatlin et al., 2007; Hardy, 2010). Significant progress has been made in partially replacing fish meal and fish oil with plant alternatives in nutritionally well balanced diets and replacement level (60-95%) depends on the species and developmental stage. Nevertheless, complete use of plant sources in carnivorous fish feeds faces several barriers such as poor palatability, inappropriate amino-acid balance (lysine and methionine deficiency), high content of carbohydrates and indigestible organic matter, presence of anti-nutritional compounds, unknown nutritional limitations, decreased final product quality in terms of n-3 LC-PUFA and environmental effects related to high phosphorus and solid waste in farm effluents (Naylor et al., 2000; Gatlin et al., 2007; Naylor et al., 2009; Hardy, 2010). A viable, facilitative strategy to overcome these constraints is to use advanced genetics and genomic tools to develop strains of fish that can tolerate higher levels of plant feedstuffs or associated components in the diet (Naylor et al., 2009; Le Boucher et al., 2012; Overturf et al., 2013).

In particular, although digestible carbohydrates with nutritive value belong to the biologically 'dispensable' class of nutrients, it is economically 'indispensable' in all fed livestock diets because it is relatively the cheapest and most abundant source of dietary energy (Wilson, 1994; Kaushik, 2001; Stone, 2003). Maximising the inclusion of digestible carbohydrates in the diets can enhance the profitability of the farming enterprise, which is true especially in the culture of carnivorous fish, where the contribution of feeds to the total variable costs is easily above 60% and can go beyond depending on the intensity of culture. But on the other hand, the important question is concerning the adaptability of the farmed fish to these dietary changes with higher levels of digestible carbohydrates or plant based ingredients.

In this context, the present research was undertaken to identify the putative genotypic differences in digestion, intestinal nutrient transport and metabolic utilisation of digestible carbohydrates between two lines ('Fat' and 'Lean') of rainbow trout (*Oncorhynchus mykiss*), divergently selected for muscle fat content using a non-destructive method. The effect of dietary digestible carbohydrate on the intermediary metabolism and performance of the two trout lines was analysed under a 'fish oil based' and a 'vegetable oil based' dietary regime.

Table 1. *Carbohydrate content of selected plant ingredients* (Adapted from Bach Knudsen, 1997).

Ingredient		Total carbohydrates (g/kg dry matter)	Available fraction* (g/kg dry matter)
Maize	Whole grain	823	707
	Feed meal	783	601
	Gluten	238	211
	Bran	791	402
	Flour	940	912
Wheat	Whole grain	823	665
	Bran	704	259
	Flour	887	829
Rye	Whole grain	850	638
	Bran	674	132
Barley	Hulled	834	603
	Dehulled	819	663
Oats	Hulled	787	481
	Feed meal	749	637
Soybean	Meal	400	104
	Protein concentrate	275	73
Rapeseed	Meal	454	84
	Cake	399	87
Cottonseed	Meal	462	38
	Cake	423	30
Linseed	Meal	493	58
Coconut	Cake	622	130
Palm	Cake	636	33
Sunflower	Cake	517	51
Peas		735	493
Faba beans		705	439
White lupins		534	43

* Digestible starch, sucrose and monosaccharides

1.2. Carbohydrates in fish nutrition

Carbohydrates are naturally occurring organic compounds that contains carbon, hydrogen, and oxygen in proportions that usually correspond to the empirical formula $(CH_2O)_n$. They are abundant in all plant based ingredients as energy reserve and structural polysaccharides (Table 1), therefore their presence in fish feed tends to rise with the paradigm shift in feed formulation towards terrestrial plant resources. Only some forms (simple sugars and starches) have nutritive value in fish nutrition, while others (fibres) have a secondary nutritional role or even a negative one (Kaushik, 2001; Stone, 2003; NRC, 2011). **In this thesis, the term 'carbohydrate' will be used to refer only to sugars and starches with nutritive value.**

The capacity of carbohydrate utilisation varies greatly among fish species, depending on their feeding habit and is generally lower than in terrestrial livestock (Wilson, 1994; Kaushik, 2001). Besides as in other animals, fish do not have a dietary requirement for carbohydrate as they can synthesize glucose efficiently from non-glucose precursors (lactate, amino acids) and they are able to survive and grow when fed diets without carbohydrates (NRC, 2011). Despite this dispensability, inclusion of carbohydrates either directly as a cheap source of energy or indirectly as a by-product of plant proteins helps to develop less expensive dietary formulations that maintain efficient growth at lower cost per unit gain (Hardy, 2010). Appropriate level of carbohydrates in the diet of all cultured fish can prevent the catabolism of expensive nutrients such as protein and lipids for energy needs and to provide metabolic intermediates for other biologically important compounds (Wilson, 1994; Stone, 2003). The absence of dietary carbohydrates (when not compensated by dietary fat) can lower protein retention and increases nitrogen excretion, due to deamination of amino acids for glucose production (Peragón et al., 1999; NRC, 2011). In addition to economical objectives (protein sparing), digestible carbohydrates in feeds also thus helps to meet environmental regulation objectives (reduced nitrogen discharge). Furthermore, starch plays the role of a binder that aids pellet binding and are useful in the manufacture of floating feeds through extrusion process, which is particularly important considering the aquatic culture medium.

1.3. Overview of metabolic utilisation of carbohydrates in teleosts

The ability of farmed fish to use dietary carbohydrates as an efficient source of energy remains a controversial subject till date (Polakof et al., 2012), further complicated by extreme diversity in feeding habits, anatomical/physiological features and farming habitats.

Optimistic arguments that support the potential for carbohydrate use in fish are the presence of major enzymes involved in starch digestion as well as those of glucose metabolism (Cowey and Walton, 1989; Krogdahl et al., 2005; Enes et al., 2009); the presence of several members of the glucose transporter family (Planas et al., 2000; Teerijoki et al., 2000; Krasnov et al., 2001), inducible glucokinase (Panserat et al., 2000c) and other glucose sensing components in central and peripheral tissues (Polakof et al., 2011a); the use of glucose as a carbon substrate for the same purposes as in other animals i.e., ATP generation through oxidative pathways and ribose 5-phosphate/NADPH production via pentose phosphate pathway (Enes et al., 2009; NRC, 2011) and in particular, the preferential use of glucose in fish brain, spleen, kidney and gills (Blasco et al., 2001; Soengas and Aldegunde, 2002); the capacity of glucose to stimulate insulin secretion (Furuichi and Yone, 1981; Mommsen and Plisetskaya, 1991; Enes et al., 2012); and sensitivity of blood glucose levels to a variety of physiological, environmental and pharmacological conditions indicating that fish do have an active glucose homeostatic system (Polakof et al., 2012).

Sceptical arguments mainly revolve around the induction of persistent hyperglycaemia following the intake of a carbohydrate rich diet or glucose injection and the varying levels of amylase activity depending on the species-feeding habit (Moon, 2001; NRC, 2011). The possible reasons behind the prolonged hyperglycemia are low potency of glucose as an insulin secretagogue compared with amino acids (Mommsen and Plisetskaya, 1991), inhibition of insulin secretion by other hormones such as somatostatin (Sheridan and Kittilson, 2004), relatively low number of muscle insulin receptors (Navarro et al., 1999), poor use of glucose in white muscle - the largest tissue (West et al., 1993), lack of inhibition of endogenous glucose production (Panserat et al., 2000a), weak hepatic lipogenesis from glucose (Hemre and Kahrs, 1997) and less complex neural regulatory network i.e., the presence of glucose excited/inhibited neurons not yet demonstrated (Polakof et al., 2011a).

Altogether, if the efficiency of carbohydrate utilisation is estimated strictly based on the rate of glucose delivery from digestion and glucose clearance from the bloodstream, fish, especially carnivores tend to be poor users of dietary carbohydrates (NRC, 2011). Even so, when comparisons are made with other vertebrate groups it is important to take into account features such as the poikilothermic nature of fish, corresponding low metabolic rate, habitat oxygen availability, domestication differences and adaptation of its metabolism to long term food deprivation (Moon, 2001; Polakof et al., 2012).

Table 2. *Comparison between trout and carp for carbohydrate utilisation markers*

Marker	Trout[a, b]	Common carp	Average body mass (g)	Nutritional status	Rearing temp. (°C)	Reference
Amylase activity - Digestive tract (U mg protein^{-1})	1.3	72.5	Trout: 114 Carp: 110	48 hours starved	-	Hidalgo et al., 1999 [a]
Glucose uptake rate - Proximal Intestine (nM min^{-1}cm^{-1})	12.5	164	Trout: 391 Carp: 193	Fed with commercial diet containing 25% digestible starch for 8 weeks	Trout: 12-15 Carp: 20-25	Buddington et al., 1987[a]
Glucose uptake rate - Middle Intestine (nM min^{-1}cm^{-1})	14.9	78				
Integrated glucose uptake capacity (nM min^{-1}g body weight^{-1})	1.8	29				
Plasma glucose level (mM)	10.5	3.5	Trout: ~150 Carp: ~150	6 hours after dietary intake of 20% digestible starch	Trout: 18 Carp: 18	Panserat et al., 2000 [a]
Glucokinase activity - Liver (mU mg protein^{-1})	36.7	9.7				
Low Km Hexokinase activity – Liver (mU mg protein^{-1})	2.1	4.8				
Plasma insulin level (ng ml^{-1})	9.6	9.1	Trout: 123 Carp: 158	18 hours after Commercial feed intake	-	Parrizas et al., 1994 [b]
Number of insulin receptors - White muscle (fM mg glycoprotein^{-1})	82	440				

[a] Rainbow trout (*Oncorhynchus mykiss*); [b] Brown trout (*Salmo trutta fario*)

1.4. Factors influencing carbohydrate utilisation

The extent to which dietary carbohydrates can be used to meet energy requirements varies greatly between and even within species, depending on a multitude of factors categorized into those related to biology (feeding habit, exercise, genotype), diet (carbohydrate source - botanical origin, molecular complexity, physical state; inclusion and feeding level; nutrient interactions) and the environment (temperature, salinity, stress).

1.4.1. Biological factors

Feeding habits

Natural feeding habit is the primary determinant of the fish's ability to use carbohydrates. Compared to herbivorous and omnivorous species like carp, tilapia and catfish, carnivorous species like trout, salmon and seabass exhibit lower - amylase activity (Hidalgo et al., 1999), intestinal brush border sucrase-isomaltase activity (Harpaz and Uni, 1999), intestinal glucose uptake rate/capacity (Buddington et al., 1987), insulin binding capacity and receptor number in the white skeletal muscle (Parrizas et al., 1994), control of blood glucose and slow return to basal level after glucose loading (Furuichi and Yone, 1981; Legate et al., 2001), regulation of gluconeogenic enzymes by dietary carbohydrates (Panserat et al., 2000a; Panserat et al., 2001b; Panserat et al., 2002a), ability to withstand higher dietary carbohydrate levels (Wilson, 1994; Kaushik, 2001) and degree of protein sparing (Hemre et al., 2002; Stone, 2003). Some results generated by comparative studies on rainbow/brown trout and common carp are presented in Table 2. The composition of the natural diet (carbohydrate content) thus has resulted in an evolution driven genetic hardwiring of the physiological response of fish to high levels of carbohydrate in feed formulations (Buddington et al., 1987).

Exercise

Sustained swimming reportedly can reduce the post-prandial hyperglycemia associated with a high carbohydrate diet (30% digestible starch) and improve protein deposition by enhancing glucose oxidation in the red and white muscle of rainbow trout (Felip et al., 2011). In fact, glucose utilisation by trout red muscle is known to increase 28 fold during peak swimming activity (West et al., 1993). Exercise induced protein sparing by dietary carbohydrates (higher nitrogen retention) has also been reported in another carnivore fish, gilthead sea bream (Felip et al., 2013). A possible reason for this phenomenon could be an increase in oxygen consumption during swimming, which can be about twice as that of resting trout, resulting in

increased use of blood-borne glucose as fuel (van den Thillart, 1986). Moreover, the relative contribution of carbohydrate to energy metabolism increases with swimming speed and decreases over time (Lauff and Wood, 1996).

Genotype

The existence of a genetic component that can determine the ability to utilise carbohydrate is unknown in fish but remains a possibility, as suggested by the variation in glucose tolerance among three natural strains of Chinook salmon (Mazur et al., 1992). Another inquisitive finding is that selectively bred fast growing families of rainbow trout have higher plasma insulin levels than their slow growing counterparts (Sundby et al., 1991). Though the authors did not specify an exact reason, it is tempting to speculate that genetic ability to secrete more insulin and ensuing better use of dietary carbohydrates (protein sparing) could be behind the higher growth observed in the fast growing families. Overall, the intra-specific difference is little known and has prospect for a detailed exploration, thus it is the main aim of the thesis.

1.4.2. Dietary factors

Molecular complexity

Most fish have the enzymes (α-amylase) to hydrolyse glucose polymers with α-glycosidic linkages, i.e., starch forms such as amylose (α1-4) and amylopectin (α1-4, α1-6). But, they generally lack enzymes that can cleave the anomeric β-glycosidic bonds or α-galactosidic bonds of non-starch polysaccharides such as cellulose, hemi-cellulose, pectins and gums, rendering them non-nutritive or detrimental (Kaushik, 2001; Rust, 2002; NRC, 2011). On the basis of degree of polymerisation, the metabolic utilisation and net energy value of simple sugars and complex starch is species dependant (Wilson, 1994; Enes et al., 2011). Simple sugars such as glucose have a higher digestibility, but digestible complex starch lead to better feed utilisation, growth rate and are also preferred for economical reasons (Pieper and Pfeffer, 1980; Kaushik, 2001; Cui et al., 2010; NRC, 2011).

Botanical origin

Starch is known to accumulate in the endosperm of cereals and in tubers as discrete granules with a size and form characteristic of the individual plant species (Buléon et al., 1998; Svihus et al., 2005; NRC, 2011). The size of the starch granule decides the contact surface area for action of digestive enzymes (smaller the better), whereas the available polymer chain ends of the starch form increases the cleavage sites for the enzyme α-amylase i.e., highly branched

amylopectin more susceptible than linear amylose (Zobel, 1988; Svihus et al., 2005; NRC, 2011). For a proven illustration, size of wheat starch granule is 22 µm, maize is 35 µm and potato is 40-100 µm and their respective digestibility in rainbow trout was 58, 34 and 5% (Bergot, 1993). Similarly, digestibility of waxy (99:1), native (75:25) and high amylose (30:70) maize starch with decreasing amylopectin to amylose proportions was 56, 34 and 24%, respectively (Bergot, 1993). In juvenile seabass and seabream, the nature of the starch (waxy vs. native maize) was reported to have no differential effect on growth performance and hepatic intermediary metabolism (Enes et al., 2006b, 2008b). Another aspect to note is the presence of α-amylase inhibitors in some cereals such as wheat, rye, triticale and sorghum, which is proven to inhibit the α-amylase of carp and tilapia (Natarajan et al., 1992).

Physical state

Hydrothermal pre-treatments such as cooking, extrusion, steaming and expansion can alter the physical state of complex starches from cereals and pulses (Kaushik, 2001; NRC, 2011). Heating to 60-80 ^{o}C in the presence of excess water, causes water to penetrate the starch granules until they swell and disrupt. Following the irreversible loss in crystalline structure, amylose and amylopectin leach from the granule and produces a viscous suspension (Enes et al., 2011; NRC, 2011). Compared to native starch, this form of gelatinised starch (>70% gelatinisation) greatly improves enzymatic degradation in the digestive tract of fish, more significantly in carnivores like trout, sea bass and sea bream, thereby maximising starch digestibility, dietary digestible energy supply and resultant protein sparing (Bergot, 1993; Dias et al., 1998; Peres and Oliva-Teles, 2002; Venou et al., 2003; Hua and Bureau, 2009).

Dietary inclusion level

Apart from source characteristics, the level of dietary inclusion is a major species-specific criterion that determines the utilisation prospects of carbohydrates. The common changes associated with increasing dietary levels of starch are accumulation of glycogen in the liver and beyond tolerable limits, decrease in starch digestibility, feed utilisation and growth performance of the fish (Wilson, 1994; Stone, 2003; Krogdahl et al., 2005; Enes et al., 2011). The reduction in digestibility may be due to the substrate overload and subsequent saturation of digestive carbohydrases or a decrease in gut transit time (Spannhof and Plantikow, 1983; NRC, 2011), whereas growth retardation can be explained by severe postprandial hyperglycemia, hepatic dysfunction (hepatomegaly) and related changes in intermediary metabolism (Kaushik, 2001; Amoah et al., 2008). The threshold limits for these

physiological disorders will decide the dietary inclusion level of carbohydrates. Based on the reported findings for different fish species, the maximum recommended levels of digestible starch inclusion in feed fall within 15-25% for salmonids and marine fish and up to 50 % for omnivorous species (NRC, 2011). Furthermore, feeding frequency and ration size are two other factors allied with dietary inclusion level that determines the absolute starch intake of the fish, which is more precisely linked to its digestibility and thus utilisation (Bergot and Breque, 1983; Kaushik, 2001; Krogdahl et al., 2004; Yamamoto et al., 2007).

Influence of other nutrients on carbohydrate metabolism and utilisation

The caloric balance in a high carbohydrate diet is usually achieved by modifying the levels of lipids and protein. Hence, carbohydrate utilisation can also be affected by other nutrients within the diet (Moon, 2001; Hemre et al., 2002; Polakof et al., 2012). Carnivorous fish such as salmonids are fed diets with relatively high levels of lipids, as it is known to have a better protein sparing effect than carbohydrates at a similar level of digestible energy intake (Brauge et al., 1994; NRC, 2011). However, high lipid diets may reduce starch digestibility as apparent from studies in Atlantic salmon (Grisdale-Helland and Helland, 1997) and this may be due to the fact that lipids can influence the velocity of all nutrients passing through the gastrointestinal tract. Starch digestibility was also affected by the type of lipid; it increased with medium chain triglycerides as compared to fish oil in Atlantic salmon (Nordrum et al., 2000; Nordrum et al., 2003).

In a short term pair feeding trial in rainbow trout, high level of dietary lipids (due to the presence of fish oil) was found to increase the expression and activity of glucose 6-phosphatase (a key enzyme involved in endogenous glucose production), subsequently resulting in an elevated postprandial glycemia (Panserat et al., 2002b). This led the authors to conclude that poor dietary carbohydrate utilisation in rainbow trout may be related at least in part to increased hepatic glucose production under conditions of high dietary fat intake. This added more substance (the role of dietary lipid level) to an earlier finding that dietary carbohydrates do not regulate hepatic gluconeogenesis in rainbow trout (Panserat et al., 2000a; Panserat et al., 2001b). The phenomenon was thoroughly examined and confirmed in rainbow trout fed high carbohydrates and different lipid levels (Figueiredo-Silva et al., 2012). The consumption of a high fat diet (blend of 14.1% rapeseed oil and 5% fish oil) for 14 days was found to cause prolonged hypergylcaemia and reduced plasma glucose clearance in response to an exogenous glucose or insulin challenge, characterised by a decrease in the

hepatic activities of lipogenic and glycolytic enzymes, concomitant increase in glucose 6-phosphatase, reduced glycogen levels in the white muscle and impaired insulin signalling. Thus, the persistent postprandial glycemia after a high carbohydrate meal stems from a metabolic interaction between dietary macronutrients rather than from high carbohydrate intake alone. Considering the progressive shift towards replacing fish oil with vegetable oil sources, it is extremely important to obtain a better understanding of the effect of these nutrient interactions in terms of carbohydrate metabolism and utilisation, which was one aspect that was addressed using a long term feeding trial in this doctoral dissertation.

Typically in fish, specific amino acids (lysine, arginine, alanine, methionine and serine) are known to possess more potent insulinotropic activities than glucose (Mommsen and Plisetskaya, 1991; Andoh, 2007). In trout hepatocytes, amino acids were also found to be important signalling molecules that regulate intermediary metabolism through activation of the mTOR pathway (Lansard et al., 2010; Lansard et al., 2011). However, the effect of dietary protein (amino acid) level on carbohydrate metabolism and utilisation is not so clear. Partial substitution of dietary protein (53%) with gluconeogenic dispensable amino acids (alanine, aspartate and glutamate) does not seem to modify glycemia or to have a negative impact on dietary carbohydrate (11-13%) utilisation in rainbow trout. In fact feeding diets with added amino acids suppressed gluconeogenic enzyme activities (Kirchner et al., 2003b). Contradictorily, a graded increase in protein levels (27 to 55%) under similar intake of lipid and carbohydrates (pair-fed) was found to elevate the activities of key gluconeogenic enzymes in rainbow trout, with lowest activity observed in the low protein group (below adequate level). But, low protein intake was also intriguingly associated with the highest postprandial glycemia (Kirchner et al., 2003a). In many other studies, the effect of protein alone on glucose metabolism cannot be delineated and ascertained (Cowey et al., 1981; Walton, 1986). Very recently, methionine restriction was shown to abolish the glucose-intolerant phenotype in rainbow trout, 6 hours post-feeding, irrespective of carbohydrate load (Craig and Moon, 2013). However, such restriction may limit growth performance. The differing proportions of dietary macronutrients may stimulate the secretion and action of several hormones to variable degrees (Moon, 2001; Hemre et al., 2002). Understanding such alterations in hormonal balance and ensuing regulation of metabolism will be a very challenging but rewarding subject for future investigations on carbohydrate utilisation.

Second to none, is the presence of certain micronutrients in the diet and their biochemical/ physiological role, but are often less understood. For example, thiamine's (vitamin B_1) well known co-enzymatic involvement in the pyruvate dehydrogenase complex and the transketolases of pentose phosphate pathway emphasizes its importance in realising the energy production from digestible carbohydrates (Gouillou-Coustans and Guillaume, 2001). In general, limited data are available on the relation between thiamine requirements and the intake of carbohydrates (Lonsdale, 2006). Chromium is known to potentiate the action of insulin in association with an organometallic glucose tolerance factor (NRC, 1997). This has been evidenced by increased blood glucose clearance in rainbow trout (Bureau et al., 1995) and metabolic utilisation of glucose in carp and hybrid tilapia (Hertz et al., 1989; Shiau and Chen, 1993). This shows that the lesser known micronutrients can well be a cornerstone.

1.4.3. Environmental factors

Temperature

The physiology of fish is invariably linked to temperature because they are ectotherms. Consequently, changes in temperature can modify the processing/fate of dietary inputs. In terms of carbohydrate utilisation, an increase in the temperature of rearing water within the optimal range is often known to improve amylase activity (Alexander et al., 2011) and starch digestibility (Médale et al., 1991; Medale et al., 1999; Yamamoto et al., 2007) leading to a differential time-course of plasma glucose i.e., relatively rapid rise and fall (Brauge et al., 1995; Medale et al., 1999; Capilla et al., 2003) , higher activity of glycolytic enzymes (Enes et al., 2006a; Couto et al., 2008), glycogen stores in the liver (Hemre et al., 1995) and finally protein sparing i.e., protein retention and growth (Enes et al., 2006a; Moreira et al., 2008). This physiological sequence of better carbohydrate use at higher temperature was constructed based on available data in carp, trout, salmon, sea bass and sea bream, but this cannot be generalised because of several discrepancies amongst the different studies. Nevertheless as Brauge et al. (1995) reported, it is certain that energy metabolism is affected by an interaction between temperature and non-protein energy sources (carbohydrate and lipid). It is important to note that even at the same temperature (18^oC) there is difference in glucose metabolism/use between the carnivorous trout and omnivorous carp (Panserat et al., 2000c).

Salinity

In euryhaline and diadromous fish, the impact of changes in the salinity of rearing water on dietary carbohydrate utilisation is less known. In rainbow trout and Atlantic salmon, starch

digestibility was found to be lower in sea water than in freshwater (Storebakken et al., 1998; Krogdahl et al., 2004), which may be due to alteration in intestinal structure or function (osmoregulatory adaptation) as a similar decrease in digestibility occurs also for other nutrients. Conversely in African tilapia, intestinal glucose transport was found to be greater in seawater fish than in those acclimated to freshwater, possibly due to enhanced Na^+ binding properties, transfer rate or number of the transport proteins (Reshkin and Ahearn, 1987). In metabolism, salinity was shown to interact with the regulation of lipogenesis from radiolabelled glucose in rainbow trout previously fed different levels of dietary starch (Brauge et al. 1995b). Other studies on carbohydrate metabolism during seawater adaptation of rainbow trout show a consistent glycogenolysis in liver, white and red muscle, with the glucose produced in white muscle being used *in situ* through glycolysis (Soengas et al., 1995a; Soengas et al., 1995b). The mobilization of energy reserves is said to be indirectly related to the high energy demand of the osmoregulatory organs, but use of exogenous glucose is limited.

Stress

Stressors like repeated handling and contamination of rearing water by pesticide, heavy metal or other pollutants cause secondary changes in the fish's glucose metabolism, often measured as increased plasma glucose concentration (Hemre et al., 2002). Besides compromising the health status of the fish, these metabolic changes may intensify the postprandial hyperglycemia and ultimately result in poor use of dietary carbohydrates.

Table 3. *Blood glucose levels of carnivorous fish challenged with either an intraperitoneal (IP) injection or an oral dose of carbohydrate* (Adapted from Stone, 2003)

Species	CHO type	Admin. mode	Dose rate	Basal level (mM)	Glucose peak (mM)	Time to Peak (h)	Duration[1*]	Tolerance	Reference
Rainbow trout	Glucose	Oral	1.0	4.5	27.8	6	> 6	Intolerant	Palmer and Ryman, 1972
Atlantic Salmon	Glucose	IP	1.0	5.0	15.0	3	> 72	Intolerant	Hemre et al., 1995
Asian sea bass	Glucose	IP	1.0	4.2	10.2	8	16	Intolerant	Anderson, 2003
European sea bass	Glucose	IP	1.0	3.6	15.5	6	24	Intolerant	Peres et al., 1999
Gilthead sea bream	Glucose	IP	1.0	3.8	20.0	3	24	Intolerant	
White sea bream	Glucose	IP	1.0	4.0	19.0	2	9	Tolerant	Enes et al., 2012
Red sea bream	Dextrin	Oral	0.2	2.6	9.8	2	> 5	Intolerant	Furuichi, 1983
Yellowtail	Dextrin	Oral	0.2	6.1	10.8	3	> 5	Intolerant	
Yellowtail kingfish	Glucose	IP	1	3.9	12.8	2	48	Intolerant	Booth et al., 2013
	Glucose	Oral	1	3.3	10.8	6	18	Intolerant	
	Glucose	Oral	3	3.4	14.4	6	23	Intolerant	
	Glucose	Oral	6	3.7	22.9	6	32	Intolerant	
Turbot	Glucose	IP	0.1	4.5	13.8	3	> 24	Intolerant	Garcia-Riera and Hemre, 1996
White sturgeon	Glucose	Oral	0.1	3.9	10.2	6	24	Intolerant	Deng et al., 2001
	Maltose	Oral	0.1	3.9	8.0	6	24	Intolerant	
	Maize dextrin	Oral	0.1	3.9	6.7	6	15	Tolerant	
	Maize starch	Oral	0.1	3.9	5.3	6	15	Tolerant	
Autralian snapper	Glucose	IP	1	2.4	18.9	3	18	Intolerant	Booth et al., 2006

CHO, carbohydrate; Admin. mode, method of administration; Dose rate, g carbohydrate kg^{-1} bodyweight; [1] Duration of hyperglycemia
[*] Basal concentrations of blood glucose were not reached at the completion of the experiment

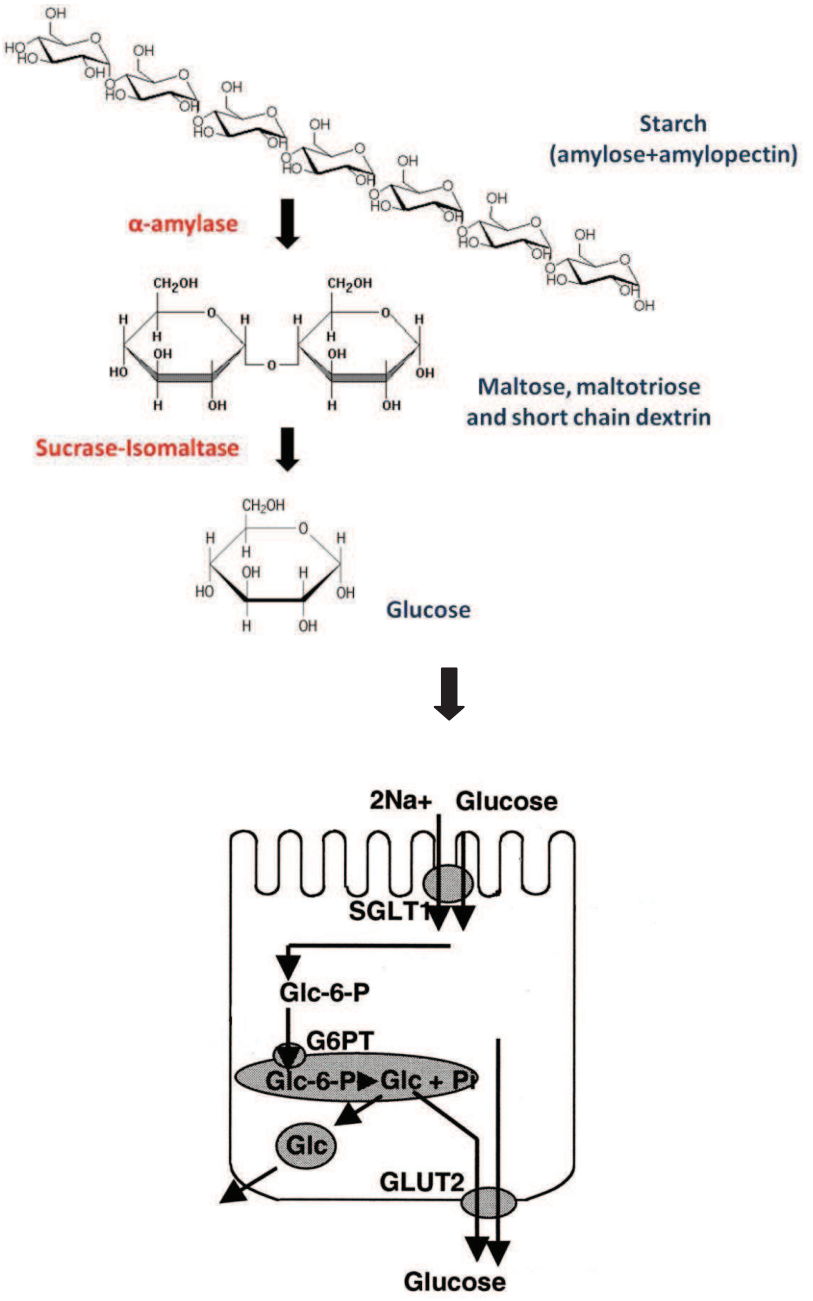
1.5. Potential limiting steps of carbohydrate use in carnivorous fish

The general fact concerning poor utilisation of carbohydrates in carnivorous fish is that, they have evolutionarily adapted their anatomy, physiology and metabolism according to their natural diet that contains very less or no starchy carbohydrates (Buddington et al., 1997; Kaushik, 2001; NRC, 2011). Consequently, after a carbohydrate rich meal they are not able to clear the glucose influx efficiently, resulting in a prolonged, high level of glucose in the blood (Wilson, 1994; Kaushik, 2001; Stone, 2003; Polakof et al., 2012). Identical and even more severe glycemic response can be observed from oral/intra-peritoneal glucose tolerance tests (GTT), and hence the clinical term 'glucose intolerant' that refers to the inability of an organism to rapidly deal with a glucose load is commonly applied to most of the carnivorous fish (Table 3). The rate of glucose loading, intensity of the blood glucose peak and clearance rate is species specific and depends on the complexity, dietary inclusion level and source of the carbohydrates (Bergot, 1979; Wilson and Poe, 1987; Hemre and Hansen, 1998; Stone, 2003). Preceding conditions like high carbohydrate intake and food deprivation also influence the outcome of the GTT (Furuichi and Yone, 1981; Mazur et al., 1992; Legate et al., 2001). Above the threshold level of glycemia, excess glucose may be excreted in the urine (glycosuria) and/or through the gills (Hemre and Kahrs, 1997; Deng et al., 2001). The most logical reason for this persistent hyperglycemia relates low body temperature, oxygen consumption and metabolic rate to the low glucose turnover and slow glucose transit observed in fish (Bergot, 1979; Moon, 2001). Other potential explanations are interactive imbalances in endocrine response/control (e.g. insulin and somatostatin), inadequate regulation of specific components of the hepatic intermediary metabolism (e.g. gluconeogenesis) and relatively meagre utilisation of glucose in the peripheral tissues i.e., muscle and adipose tissue (Moon, 2001; Enes et al., 2009; Polakof et al., 2012). The following section will detail the existing physiological and metabolic mechanism and the potential limitations that hinder the use of dietary carbohydrates in farmed carnivorous fish.

1.5.1. Digestion of starch and intestinal glucose transport

In spite of large variations in the structural and functional anatomy of the gastrointestinal tract (GIT), all the fish species investigated to date, possess the enzymatic apparatus for hydrolysis and absorption of simple and more complex carbohydrates (Krogdahl et al., 2005). The starch components, amylose and amylopectin are hydrolysed by α-amylase (an endoglucosidase secreted by the exocrine pancreas) to shorter oligosaccharides (short chain

Figure 2. *Schematic representation of starch digestion and glucose absorption in fish gastrointestinal tract*



This figure illustrates the hydrolytic steps involved in the breakdown of complex starch to glucose in the fish gastrointestinal tract by the action of pancreatic α-amylase and intestinal disaccharidases, followed by glucose absorption/transport across the apical (SGLT1) and basolateral (GLUT2) membranes of the enterocytes via specific glucose transporters. Also, an alternate means of exporting glucose to the bloodstream independent of GLUT2 is shown.

dextrins, maltotriose and maltose). These residues are further hydrolysed by various brush border enzymes (disaccharidases or glucosidases) into their constituent monosaccharides (mainly glucose), which can be transported across the villi (Kaushik, 2001; Krogdahl et al., 2005; NRC, 2011). Trans-cellular transport of glucose from the intestinal lumen to the blood stream occurs through specific transporters in the enterocyte membranes (Figure 2), namely the electrogenic, Na^+ dependent glucose symporter (SGLT1) in the brush border/apical membrane and the facilitative, Na^+ independent glucose transporter (GLUT2) in the basolateral membrane (Collie and Ferraris, 1995; Bakke et al., 2010). The existence of an alternate metabolic pathway for the export of glucose to the blood independently of GLUT but dependent on phosphorylation of glucose by hexokinase and further dephosphorylation by glucose 6-phosphatase (Mithieux, 2005), is also possible in fish. Nevertheless, paracellular passage of monosaccharides appears to be negligible (Ferraris et al., 1990).

The uniqueness of fish is that the entire length of the post-gastric gut is capable of active nutrient transport. The proximal regions of the intestine generally contribute more than the distal regions (Ferraris and Ahearn, 1984; Bakke-McKellep et al., 2000) and the glucose transporters show varying characteristics along the intestinal tract (Ahearn et al., 1992). Coherent with this, the carbohydrase activity is also known to decrease from the proximal to the more distal parts of the intestine (Stone, 2003; Krogdahl et al., 2005).

In carnivorous fish, the GIT is usually short, simple and less voluminous, apt for processing a highly digestible, nutrient dense diet that is high in protein and low in carbohydrate (Buddington et al., 1997). The main reported constraints for starch digestion and absorption in carnivores are low activity levels of α-amylase and disaccharidases (Ugolev and Kuz'mina, 1994; Hidalgo et al., 1999; Kuz'mina et al., 2008), inhibition of digestive enzymes by high level of carbohydrates (Spannhof and Plantikow, 1983; Buddington and Hilton, 1987; Krogdahl et al., 1999), low rate/capacity of intestinal glucose uptake (Buddington et al., 1987) and lack of phenotypic flexibility to modulate enzyme and transporter levels to match dietary starch levels (Buddington and Hilton, 1987; Buddington et al., 1997; Krogdahl et al., 1999; Karasov et al., 2011). Another reason can be the very low bacterial diversity or absence of amylolytic bacteria in the intestinal microbiome (Karasov et al., 2011; Ray et al., 2012).

In detail, firstly, compared to omnivorous tilapia, the total carbohydrase activity in carnivorous fish such as Atlantic salmon, rainbow trout, European sea bass and gilthead sea bream is 9, 22, 31 and 33%, respectively (Papoutsoglou and Lyndon, 2005). Particularly, the

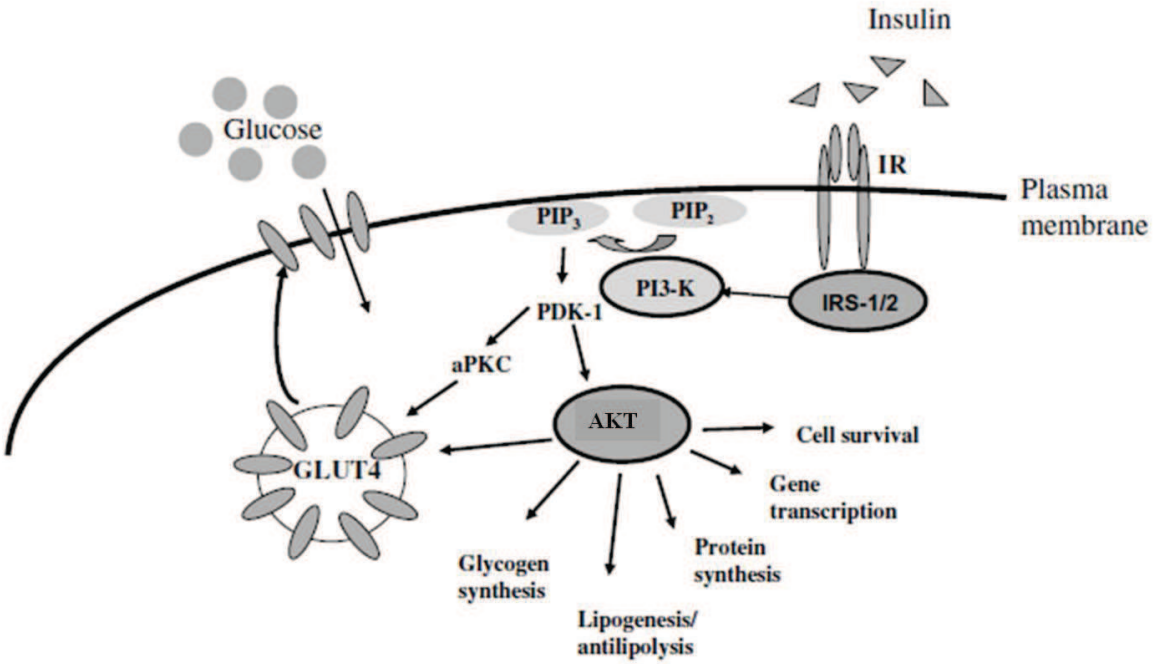
low amylase activity in Atlantic salmon can be due to the deletion of seven amino acids in one of the large loops of the enzyme in relatively close proximity to the active site that could impair substrate binding (Frøystad et al., 2006). Secondly, the inhibition of amylase activity by high carbohydrate levels can be imputed to adsorption of raw starch with the enzyme, possible presence of amylase inhibitors and accelerated intestinal transit (Spannhof and Plantikow, 1983). However, this does not apply to gelatinised starch. Thirdly, the low rate of glucose absorption can be explained partly by lower densities of transporters and partly by smaller amounts of absorptive tissue (Collie and Ferraris, 1995). Fourthly, in the wild, carnivores do not switch diets variedly like omnivores or switch between diets that differ little in substrates (Karasov et al., 2011). Because of this consistent natural diet with a low carbohydrate content, they apparently lost or never developed the capacities to adaptively modulate digestive characteristics in response to changes in diet composition i.e., high carbohydrate content (Buddington and Hilton, 1987; Buddington et al., 1997).

Digestion and intestinal uptake are thus the primary limiting steps for the efficient utilisation of a carbohydrate rich diet in carnivorous fish. Nevertheless, it is important to note that the total efficiency of carbohydrates as an energy source is not only linked to its digestibility. Even when fish digest starch well, the metabolic utilisation of absorbed glucose can be low.

1.5.2. Hormonal regulation of glucose homeostasis

Insulin and glucagon are the two major pancreatic endocrine hormones that regulate blood glucose levels and the underlying metabolism in higher vertebrates. Fish, though prone to hyperglycaemia, definitely do have these hormones with some functional similarities, but many other distinct features (Moon, 2004). Piscine insulin is structurally akin to typical vertebrate insulin, containing 51 to 58 amino acid residues, forming A and B chains. These chains are connected by two interchain disulfide bridges and one intrachain disulfide bridge through invariant cysteine residues (Mommsen and Plisetskaya, 1991). However, due to immunological differences, homologous radioimmunoassay had to be developed to measure them properly. Plasma insulin levels in fish ranges 0.2 to 5 nmol/l or even higher following a meal and it is secreted in response to several stimuli, mainly nutritional (feed intake). It performs a wide array of functions in fish (from intermediary metabolism to glucoregulation in specific tissues) mediated by insulin receptors belonging to the tyrosine kinase family, through conserved signal transduction processes e.g. IRS–PI3K–Akt/PKB (Figure 3; Mommsen and Plisetskaya, 1991; Navarro et al., 2002; Caruso and Sheridan, 2011).

Figure 3. *Insulin signalling via the PI3K-dependent pathway* (Adapted from Eriksson, 2007)



Insulin binding to its receptor will lead to the activation of a cascade of signalling proteins, which mediates/exerts the downstream effects of insulin on intermediary metabolism and activation of glucose transport. GLUT4, glucose transporter 4; IR, insulin receptor; IRS1/2, insulin receptor substrates 1 and 2; PDK1, phosphatidylinositol dependent protein kinase 1, PI3K, phosphatidylinositol-3-kinase; aPKC, atypical protein kinase C; AKT, protein kinase B PIP2, phosphatidylinositol-3,4-phosphate; PIP3, phosphatidylinositol-3,4,5-phosphate.

The existence of insulin sensitivity and intact functional mechanisms in carnivorous rainbow trout has been demonstrated by administering exogenous insulin (*in vivo* and *in vitro*) in several studies. Insulin treatment was found to reduce plasma glucose levels (Cowey et al., 1977; Polakof et al., 2010b) ; activate IRS and Akt, key elements in the signalling pathway (Plagnes-Juan et al., 2008; Seiliez et al., 2011); stimulate glucose uptake in the peripheral tissues (Capilla et al., 2004; Polakof et al., 2010b); increase oxidative clearance of glucose (Ablett et al., 1981) by activating glycolytic enzymes (Cowey et al., 1977; Petersen et al., 1987); enhance glycogenesis and lipogenesis (Ablett et al., 1981; Polakof et al., 2010a; Polakof et al., 2011b,c); and suppress gluconeogenesis and fatty acid oxidation potential (Petersen et al., 1987; Plagnes-Juan et al., 2008; Polakof et al., 2010b; Polakof et al., 2011b). In brief, trout exhibits most of the classic metabolic adjustments employed by mammals to efficiently utilise glucose in the appropriate insulin context.

Feeding rainbow trout with a high carbohydrate diet induces the expression of major components of the glucosensing system in Brockmann bodies, which is a distinct grouping of pancreatic endocrine cells near the gall bladder that secretes insulin (Polakof et al., 2008). Correspondingly, increase in plasma insulin levels as high as 8.6 nM is often found to accompany the carbohydrate rich diet induced hyperglycemia (Hilton et al., 1987; Gutierrez et al., 1989; Banos et al., 1998). This is further linked to an increase in the number of muscle insulin receptors that signifies greater insulin binding capacity (Gutierrez et al., 1989; Banos et al., 1998). All these substantiate the insulinotropic potency of dietary carbohydrates, even if it is relatively less than that of certain amino acids (Mommsen and Plisetskaya, 1991). Furthermore, data presented in a recent review (Caruso and Sheridan, 2011) suggested that in rainbow trout adapted to a high carbohydrate diet, the most glucose tolerant fish displayed the highest insulin levels, whereas the least glucose tolerant fish displayed the lowest insulin levels. Such differences in insulin levels were inversely correlated with plasma somatostatin levels. This is consistent with the fact that insulin secretion is inhibited by hypersomatostatinemia and also that somatostatin secreting cells of the trout pancreatic islets are more sensitive to glucose than the insulin secreting cells (Harmon et al., 1991; Eilertson and Sheridan, 1995; Sheridan and Kittilson, 2004). Through these findings, it is apparent that secretion and physiological action of insulin may depend on a maze of complex interactions with other hormones (Mommsen and Plisetskaya, 1991; Moon, 2001). As such, the adequacy of inherent insulin secretion to ameliorate hyperglycemia after a carbohydrate rich meal remains enigmatic in carnivorous fish. Furthermore, the very low number of insulin receptors

per microgram of membrane protein in trout muscle and the resultant low insulin receptor binding could limit insulin action in the peripheral tissue metabolism even when plasma insulin levels are high (Gutiérrez et al., 1991; Navarro et al., 1999).

Apart from insulin, the proglucagon derived glucagon and glucagon-like peptides play a significant part in altering glycemia and glucose metabolism. Glucagon is a small peptide consisting of 29 amino acids in most fish species (conserved) and it is released into circulation mainly by pancreatic alpha cells. The metabolic effects of glucagon in fish are mediated via a G protein-coupled receptor transduction system and it generally promotes cellular glucose production and elevates plasma glucose in fish, i.e., antagonistic role to insulin. These actions appear to result from the activation of specific enzymes involved glycogenolysis, gluconeogenesis and lipolysis (Plisetskaya and Mommsen, 1996; Moon, 1998; Navarro et al., 1999). After feed intake, a pattern of biphasic increases in circulating glucagon has been consistently observed in several fish species and plasma values usually range between 0.01 and 4 ng ml^{-1} (Plisetskaya and Mommsen, 1996; Navarro et al., 2002). Concerning the effect of diet composition, the postprandial glucagon levels was found to be inversely related to the carbohydrate content of the diet in rainbow trout (del Sol Novoa et al., 2004), but not in cod (Hemre et al., 1990). The adaptive response of trout glucagon to dietary carbohydrate and associated hypergycemia was independent of insulin secretion, which did not respond to dietary stimulus in this study. This emphasizes the point that the regulation of these hormones is dissociated and the inter-relationship between them is not well defined (Plisetskaya and Mommsen, 1996). Concerning glucagon like peptides (GLP-1), piscine GLP-1 is hyperglycaemic and dramatically contradicts the mammalian isoform, which is known to exert glucostatic incretin function (promote insulin secretion). It is biologically a very potent hormone in fish and plasma concentrations are higher than those of glucagon. It appears to supplant or supplement the physiological effect of glucagon, thus opposing the effects of insulin (Plisetskaya and Mommsen, 1996; Mommsen, 2000). Such major deviations in hormonal function that characterizes fish physiology may underlie glucose intolerance in fish. Beyond this, several other hormones such as insulin-like growth factor, growth hormone, somatostatins, cortisol and catecholamines may play a critical role in glucose homeostasis and needs to be thoroughly examined in the wholesome context of carbohydrate utilisation in fish (Moon, 2001; Nelson and Sheridan, 2006).

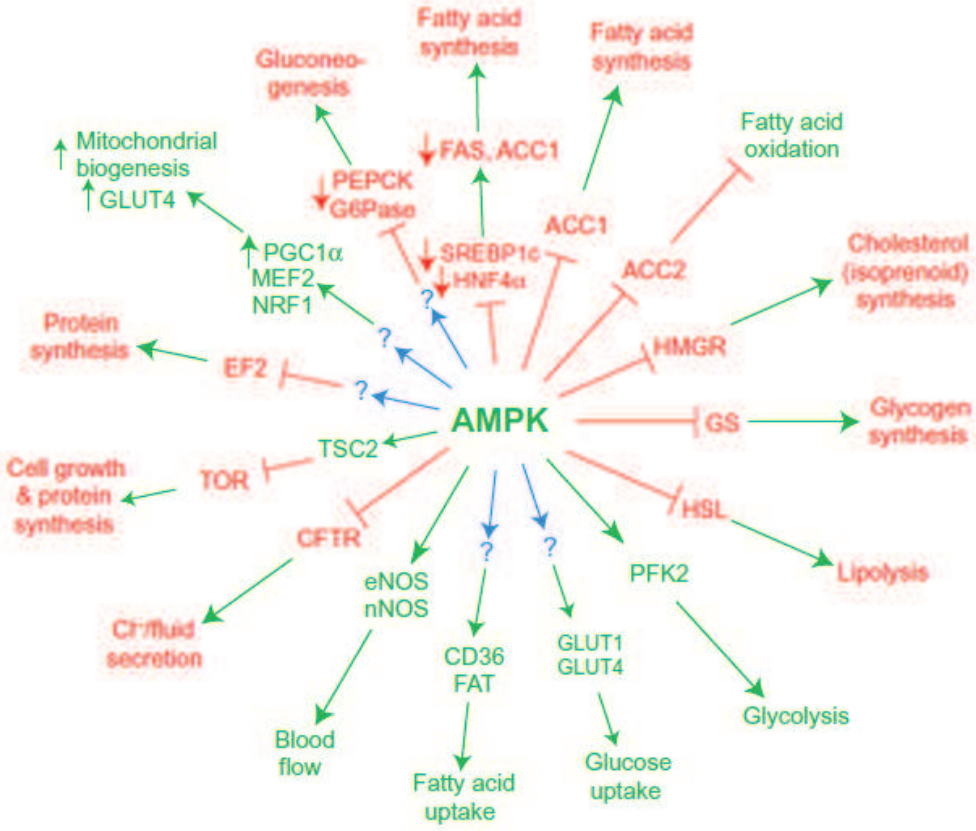
1.5.3. Glucose transport/uptake into the cells

The first and the basic step in carbohydrate utilisation is the transport of glucose from the bloodstream into the cells of the liver, muscle, adipose tissue and several other tissue/organs. Most of the cellular glucose uptake occurs passively, through the members of the facilitative glucose transporter family (solute carriers SLC2A, protein symbol GLUT). These transporters utilise the diffusion gradient of glucose across plasma membranes. The various isoforms (13) exhibit different substrate specificities, kinetic properties and tissue expression profiles (Joost and Thorens, 2001; Wood and Trayhurn, 2003; Zhao and Keating, 2007). Hitherto, four members of the class 1 sub-family of glucose transporters (GLUT1-4) have been cloned and identified in different species of fish.

GLUT1 is almost ubiquitous, responsible for the constitutive, non-insulin dependent glucose delivery into the cells and have been characterised in rainbow trout, Atlantic cod, carp and tilapia (Teerijoki et al., 2000, 2001; Hall et al., 2004; Hrytsenko et al., 2010). GLUT2 occurs primarily in liver, intestine and kidney of the rainbow trout and Atlantic cod (Krasnov et al., 2001; Panserat et al., 2001a; Hall et al., 2006). In liver, it seems to serve the bi-directional movement of glucose, depending on the dietary or hormonal status (Panserat et al., 2001a). Further in rainbow trout pancreas i.e., Brockmann bodies, it has been reported to be involved in glucose-sensing mechanism (Polakof et al., 2007). GLUT3 is substantially expressed in the kidney of grass carp and Atlantic cod, in contrast to brain in mammals, and it also appears to be distributed ubiquitously (Zhang et al., 2003; Hall et al., 2005). GLUT4 is the only insulin sensitive member of the class 1 sub-family and it is expressed in insulin sensitive tissues (heart, muscle and adipose tissue) of brown trout, coho salmon and Atlantic cod (Planas et al., 2000; Capilla, Encarnación et al., 2004; Hall et al., 2006).

In mammals, GLUT4 plays an important role in glucose homeostasis mediating insulin action to increase glucose uptake in insulin-responsive peripheral tissues. In the basal state, GLUT4 is located in intracellular compartments and upon insulin stimulation is recruited to the plasma membrane allowing glucose entry into the cell (Bryant et al., 2002). A similar intracellular GLUT4 translocation mechanism that enhances glucose uptake has been found to exist in carnivorous trout skeletal muscle cells and adipocytes (Capilla et al., 2002; Capilla, Encarnación et al., 2004; Díaz et al., 2007; Díaz et al., 2009). However, trout GLUT4 was found to have comparatively lower affinity for glucose, lesser extent of insulin stimulated recruitment to the cell surface (sequestration characteristics) and also showed differences in

Figure 4. *Metabolic targets of AMPK signalling in the cell* (Adapted from Hardie, 2004)



Target proteins and processes activated by AMPK activation are shown in green, and those inhibited by AMPK activation are shown in red.

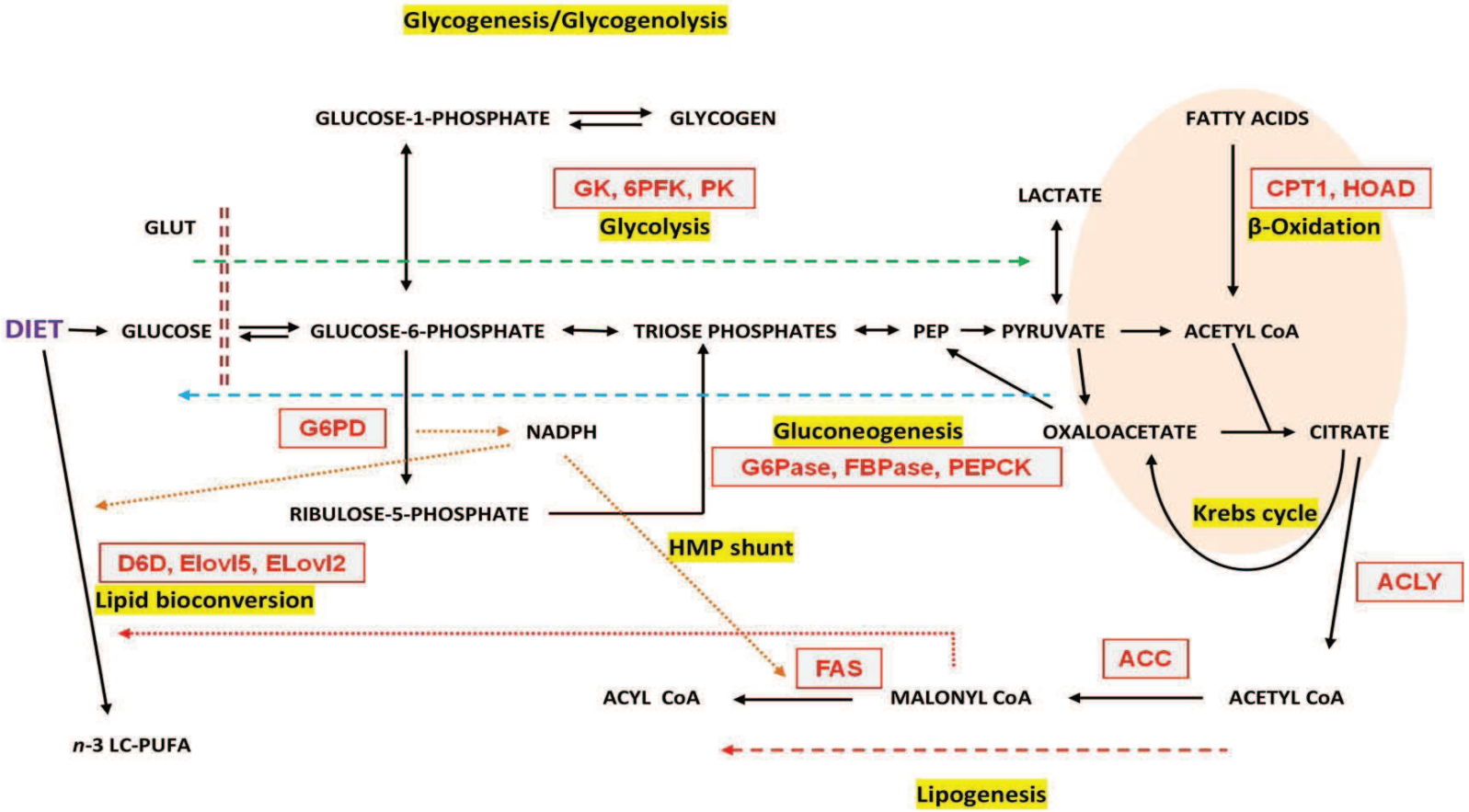
the sequence of protein motifs known to be critical for the insulin responsive trafficking (Díaz et al., 2007; Capilla et al., 2010). On the whole, it is clear that not the absence of a particular transporter (Wright Jr et al., 1998), but the above mentioned differences in kinetic characteristics of GLUTs is the possible partial explanation for glucose intolerance in trout. Concerning dietary carbohydrate utilisation, very little is known about dietary regulation of glucose transporters. In few studies, GLUT4 expression in the white muscle of rainbow trout was reported to be inert to a carbohydrate rich meal, suggesting poor ability of the peripheral tissues to adapt to a high influx of glucose (Capilla et al., 2002; Panserat et al., 2009). Further investigations are necessary to address this knowledge gap.

1.5.4. Intracellular energy/nutrient sensors

Once glucose is taken up inside the cells, it signals a shift in the nutritional status of the cell. The ensuing metabolism and use of glucose is then regulated by intracellular metabolic sensors (Figure 4). Two such sensors that are implicated in glucose homeostasis and actively being investigated in fish are AMP activated protein kinase (AMPK) and target of rapamycin (TOR). AMPK functions as a metabolic master switch, by which cells in both vertebrates and lower organisms sense and decode changes in energy status (AMP/ATP ratio). It integrates nutritional and hormonal (insulin) signals to promote energy balance by switching on catabolic pathways and switching off ATP-consuming pathways, both by short-term effects on phosphorylation of regulatory proteins and by long-term effects on gene expression (Rutter et al., 2003; Hardie et al., 2012). Similarly, TOR signalling cascade is known to integrate nutrient availability, growth factors and energy status to control cell growth by regulating translation and transcription processes in response to nutrients (Martin and Hall, 2005; Wullschleger et al., 2006). In short, AMPK is activated when energy level in the cell is low (fasted state); whereas TOR is activated in the presence of nutrients (fed state).

In rainbow trout, the activation of AMPK and TOR signalling cascade have been characterized both in the muscle and the liver (Seiliez et al., 2008; Lansard et al., 2009; Polakof et al., 2011c; Magnoni et al., 2012). Feed intake was found to decrease AMPK phosphorylation in liver, with a concomitant increase of TOR phosphorylation in liver and muscle. This supports the idea that the function of these nutrient sensors is conserved in fish (Seiliez et al., 2008; Lansard et al., 2009; Polakof et al., 2011c). The pharmacological activation of AMPK by AICAR (an analogue of AMP) and metformin induced glucose uptake and oxidation in trout myotubes (Magnoni et al., 2012). Similarly, acute *in vivo*

Figure 5. *Schematic representation of the major metabolic pathways involved in hepatic intermediary metabolism*



The target metabolic enzyme markers of the glycolytic, gluconeogenic, fatty acid synthesis, oxidation and bioconversion pathways analysed in the present study are shown in red boxes.

treatment with rapamycin, a specific inhibitor of the TOR pathway, resulted in the decrease of expression and activity of key glycolytic and lipogenic enzymes (Dai et al. unpublished). These studies suggest that downstream targets and actions of AMPK and TOR in fish are consistent with what is known in mammals, particularly the consequences on glucose metabolism. Further, macronutrient composition of the diet, i.e., reduction of protein content in favour of digestible carbohydrates was found to impair the activation of TOR (Akt/TOR) signalling pathway in the liver and muscle of rainbow trout and this was subsequently reflected in the postprandial regulation of several genes related to glucose, lipid and amino acid metabolism (Seiliez et al., 2011). All these data signifies the role of AMPK and TOR signalling cascade in the regulation of intermediary metabolism. Therefore a better understanding of this molecular basis for gene regulation is important for diet optimisation.

1.5.5. Hepatic intermediary metabolism

The liver plays a central role in controlling glucose homeostasis by serving as a consumer as well as a producer of glucose. The hepatocytes express several enzymes that are alternatively turned on or off depending on the blood glucose levels. When glucose concentrations are elevated (fed state), enzymes of the anabolic pathways of glucose disposal (glycolysis), glycogen synthesis from glucose (glycogenesis) and *de novo* synthesis of lipids (lipogenesis) are activated. When glucose concentrations are depressed (fasted state), enzymes of the *de novo* glucose synthesis (gluconeogenesis) and breakdown of glycogen reserves (glycogenolysis) are activated to supply the glucose requirements. Therefore, the net hepatic glucose uptake, which results from simultaneous regulation of glucose-producing pathways and of glucose disposal pathways, is a key determinant of blood glucose concentrations and ultimately the efficiency of dietary carbohydrate utilisation (Pilkis and Granner, 1992; Postic et al., 2004). In fish, all these metabolic pathways and component enzymes are known to exist (Figure 5), however the net regulation by nutritional and hormonal factors differs (Cowey and Walton, 1989; Hemre et al., 2002; Enes et al., 2009).

Glycolysis

It is the only major route of glucose catabolism through which progressive oxidation of one molecule of glucose into two molecules of pyruvate occurs, with a net yield of two ATP via substrate phosphorylation and two NADH [Pyruvate further goes through Kreb's cycle and oxidative phosphorylation (NADH through the latter) to produce energy (ATP)]. The flux of the glycolytic pathway is known to be regulated at three rate limiting steps catalysed by the

Table 4. *Effect of dietary carbohydrates on glucose metabolic enzyme activities (mU/mg protein) in rainbow trout* (Adapted from Enes et al., 2009)

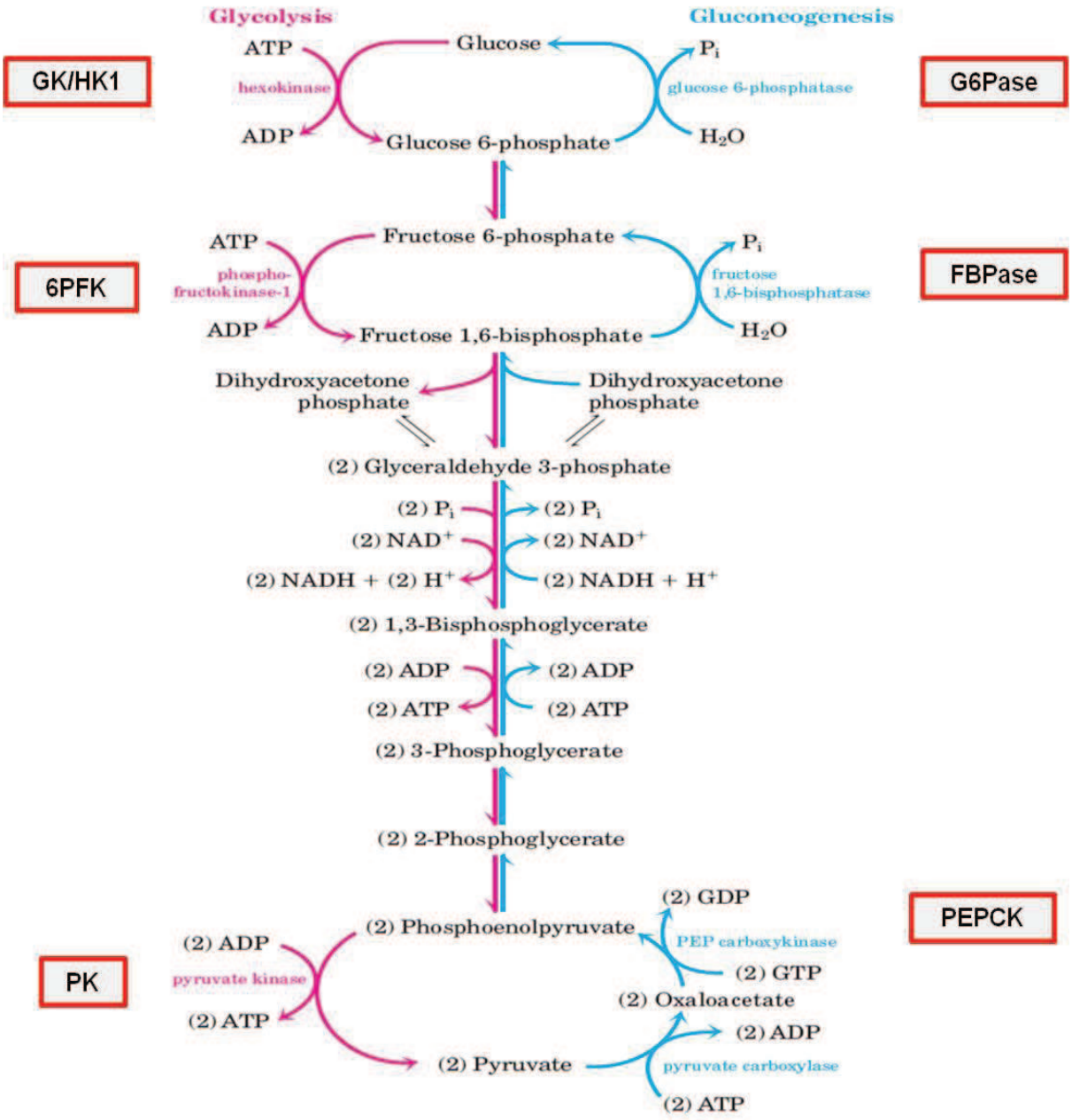
Carbohydrate source	Inclusion (%)	HK	GK	6PFK1	PK	G6Pase	Water temp. (^{o}C)	Assay temp. (^{o}C)	Reference
Dextrose (dextrinised)	26	0.4	-	12.5	200	-	12	NM	Fideu et al. (1983)
	77	0.6	-	13.8	200	-			
Control diet	0	0.4	3.3	-	-	-	18	37	Panserat et al. (2000a)
Peas starch (extruded)	20	2.1	36.7	-	-	-			
Control diet	0	-	-	-	-	15	18	30	Panserat et al. (2000b)
Peas starch (extruded)	20	-	-	-	-	22			
Control diet	0	2.3	6.2	-	-	-	8	37	Capilla et al. (2003)
Wheat starch (extruded)	20	1.9	14.3	-	-	-			
Wheat starch (extruded)	40	2.3	18.8	-	-	-			
Peas starch (extruded)	20	3.2	27.5	-	-	-			
Peas starch (extruded)	40	4.9	44.8	-	-	-			
Control diet	0	2.8	1.4	-	-	-	18	37	
Wheat starch (extruded)	20	3.7	11.8	-	-	-			
Wheat starch (extruded)	40	4	29.5	-	-	-			
Peas starch (extruded)	20	8.6	29.4	-	-	-			
Peas starch (extruded)	40	6.8	33	-	-	-			

HK, hexokinase; GK, glucokinase; 6PFK1, 6-phosphofructo-1-kinase; PK, pyruvate kinase; G6Pase, glucose-6-phosphatase; G6PD, glucose 6-phosphate dehydrogenase; NM, not mentioned

enzymes hexokinase, 6-phosphofructo-1-kinase and pyruvate kinase (Pilkis and Granner, 1992). The first reaction of cellular glucose utilisation is the phosphorylation of glucose into glucose 6-phosphate, catalysed by hexokinases (HK), which thus traps glucose in the cell for further processing downstream or through pentose phosphate pathway or glycogenesis. In mammals, four closely related hexokinase isozymes have been described. Three of them (HK I-III; EC 2.7.1.1) have a relatively high affinity (low Km) for glucose, differs in tissue distribution and are inhibited by high concentrations of glucose-6-phosphate. The fourth hexokinase, also known as glucokinase (GK; EC 2.7.1.2) is characterised by a low affinity (high Km) for glucose and a lack of inhibition by glucose-6-phosphate, thus allowing accumulation of glucose within the cells. It is expressed in the hepatic parenchyma cells and in the pancreatic β-cells, and it is under nutritional and hormonal control (Printz et al., 1993).

Early studies in fish suggested that the prolonged hyperglycemia observed after consuming a carbohydrate rich meal or after a glucose tolerance test might result from a limited glucose phosphorylation by HK and/or from the absence of an inducible hepatic GK (Cowey et al., 1977; Walton and Cowey, 1982). This hypothesis was refuted later on, as the presence of a glucokinase-like enzyme was detected in the liver of Atlantic salmon, rainbow trout, common carp and gilthead seabream (Borrebaek et al., 1993; Tranulis et al., 1996; Blin et al., 1999), cloned and characterised (Caseras et al., 2000; Panserat et al., 2000b). Since then, expression and activity of HK and GK have been detected in all fish species examined so far. The expression of HK1 isoform was detected in many tissues, whereas GK expression was restricted to the liver and the brain (Blin et al., 2000; Soengas et al., 2006). The activity of HK does not seem to be under nutritional regulation (Table 4); this can be because of its low Km and resulting saturation even at the basal blood glucose level (Wilson, 1994). On the other hand, hepatic GK expression and activity (high Km) strongly respond to changes in blood glucose levels due to dietary carbohydrate supply (Panserat et al., 2000c; Enes et al., 2009). The response of hepatic GK was found to increase proportionately with dietary starch content even in carnivorous rainbow trout, sea bass and sea bream (Capilla et al., 2003; Enes et al., 2006b, 2008a). Recently, a post-transcriptional GK regulatory protein (GKR) that inhibits/controls GK activity has been identified in rainbow trout, carp and goldfish, with biochemical and functional characteristics similar to that of mammals (Polakof et al., 2009). Trout GKR was the most potent among the analysed fish species, but functionally less active than the mammalian isoform. Could this protein have any role in glucose intolerance in carnivorous fish? This needs to be investigated. Moreover, no link between postprandial

Figure 6. *Glycolysis and gluconeogenesis pathway* (Adapted from Lehninger et al., 2005)



The reactions of glycolysis are shown on the left side in red; the opposing pathway of gluconeogenesis is shown on the right in blue. The three substrate cycles involving the rate limiting enzymes of glycolysis (hexokinase, HK; 6-phosphofructokinase-1, 6PFK; pyruvate kinase, PK) and gluconeogenesis (Phosphoenolpyruvate carboxykinase, PEPCK; fructose 1,6-bisphosphatase, FBPase; glucose-6-phosphatase, G6Pase) are also shown.

insulin levels and GK activity has been found in carp, perch and trout (Borrebaek et al., 2003; Capilla et al., 2003, Capilla et al., 2004). But, new evidence from *in vitro* studies in carnivorous gilthead sea bream suggests the existence of a mechanism in which, the transcription factor specificity protein Sp1 mediates insulin activation of GK transcription (Egea et al., 2007; Egea et al., 2008). This needs to be investigated under *in vivo* conditions under a high carbohydrate dietary regime. Overall, GK in fish liver have been proved to exist with adaptive response to carbohydrate rich diets, but more is to be known about its transcriptional and post-transcriptional regulation.

The other two rate limiting steps in glycolysis is the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate, catalysed by 6-Phosphofructo-1-kinase (6PFK; EC 2.7.1.11) and the final glycolytic reaction, i.e., transformation of phosphoenolpyruvate (PEP) to pyruvate, catalysed by pyruvate kinase (PK; EC 2.7.1.40). Nutritional status, meal frequency and high carbohydrate diets are known to affect hepatic 6PFK activity in fish (Enes et al., 2009). However, it is not always responsive to dietary carbohydrates as GK. Similarly, the nutritional regulation of PK is not completely convincing in fish, as several studies dispute the responsiveness of this enzyme to high level of dietary carbohydrates (Hilton and Atkinson, 1982; Fideu et al., 1983; Panserat et al., 2001a; Enes et al., 2009; Skiba-Cassy et al., 2013). However, experiments conducted with ^{14}C glucose in fish demonstrated that the major part of the administered glucose was catabolised, even if slowly due to the low metabolic rate (Brauge et al., 1995; Hemre and Kahrs, 1997). This clearly indicates that the enzymatic machinery required for catabolism of glucose is functional in fish, however the regulatory mechanisms maybe inadequate.

Gluconeogenesis

Fish are known to possess a very efficient gluconeogenic pathway, which comprise a series of metabolic reactions that results in the synthesis of glucose from non-glucose substrates such as lactate, glycerol (derived from the hydrolysis of triacylglycerols) and α-ketoacids (derived from the catabolism of glucogenic amino acids). While glycolysis occurs in every tissue, gluconeogenesis is specific to the liver, kidney and intestine. Hepatic glycolysis and gluconeogenesis share several enzymes which catalyse the reversible reactions that are close to equilibrium under physiological conditions. Nevertheless, there are three unidirectional regulatory steps (glycolysis - gluconeogenesis substrate cycles; Figure 6) catalysed by the enzymes phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and glucose-6-

phosphatase (Suarez and Mommsen, 1987; Pilkis and Granner, 1992; Lemaigre and Rousseau, 1994; Enes et al., 2009). To elaborate, phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) catalyses the conversion of oxaloacetate to phosphoenolpyruvate (the first step of gluconeogenesis), exists as two isoforms, cytosolic and mitochondrial, with similar kinetic properties but encoded by separate genes. Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) catalyses the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate, in an intermediate step, exists as several isoforms, all cytosolic. Glucose-6-phosphatase (G6Pase; EC 3.1.3.9) catalyses the dephosphorylation of glucose-6-phosphate to glucose, i.e., the last step of gluconeogenesis as well as glycogenolysis, so this microsomal enzyme plays a key role in glucose homeostasis.

All the three rate limiting enzymes of gluconeogenesis are known to be regulated by both hormonal and nutritional factors. For instance in higher vertebrates, glucagon and starvation are known to stimulate them, whereas insulin, refeeding and high dietary carbohydrate levels inhibits them (Pilkis and Granner, 1992). Hormonal and nutritional regulation of these enzymes in omnivorous fish such as the common carp often corresponds to higher vertebrates (Shikata et al., 1994; Sugita et al., 1999; Sugita et al., 2001; Panserat et al., 2002a). On the contrary, nutritional status and carbohydrate content of the diet do not affect any of these enzymes in carnivorous fish such as the rainbow trout, sea bass and sea bream, (Panserat et al., 2001c; Panserat et al., 2001b; Caseras et al., 2002; Enes et al., 2006b; Fernández et al., 2007; Moreira et al., 2008). Furthermore, high protein diet and glucagon are found to have more influence on gluconeogenic enzyme activities than high carbohydrate diet and insulin (Cowey et al., 1977; Foster and Moon, 1990; Kirchner et al., 2003a; Kirchner et al., 2005). Overall, this implies that the uncontrolled hepatic endogenous glucose production in carnivorous fish, regardless of the carbohydrate content of the diet, may intensify glucose intolerance and lead to poor use of carbohydrates.

Glycogen metabolism

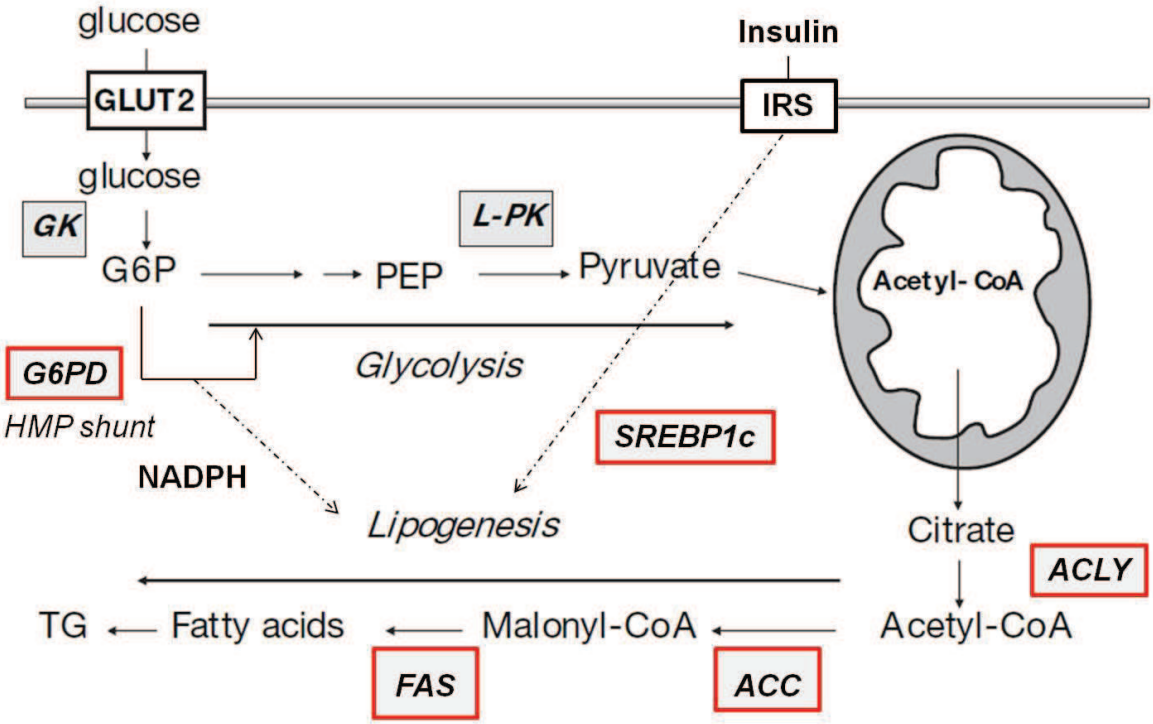
Similar to higher vertebrates, fish can store carbohydrates in the form of glycogen and by far liver is the main storage site (per unit of tissue), followed by the red muscle, brain and skeletal white muscle. Glycogen concentration in the liver can reach up to 200 mg/g of fresh tissue, depending on the feeding status and the species, whereas in white muscle it varies between 0.4 to 2 mg/g tissue. Nevertheless, on a whole fish basis, total muscle glycogen may be higher than liver glycogen, as muscle represents at least 50% of body weight. The hepatic

glycogen content serves as a universal reserve of glucose which can be used under conditions of food deprivation or low carbohydrate intake, while muscle glycogen appears to have a closed role for its performance alone (Kaushik, 2001; Hemre et al., 2002; NRC, 2011). In many studies, a linear relationship between carbohydrate intake and hepatic glycogen content have been reported (Kaushik, 2001; Enes et al., 2009). For instance, glycogenesis originating from dietary starch was found to contribute up to 69% of the liver glycogen pools in gilthead sea bream, using ^{13}C isotope-labelled starch (Ekmann et al., 2013). Concerning the rate limiting enzymes in glycogen metabolism, the synthesis of glycogen (glycogenesis) is catalysed by glycogen synthase (GSase; EC 2.4.1.11) and the breakdown of glycogen (glycogenolysis) is catalysed by glycogen phosphorylase (GPase; EC 2.4.1.1). Both these enzymes are activated through phosphorylation (GPase) and dephosphorylation (GSase) reactions (Enes et al., 2009). An *in vivo* study in rainbow trout hepatocytes indicated that both the glycogen synthetic and breakdown pathways are active concurrently and any subtle changes in the glycogen phosphorylase to synthase ratio may determine the hepatic glycogen content (Pereira et al., 1995). Nutritional status, carbohydrate intake, glucagon and insulin treatment are known to influence GPase activity (Foster and Moon, 1990; Puviani et al., 1990; Pereira et al., 1995; Soengas et al., 1996; Borrebaek and Christophersen, 2000; Sugita et al., 2001). While feed deprivation and glucagon induces GPase activity, dietary intake of carbohydrates and insulin decreases it. Insulin effect on GPase activity is not always the same (Sundby et al., 1991; Polakof, S et al., 2010a). On the other hand, hormonal and nutritional control of GSase appears to be limited. Effect of dietary carbohydrates on GSase activity has not been demonstrated, but indirect evidence from high glycogen levels indicates that it is functional and responsive to dietary manipulations.

Lipid biosynthesis

The capacity of higher vertebrates to store surplus carbohydrates (consumed in excess of oxidative needs) as glycogen is limited and there is no other direct mechanism for storage. Therefore, the major choice for the animal (other than excretion) is to resort to *de novo* lipogenesis (DNL; a kind of metabolic safety valve) for storing excess carbohydrate energy as lipids in the liver and adipose tissue (Hellerstein et al., 1996). DNL is a highly regulated, complex cytosolic polymerization process that begins with acetyl-CoA as the initiating primer, followed by synthesis/activation of malonyl-CoA and repeated sequential addition/condensation of malonyl-CoA to the growing acyl chain to finally form the 16-carbon fatty acid palmitate. Acetyl-CoA carboxylase (ACC; EC 6.4.1.2) and the large multi-

Figure 7. *Hepatic de novo lipid biosynthesis pathway* (Adapted from Postic et al. 2004)

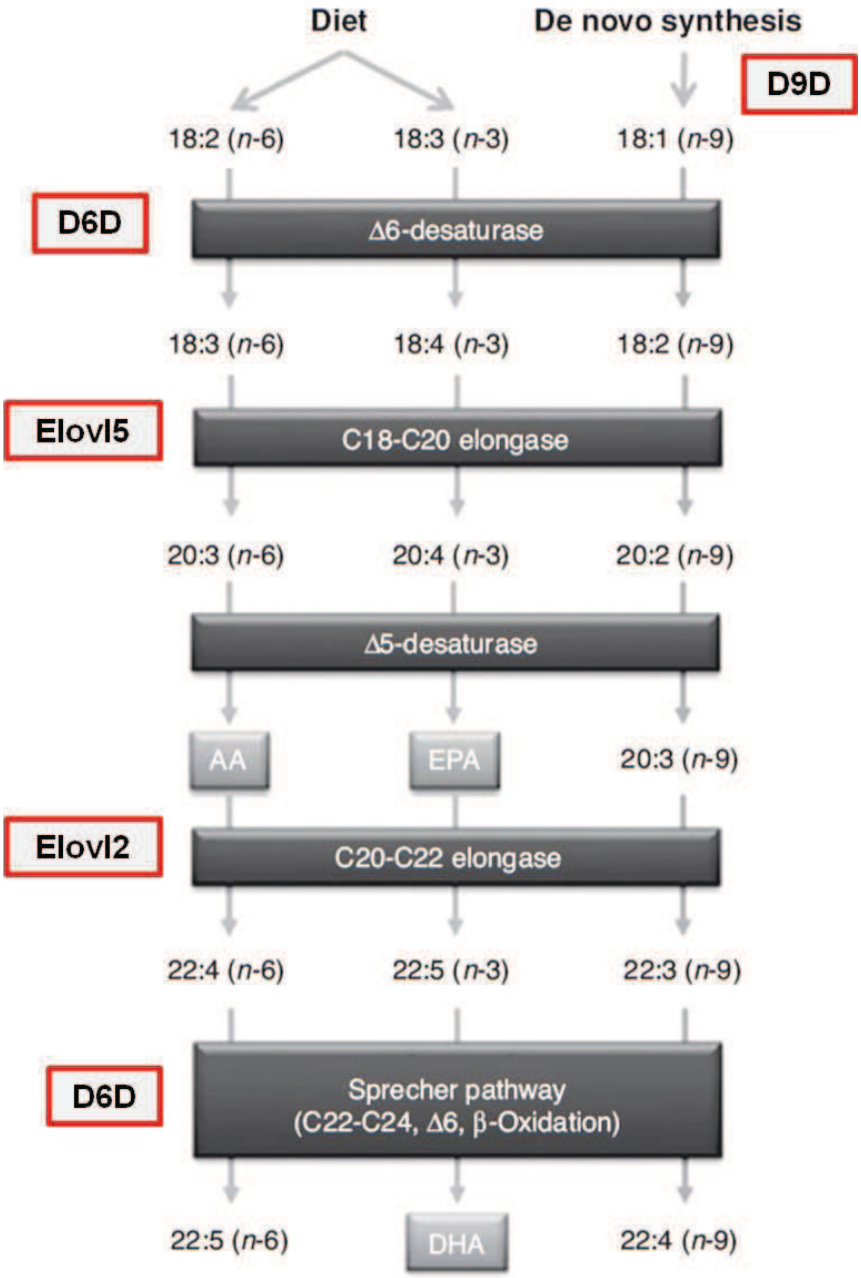


This figure illustrates the flux of carbon from glucose into *de novo* lipogenesis through glycolysis. The rate limiting steps catalysed by ATP citrate lyase (ACLY), acetyl CoA carboxylase (ACC), fatty acid synthase (FAS) and the insulin mediated transcriptional regulation of lipogenesis through SREBP1c is shown. Further, the supply of key reducing equivalent NADPH generated in the oxidative branch (glucose-6-phosphate dehydrogenase; G6PD) of the hexose monophosphate shunt, is critical for this process.

subunit fatty acid synthase (FAS; EC 2.3.1.85) are the key regulatory enzymes of DNL (Hellerstein et al., 1996; Strable and Ntambi, 2010). Glucose, as one of the main precursors of acetyl-CoA can directly provide carbon backbones to DNL via glycolysis, pyruvate dehydrogenase and ATP citrate lyase (ACLY; EC 2.3.3.8) enzymes (Figure 7). But more importantly, when glucose is oxidised through the 'alternate' pentose phosphate pathway, it yields NADPH, the key reducing co-factor which is absolutely essential for DNL. Specifically, glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGD; EC 1.1.1.44) are the two NADPH generating enzymes of the pentose phosphate pathway's oxidative branch (Girard et al., 1997; Towle et al., 1997). In mammals, a carbohydrate rich diet intake was found to stimulate all these DNL and NADPH yielding enzymes, consistently (Hellerstein et al., 1996).

In the same way as in higher vertebrates, DNL pathway has been proved to exist in fish (Sargent et al., 2002). The link between dietary carbohydrate intake and hepatic DNL has been demonstrated in several fish species through fat deposition (lipid retention above 100%) or elevated activities/expression of NADPH yielding (mainly G6PD) and lipogenic enzymes (Lin et al., 1977a; Hilton and Atkinson, 1982; Likimani and Wilson, 1982; Kaushik et al., 1989; Brauge et al., 1995; Alvarez et al., 2000; Hemre, 2002; Dias et al., 2004; Panserat et al., 2009). However, few studies that examined the recovery of radio-labelled glucose or stable isotope labelled starch into lipid fractions in carnivorous fish such as Atlantic salmon, Atlantic cod, hybrid striped sea bass and gilthead sea bream led to the conclusion that the amount of DNL from glucose is quite limited (Hemre and Kahrs, 1997; Hemre and Storebakken, 2000; Rawles et al., 2008; Ekmann et al., 2013). According to these studies, the possible explanation for enhanced fat deposition in fish fed carbohydrate rich diets may be then that glucose could increase lipid deposition from dietary lipid by being oxidised instead of dietary lipid, thus reducing the contribution of lipid to oxidative metabolism, as reported in humans (Hellerstein, 2001; McDevitt et al., 2001). It was also suggested that the role of carbohydrates in DNL is more for the production of cytosolic reducing equivalents (NADPH) than for the delivery of carbon backbones (acetyl-CoA) for DNL (Hemre et al., 2002). However, these two postulations cannot sufficiently explain the >100% lipid retention observed in rainbow trout fed a diet with 38% digestible carbohydrates (Kaushik et al., 1989). The contribution of glucose to lipid synthesis and its role in the regulation of lipid oxidation deserve further attention, as rightly pointed out in NRC (2011). Thorough investigations of the glucose-fatty acid cycle in fish (Randle, 1998; Hue and

Figure 8. *Fatty acid bioconversion pathway* (Adapted from Skiba-Cassy et al., 2012)



This figure illustrates the key desaturation/elongation steps and the corresponding enzymes involved in the synthesis of polyunsaturated fatty acids such as AA, arachidonic acid (20:4 *n*-6); EPA, eicosapentaenoic acid (20:5 *n*-3); DHA, docosahexaenoic acid (22:6 *n*-3). The target enzyme markers analysed in the present study were Δ6 fatty acyl desaturase (D6D), Δ9 fatty acyl desaturase (D9D) and elongases (Elovl5 and Elovl2).

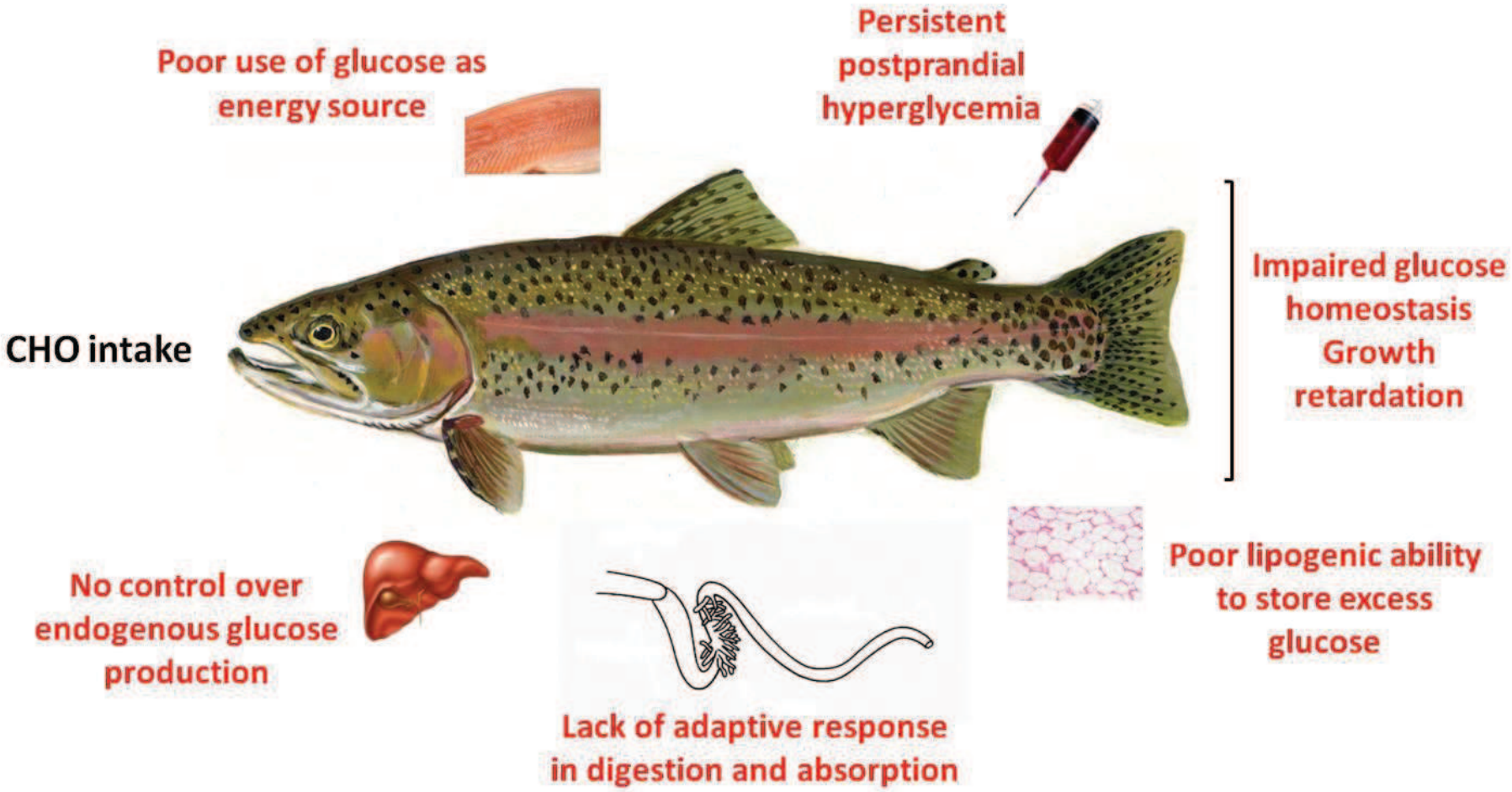
Taegtmeyer, 2009) are needed to enlighten. Furthermore, recent studies using metformin, an anti-diabetic drug that reduces blood glucose level (Panserat et al., 2009) or selected rainbow trout genotypes (Skiba-Cassy et al., 2009) have provided evidence of a relationship between the expression of lipogenic enzymes and the regulation of glucose homeostasis. Overall, DNL could be an important aspect of maximising carbohydrate utilisation in carnivorous fish.

Concerning regulation, the DNL enzymes are controlled at the transcriptional level through several factors such as the sterol regulatory element-binding protein-1c (SREBP-1c), liver X receptor (LXR) and the carbohydrate response element binding protein (ChREBP). In particular, the role of SREBP-1c in mediating the hormonal (insulin and glucagon) and nutritional (dietary carbohydrates) regulation of genes encoding the lipogenic (ACLY, ACC, FAS, D9D) and NADPH yielding (G6PD, 6PGD) enzymes has been conclusively demonstrated in a number of mammalian studies and moreover, there is specific evidence for the presence of glucose responsive element in the promoter region of lipogenic genes (Girard et al., 1997; Towle et al., 1997; Horton et al., 2002; Strable and Ntambi, 2010).

In rainbow trout liver, similarly, SREBP-1c expression was found to be induced by insulin treatment (Lansard et al., 2010; Polakof et al., 2010) and re-feeding (Skiba-Cassy et al., 2009), which was further linked to elevated expression of lipogenic enzymes. However, the regulatory response of SREBP-1c to dietary carbohydrates appears to be poor (Seiliez et al., 2011). Moreover, glucose responsive elements can be expected in the upstream regions of the lipogenic genes in fish (Hemre et al., 2002), but it is not yet identified. The regulatory mechanisms involving ChREBP is another critical area that needs to be understood not only in terms of DNL, but for the entire carbohydrate metabolism in fish.

Finally, it is probable that this well known association of carbohydrate intake and DNL may extend also to fatty acid bioconversion (n-3 LC-PUFA biosynthesis), through the supply of reducing equivalents and substrates (Poisson and Cunnane, 1991; Cook and McMaster, 2002). In fact, carbohydrate intake was found to increase the expression of Δ6 fatty acyl desaturase (D6D; EC 1.14.19.3) in rainbow trout (Seiliez et al., 2001). This elicited our interest to study the effect of dietary carbohydrates on the key enzymes involved in fatty acid bioconversion (Figure 8) and the related fatty acid phenotype.

Figure 9. *Summary of major metabolic constraints that hinder dietary carbohydrate utilisation in carnivorous fish*



1.5.6. Glucose utilisation in peripheral tissues

In mammals, skeletal muscle and adipose tissue are the two principal insulin sensitive peripheral sites that are uniquely designed for use and disposal of a glucose load (Zierler, 1999). However in fish, the situation seems to be contradicting and limited utilisation of available glucose in the peripheral tissues could probably be the key reason for the persistent hyperglycemia (Moon, 2001). The major uncertainties were concerning the number of insulin receptors (IR), presence of insulin dependent glucose transporters (GLUT4), their trafficking properties and finally the capacity of glucose phosphorylation, oxidation and storage.

Compared to mammals, fish has very less IR per unit weight of all the major insulin sensitive tissues, which suggests an overall lower insulin binding capacity. Moreover, the IR number differs among fish species based on feeding habits, i.e., lesser in carnivorous trout than in omnivorous carp (Parrizas et al., 1994) and also differs between tissues i.e., lesser in muscle than in the liver (Navarro et al., 1999), which suggests very low insulin sensitivity in the muscle of carnivorous fish with possible consequences for the regulation of glucose metabolism. Intake of dietary carbohydrates was however found to enhance IR number in rainbow trout muscle without modifying its affinity (Banos et al., 1998). Alas, metabolic implications were not investigated further in this study. Chronic treatment with physiological dose of insulin was found to activate Akt signalling in the muscle of rainbow trout fed high carbohydrate diet, but downstream regulation of glycolytic gene expression remained unaltered (Polakof et al., 2010), indicating incomplete functional mechanism.

Similarly, the rate of glucose transport in fish muscle is at the very low end of determined values for other vertebrates (Hemre et al., 2002). Recent studies have demonstrated the existence of insulin sensitive GLUT4 in trout muscle that undergoes translocation from intracellular vesicles to the plasma membrane (Diaz et al., 2007, 2009). However, the kinetics and trafficking characteristics of GLUT4 that affects the whole sequestration process appears to be relatively less effective in fish when compared to mammals (Capilla et al., 2010). White muscle GLUT4 expression was not regulated by dietary carbohydrates (Capilla et al., 2002).

Contribution of skeletal muscle disposal of glucose was less than 15% of the total glucose turnover in rainbow trout (West et al., 1994) and from a tissue wise comparison in Atlantic cod, white muscle was found to have the lowest glucose utilisation ability (Hemre and Kahrs, 1997). Both these observations could be related with low activities of glycolytic enzymes in

muscle (Knox et al., 1980). Further, the presence of carbohydrates in the diet was not found to affect the activity of glycolytic enzymes in white muscle of rainbow trout (Panserat et al., 2009), eventually suggesting its lack of ability to adapt to changes in blood glucose level. Concern over the poor response in muscle glucose metabolism originates from the fact that it represents 50% of total body weight and thus the largest disposal unit for any metabolite.

From the adipose tissue of coho salmon, insulin sensitive GLUT4 have been cloned and characterised. It is structurally and functionally homologous to mammalian GLUT4, but has a lower affinity for glucose (Capilla et al., 2004). The presence of a facilitative glucose transporter has also been ascertained in the adipose tissue of rainbow trout (Bouraoui et al., 2010). Even so, glucose metabolism was not found to be coherent in rainbow trout treated with insulin and fed a high carbohydrate diet (Polakof, S et al., 2010b). Expression of glycolytic and lipogenic genes showed inconsistent regulation. But contrary to early studies in salmonids that stated lipogenic enzyme activities in the adipose tissue are low and unresponsive (Lin et al., 1977), chronic insulin treatment in rainbow trout was found to enhance the activity of FAS in a dose dependent manner, concurrently with a decrease in the expression of β-oxidation enzyme CPT1 isoforms (Polakof et al., 2011b). Despite several limitations, all these new findings opens up possibilities and any improvement in glucose metabolism in the peripheral tissues will definitely have a pronounced effect on the overall utilisation of dietary carbohydrates. Therefore, it is imperative to conduct inclusive investigations on glucose metabolism in liver, muscle and the adipose tissue to gain wholesome understanding. This concern was also addressed in the present doctoral study.

1.6. Comparison of carbohydrate metabolism in different carnivorous vertebrates

In their natural habitat, obligate carnivorous animals such as cat, mink (mammals), shrike, barn owl (birds), trout, sea bass, sea bream, salmon and yellow tail (fish) consume prey high in protein with moderate amounts of fat and minimal carbohydrates and are therefore metabolically adapted to lower glucose utilization and higher protein metabolism. The benefit of phenotypic or metabolic flexibility is low or nil in carnivores and they are known to exhibit a greater and prolonged hyperglycemia than their omnivorous or herbivorous counterparts, following a glucose challenge or a high carbohydrate diet (Myers and Klasing, 1999; Panserat et al., 2000c; Hewson-Hughes et al., 2011).

A closer comparison between cat and trout show several commonalities in their ability to digest and metabolise carbohydrates. As in trout, cat has a relatively short colon and small intestine, low activity of pancreatic amylase and other intestinal carbohydrases, non-adaptive intestinal glucose transport, amino acids are more potent stimulators of insulin secretion and endogenous glucose production through gluconeogenesis is high and unregulated (Verbrugghe et al., 2012). However in cat, hepatic glucokinase (GK) activity/expression is minimal or absent (Washizu et al., 1999; Tanaka et al., 2005) and hepatic *de novo* lipogenesis does not use carbon backbones from glucose at all (Richard et al., 1989; Verbrugghe et al., 2013), which contradicts findings in trout that GK is indeed induced by dietary carbohydrates (Panserat et al., 2000c) and *de novo* lipogenesis can occur from exogenous glucose (Brauge et al., 1995). In other carnivores, findings on glycolysis are similarly not consistent. The glucose intolerant phenotype of barn owl is linked to low hepatic GK activity (Myers and Klasing, 1999), whereas glycolytic enzyme activities of mink are even higher than in omnivorous rat (Sorensen et al., 1995).

Nevertheless, cutting across vertebrate classes, two features are highly consistent in carnivores: (1) the ratio of glucose to proline absorption in the intestine is always lower compared to omnivores and herbivores of the respective classes (Karasov and Diamond, 1988; Karasov et al., 2011), suggesting low adaptation in intestinal glucose uptake (2) most importantly, gluconeogenesis is high and inadequately regulated even in the presence of exogenous glucose (Sorensen et al., 1995; Myers and Klasing, 1999; Washizu et al., 1999; Panserat et al., 2000a; Tanaka et al., 2005; Enes et al., 2011). Recently, it was suggested that cats do not have a higher protein requirement per se, but rather a high endogenous glucose demand that is met by obligatory amino acid-based gluconeogenesis (Eisert, 2011),

Table 5. *Nutrient requirements of rainbow trout* (adapted from NRC 2011 and Hardy, 2002)[a]

Protein/Energy components		Non-Energy components	
Amino acids (%)		*Fat soluble vitamins*	
Arginine	1.5	A (mg/kg)	0.75
Histidine	0.8	D (µg/kg)	40
Isoleucine	1.1	E (mg/kg)	50
Leucine	1.5	K (mg/kg)	R
Lysine	2.4	*Water soluble vitamins (mg/kg)*	
Methionine + cystine	1.1	Thiamin	1
Phenylalanine + tyrosine	1.8	Riboflavin	4
Threonine	1.1	Vitamin B_6	3
Tryptophan	0.3	Pantothenic acid	20
Valine	1.2	Niacin	10
Fatty acids (%)		Biotin	0.15
18:3 n-3	0.7-1.0	Vitamin B_{12}	R
n-3 LC-PUFA[b]	0.4-0.5	Folic acid	1
18:2 n-6	1.0	Choline	800[d]
Cholesterol (%)	NT	Myoinositol	300[d]
Phospholipids (%)	NT (4-14)[c]	Vitamin C	20
		Macrominerals (%)	
Digestible energy (kcal/kg)	4200	Calcium	NR
Digestible protein (%)	38	Phosphorus	0.7
Digestible lipids (%)	16-24	Magnesium	0.05
Carbohydrates (%)	NR	Sodium	NR
		Potassium	NT
		Chlorine	NT
		Trace elements (mg/kg)	
		Copper	3
		Iodine	1.1
		Iron	NT
		Manganese	12
		Selenium	0.15
		Zinc	15

NT, not tested.
NR, not required under practical conditions; R, required in diet but quantity not determined.
[a] Values are expressed in dry matter basis and represent near 100% bioavailability.
[b] 20:5 n-3 and 22:6 n-3.
[c] Values in parentheses represent requirements reported for larval/early juvenile stages.
[d] Diet without phospholipids

which appears to be true even in trout (Kirchner et al., 2003a). Supporting this, recently in European sea bass, the contribution of endogenously derived glucose to the total blood glucose levels was found to be very high even in fed state (Viegas et al., 2013). Thus, the clear linkages in the carnivorous characteristics of different vertebrate classes enable rainbow trout and other carnivorous fish to serve as a good carnivorous model in general nutritional studies focussing on glucose metabolism and use.

1.7. Rainbow trout: The ideal carnivorous fish model to investigate new strategies to improve dietary carbohydrate utilisation

In particular, the rainbow trout (*Oncorhynchus mykiss* Walbaum; family: Salmonidae) in their natural habitat prey on zooplankton as fry, followed by insects, crustaceans and other fish as they grow. So, they are more prone to use dietary protein/amino acids as the major source of metabolic energy and restricted in their ability to use carbohydrates. Consequently in culture, inclusion of carbohydrates in trout diets is always limited at a level lower than 20% to avoid metabolic complications and growth retardation. To change this scenario, we need to gain a better understanding of its glucose metabolism. Being one of the most intensively studied, widely cultured and best understood fish species in terms of nutritional requirements (Table 5), physiology and biology, the vast amount of pre-existing knowledge serves as a solid platform for advanced research. In addition, the well characterised chromosomal, protein, molecular and quantitative genetic variation in rainbow trout, makes it suitable for a cross-disciplinary approach such as diet × genotype interaction studies (Gall and Crandell, 1992; Thorgaard et al., 2002; Hardy, 2002; Panserat et al., 2013).

1.8. Diet and genotype interaction: Is it significant for sustainable aquaculture?

Following the recognition that nutrients have the ability to interact and alter genetic mechanisms underlying an organism's physiological functions, the field of nutrition has been revolutionised and studies have begun to unravel the complex relationship between nutritional molecules, genome, genetic polymorphisms, and the biological system as a whole. The inter-relationship or interaction between diet and genotype is known to be bidirectional. On one hand, diet (nutrients) can affect gene expression and on the other hand, genes (genotype) can alter the effect of dietary intake. The former is known as nutrigenomics and it involves the use of functional genomic tools to probe a biological system following a nutritional stimulus (often employed in fish nutrition studies these days). The latter is known as nutrigenetics and it describes how an individual's genetic makeup coordinates their physiological or cellular response to dietary components, in terms of digestion, absorption, metabolism and partitioning of nutrients (Mutch et al., 2005; Rimbach and Minihane, 2009).

In humans, nutrigenetics denotes 'personalised nutrition', a concept in which genotyping is used as a means to define dietary recommendations to suit the individual, mainly to prevent chronic diseases due to single nucleotide polymorphisms, copy number polymorphisms or epigenetic modifications (Kaput and Rodriguez, 2004; Joost et al., 2007; Gibney and Walsh, 2013). In companion animals like dogs, there are instances where diets are formulated according to genotype (specific diet for large breed dogs) and could serve as a frontrunner to many farmed animals in the future (Swanson et al., 2003).

In fish nutrition, few studies have tested the concept even before the term 'nutrigenetics' was coined. These early studies investigated nutrient by genotype interactions based on phenotypic responses. For instance, family specific growth response to varying levels of dietary carbohydrates was evaluated in rainbow trout (Austreng et al., 1977; Edwards et al., 1977; Refstie and Austreng, 1981). Little variation between different fish families and no interaction between fish family and diets was found for growth and other performance indices, this led to the suggestion that prospects for selectively breeding strains of rainbow trout specifically better able to utilise carbohydrates are not promising. Later on it was found that, three natural strains of chinook salmon responded to high levels of digestible starch inclusion in the diet with different degrees of growth, feed efficiency (degrees of reduction) and accumulation of liver glycogen. Remarkably, they exhibited clear differences in glucose tolerance though not well correlated with plasma insulin levels (Mazur et al., 1992). Another

minor diet dependent familial variance was observed in rainbow trout and was found to result mainly from relative performances with carbohydrate (Blanc, 2002). Phenotypic response criterions thus indicate a mixed result for genotype by diet interaction in carbohydrate utilisation in carnivorous fish; however, all these studies (except Mazur et al., 1992) were limited in the spectrum of analysis. With the recent advances in biochemistry and molecular biology, integrating molecular and phenotypic response will be an ideal situation to explore diet × genotype interactions in glucose metabolism and use with the objective of characterising fish genotypes or families with better ability to use dietary carbohydrates.

Apart from carbohydrates, family based responses in dietary protein utilisation have been investigated. Based on family × diet interactions observed for growth indices, it was suggested that prospects for selectively breeding strains of rainbow trout specifically better able to utilise protein are promising (Austreng and Refstie, 1979). These observations were further strengthened by the finding that trout of families with better growth rates and food conversion ratios excreted much less nitrogen (Kaushik et al., 1984), clearly indicating familial variation in biochemical utilisation of dietary proteins. Other studies reported genotypic differences in gastrointestinal protease activity (Torrissen and Torrissen, 1984) and apparent protein digestibility estimates (Medale, 1993; Valente et al., 1998; Rasmussen and Jokumsen, 2009; Rungruangsak-Torrissen et al., 2009), correlated with growth. But no elaborate attempts have been made to elucidate the physiological mechanisms further.

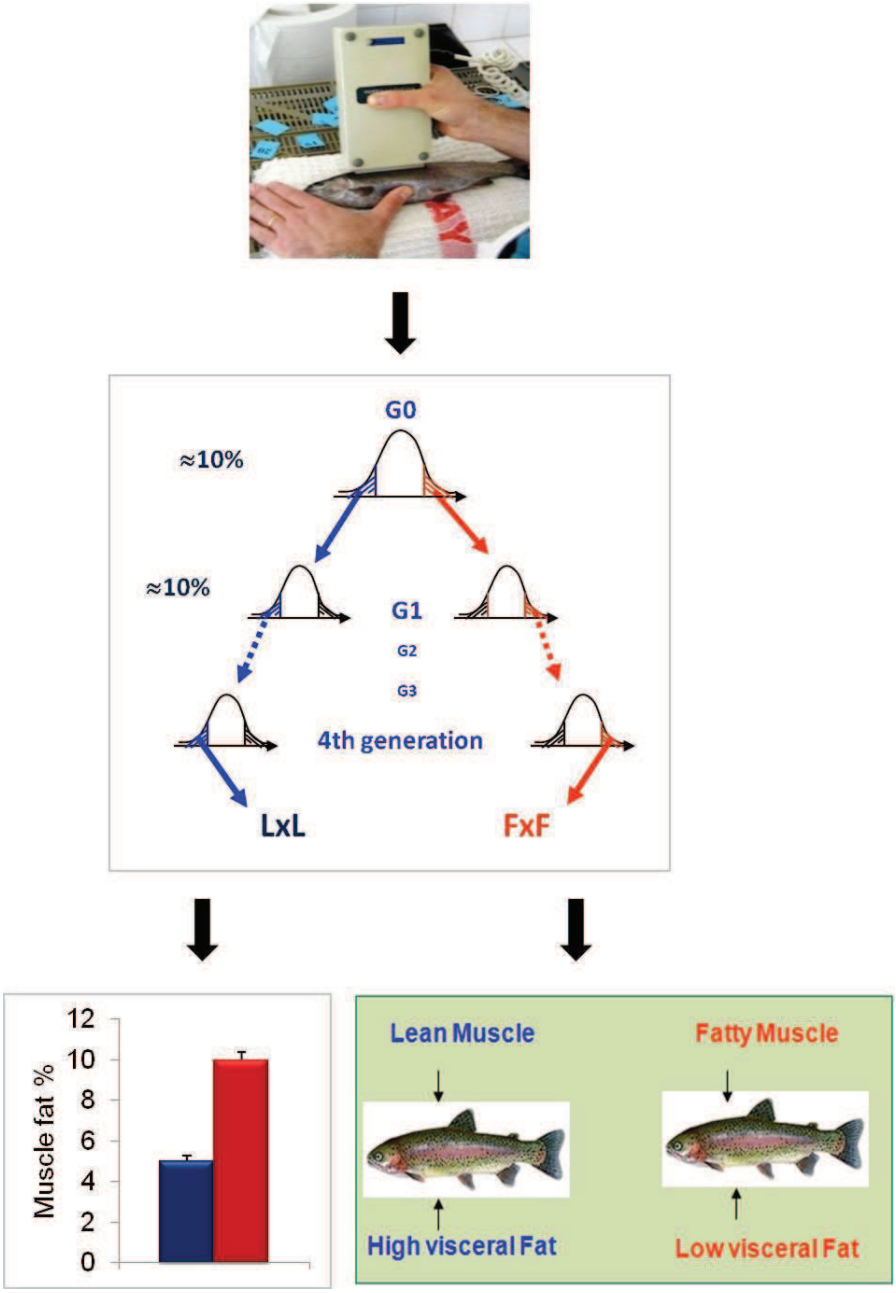
With the changing focus on plant based diet formulations, many recent studies explored diet × genotype interaction in growth traits to develop strains with better adaptability to survive and grow when fed diets with partial or complete substitution of fish meal/oil by plant proteins and oils. Though few studies in rainbow trout, European sea bass and white fish have not yielded positive results (Palti et al., 2006; Quinton et al., 2007; Le Boucher et al., 2013), others have reported substantial genetic variation in rainbow trout for utilising plant-based diets (Pierce et al., 2008; Dupont-Nivet et al., 2009; Le Boucher et al., 2011), with ultimately the possibility to select fish with better growth and survival when fed complete plant based diets (Le Boucher et al., 2012; Overturf et al., 2013). Diet × genotype interaction has also been analysed in terms of meat quality parameters such as muscle lipid content (Kolditz et al., 2008a) and n-3 LC-PUFA content (Bell et al., 2010). Energy rich feed was suggested to be a potential driver for the positive genetic relationship between growth and lipid deposition (Kause et al., 2007), but not many interactions between dietary energy content and genotype

were found in metabolic aspects (Kolditz et al., 2008a). Concerning n-3 LC-PUFA content, genetics was found to affect lipid deposition and metabolism and suggestions were made that breeding programmes could select for fish that retained more n-3 LC-PUFA in their flesh, particularly when fed diets low in these fatty acids (Bell et al., 2010; Leaver et al., 2011). More recently transcriptomic techniques have been employed to screen metabolic markers that respond to dietary alterations in fish meal/oil, depending on the genetic background of the fish (Geay et al., 2011; Morais et al., 2011a; Morais et al., 2012). Diet × genotype interactions have been observed in markers related to protein turnover and immune response in sea bass liver (Geay et al., 2011); transcriptional regulation of lipid metabolism and in particular, markers related to pathways of cholesterol, lipoprotein and LC-PUFA synthesis in Atlantic salmon liver and intestine (Morais et al., 2011a, b; Morais et al., 2012).

From all these findings, it is obvious that most fish species have large amount of intra-specific genetic variations within populations (wild as well as domesticated) and even within selectively bred families. These evidences of genetic variation support the future prospects to investigate the nutrigenetics concept, in terms of better utilisation of specific nutrients such as carbohydrates or whole diets. Compared to wild strains, selectively bred fish can be good models to study nutrient and genotype interaction. It offers not only basic genetic information and control over genetic variation, but also the scope to undertake sustained, long term studies (trans-generational) with almost similar genetic material.

Less than 10% of aquaculture production is based on genetically improved stocks at present, despite the fact that genetic gains reported for aquatic species are substantially higher (12.5% per generation in growth rate) than that of other farmed animals (Gjedrem et al., 2012). Until recently, selection traits in fish breeding programmes were mainly concentrated on output factors such as growth, age at maturation, meat composition, nutritional quality and disease resistance. But now, traits that directly affect input costs, such as those related to the efficiency of feed utilisation are receiving attention following exhaustive evidence (Silverstein et al., 1999; Thodesen et al., 1999; Henryon et al., 2002; Kolstad et al., 2004). Fish are also known to possess moderate to high heritability for many of these production traits (Gjedrem, 2000; 2010). All these facts points out the necessity to use selective breeding as a tool to improve the biological efficiency of aquaculture production. Weller (2006) stated that investment in breeding is unique because genetic gains are eternal and cumulative. They are never “used up”, and never “wear out”, indeed a remarkable point to ponder.

Figure 10. *Divergent selection procedure used to develop the fat and lean rainbow trout lines* (Adapted from Quillet et al., 2005 and Kolditz et al., 2008a)



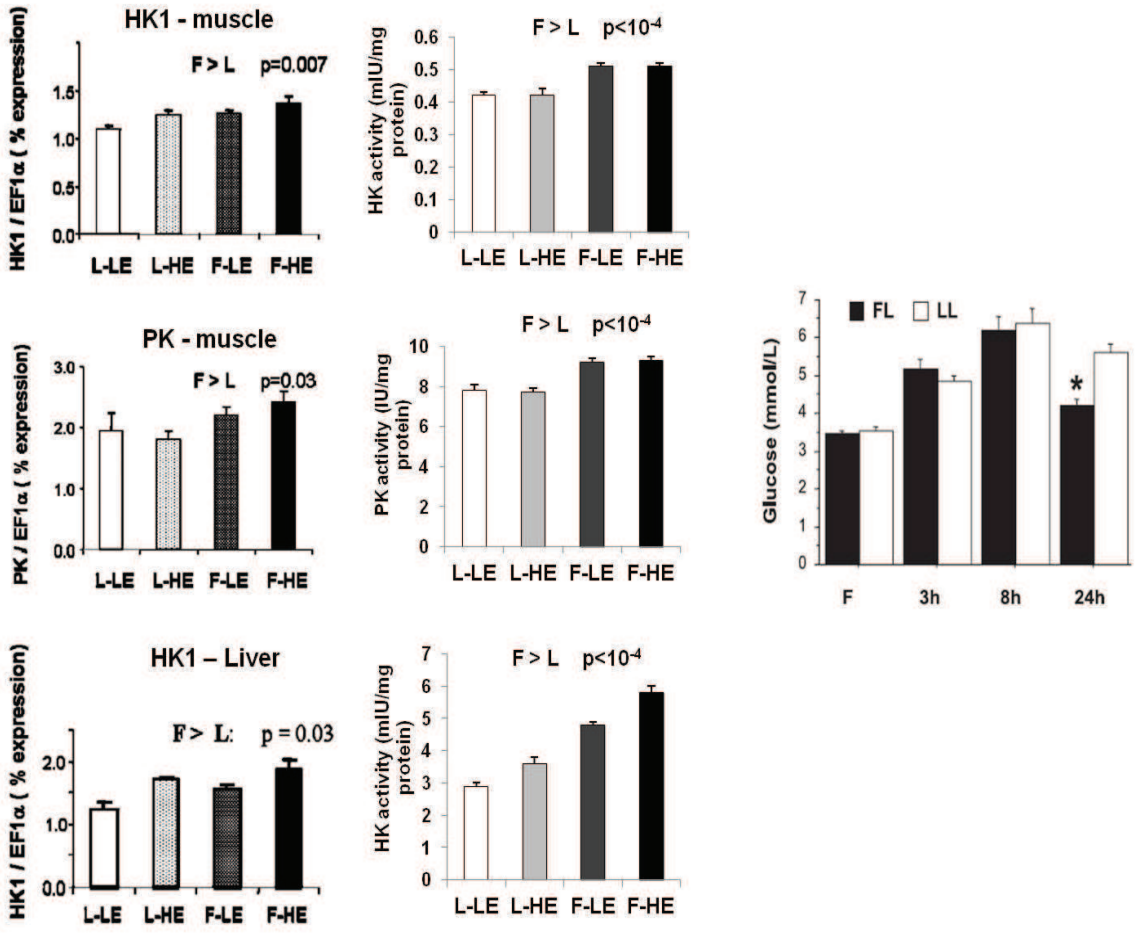
The divergent selection technique involved the non-invasive measurement of pre-dorsal muscle lipid content using a Distell Fish Fatmeter. The selection process was aided by a distribution curve of fat index obtained from random sampling of 100 fish, through which the thresholds for about 10% upward and/or downward selection were determined. At the third generation, muscle fat content of the F line was twice as that of the L line, whereas the visceral fat content was inversely higher in the L line as compared to the F line.

1.9. The INRA 'Fat' and 'Lean' rainbow trout lines

By using a non-invasive/non-destructive method i.e., Distell fish fat meter (Figure 10), Quillet et al. (2005) performed a two-way mass selection for muscle lipid content in one year old pan-sized fish belonging to the spring-spawning INRA experimental strain of rainbow trout (PEIMA, Finistere, France). Relative fat index [FI=Fat/log (W), where W is the body weight of the fish] was used as the operating trait for selection, to enable best correction for absolute weight. The resultant offspring after two generations of selection was a fat line (F) with higher muscle lipid content (upward selection) and a lean line (L) with lower muscle lipid content (downward selection). The mean realized heritability estimate (FI=0.25) indicated that selection for muscle lipid content in trout using the fat meter was efficient.

To study differences in metabolism, the two trout lines obtained after three generations of selection were fed diets differing in lipid content (10 and 23%), from the first feeding up to 6 months (Kolditz et al., 2008a). Feed intake was similar in both lines, but the L line grew better than the F line (mean weight range). At the end of the feeding trial, the trout lines were distinguished by very different muscle fat content (from 4.2 to 10% wet weight). L line fish was found to deposit fat preferentially in visceral adipose tissue, whereas the F line fish stored fat in the muscle, so that the whole body fat content was similar at this generation. Compared to the L line, the F line showed high activity and expression of glycolytic enzymes in both muscle and liver, suggesting enhanced glucose utilisation in the F line. Corresponding to this, hepatic expression of mitochondrial and peroxisomal fatty acid oxidative enzymes was lower in the F line than in the L line. Thus the lines appeared to vary in their use of energy substrates. Skiba-Cassy et al. (2009) added a strong phenotypic credence to the above findings, as they reported that 24 hours after a meal (with 10% carbohydrate) glycemia returned to basal level in F line, but remained elevated in the L line (the measurement was done in fasted-refed state). Moreover, refeeding activation of hepatic TOR signalling cascade and expression of lipogenic enzymes and transcription factor (SREBP1, FAS and G6PD) was found to be enhanced in the F line. This led the authors to conclude that genetic selection for high muscle fat content resulted in over-activation of the TOR signaling pathway-associated lipogenesis and probably also improved utilisation of glucose. All these coherent results suggested a better ability of the F line to metabolize glucose than the L line. Based on this, the two trout lines were considered to be excellent biological material to investigate genotype specific differences (diet × genotype interactions) in the utilisation of digestible starch.

Figure 11. *Previous findings that justify the objective of the present study* (Adapted from Kolditz et al., 2008a and Skiba-Cassy et al., 2009)



The fat line shows higher expression and activity of glycolytic enzymes (HK: hexokinase and PK: pyruvate kinase) in the muscle and liver as compared to the lean line. Similarly, the postprandial plasma glucose levels of the fat line are significantly lower than the lean line at the 24 h time point, suggesting a better regulation of glycemia.

1.10. Hypothesis and objectives of the thesis

The hypothesis of the thesis was that, **"the fat (F) line possess a higher ability to metabolize dietary carbohydrates than the lean (L) line as a correlated genetic response when selected for higher muscle fat content"**. It was rationalised and founded on the outcome of previous studies that suggested differential utilisation of energy substrates in these two rainbow trout lines (Figure 11; Kolditz et al., 2008a; Skiba-Cassy et al., 2009).

To verify the hypothesis, the following objectives were laid, investigated and sectioned.

1. Analyse the genotype specific response in "digestion and intestinal nutrient transport" to a diet with or without carbohydrates (20% gelatinised starch). The main idea was to ascertain the differences in starch digestibility and transcript abundance of intestinal glucose transporters between F and L line. Concurrently, the genotypic potential pertaining to digestibility and intestinal transport of other macronutrients and fatty acid bioconversion was to be demarcated at the transcriptional level, in relation to dietary carbohydrates. **(Publication 1)**

2. Decipher the genotype specific response in "metabolism, growth performance and body composition" to a diet with or without carbohydrates (20% gelatinised starch). The main idea was to identify metabolic markers [from glycolysis, gluconeogenesis, lipogenesis, β-oxidation and fatty acid bioconversion in the liver (the centre of intermediary metabolism), muscle and adipose tissue (peripheral glucose sensitive tissues), primarily at the molecular level] that can define the genetic predisposition of the two lines to use dietary carbohydrates. We were also interested to know the differences in phenotypic responses such as growth, feed utilisation, nutrient retention and postprandial plasma metabolites between the two trout lines. **(Publication 2)**

3. Investigate the "effect of dietary carbohydrate on metabolism and fatty acid composition of the two trout lines fed a vegetable oil based diet". This objective was framed based on the results obtained from the preceding studies. The main idea was to know whether dietary carbohydrates stimulate fatty acid bioconversion in a low dietary n-3 LC-PUFA environment and if so, to check the specific influence of the genetic background of the fish. However, on a comparative inter-study basis, this study was bound to provide critical insights on the effect of fish oil replacement with vegetable oils on carbohydrate metabolism in the two trout lines. **(Publication 3)**

Chapter 2

MATERIAL AND METHODS

Figure 12. *The experimental flow through rearing system setup used for conducting feeding trials at the INRA experimental farm, Donzacq*

Figure 13. *The automatic faecal collection system used for conducting digestibility study at INRA, St-Pee-sur-Nivelle*

2.1. Experimental fish and rearing systems

The study was conducted with juvenile rainbow trout (*Oncorhynchus mykiss,* Walbaum 1792) from two INRA experimental lines, namely fat line (F) and lean line (L), obtained after four generations of divergent selection for high or low muscle fat content using a non-invasive/non-destructive method (Distell Fish Fatmeter, Fauldhouse, West Lothian, UK), as detailed by Quillet et al. (2005). In the first experiment (paper 1), fish of mean initial weight 130 g were reared in tanks kept in a thermo-regulated, re-circulating water system (INRA, St-Pée-sur-Nivelle, France) at a constant water temperature of 17 °C and a controlled photoperiod (10 h L:14 h D). For the next two experiments (paper 2 and 3), fish of mean initial weight 27 and 120 g, respectively were reared in the INRA flow-through experimental facilities at Donzacq (Landes, France; Figure 12) at a constant water temperature of 17 °C, under natural photoperiod during Spring-Summer. They were fed a standard trout commercial diet (T-3P classic, Skretting, France) during the acclimatisation period.

2.2. Experimental diets

The experimental diets were prepared in our own facilities (INRA, Donzacq, Landes, France) as extruded pellets. One pair of diet (Table 6), formulated with fish oil was used for the first two experiments, namely 0C (without carbohydrate) and 20C (with carbohydrate). Another pair of diet (Table 7), formulated with vegetable oils was used for the third experiment, namely VOC- (vegetable oil based diet without carbohydrate) and VOC+ (vegetable oil based diet with carbohydrate). Gelatinised starch was included as the carbohydrate source in the diets with carbohydrates. The 0C and VOC- diet contained <1% starch, whereas the 20C and VOC+ diet contained 16-17 % starch. The increase in dietary starch content was accompanied by a decrease in the proportion of protein in the 20C and VOC+ diet. The vegetable oil mix used in the second set of diet formulation comprised of linseed oil, palm oil and rapeseed oil in the ratio 50:30:20, respectively, to supply sufficient amounts of essential fatty acid substrates (18:2 n-6, linoleic acid and 18:3 n-3, α-linolenic acid) in a well balanced mixture, for the synthesis of n-3 LC-PUFA. For the digestibility trial, Cr_2O_3 was incorporated at 1% level in the diets as an indigestible marker for measuring the ADC of diet components.

2.3. *In vivo* digestibility study

Six groups of 12 fish from each trout line were placed in 60 L cylindro-conical tanks supplied with well aerated water at a regulated flow rate of 4 L/min (Figure 13). Triplicate groups of each genotype were hand fed either of the diets (0C or 20C) containing Cr_2O_3, twice a day to

Table 6. *Composition of first pair of diet (Experiments 1 and 2)*

	0C	20C
Ingredients, %		
Fish meal[1]	81	59
Gelatinized starch[2]	0	20
Fish oil[3]	16	18
Binder[4]	1	1
Mineral mix[5]	1	1
Vitamin mix[6]	1	1
Analytical composition (Experiment 1)		
Dry matter, %	96.1	95.9
Protein, % DM	62.9	45.7
Lipid, % DM	22.1	20.3
Starch, % DM	< 1	15.7
Energy, kJ/g DM	23.7	23.1
Ash, % DM	12.9	10.1
Analytical composition (Experiment 2)		
Dry matter, %	94.0	94.0
Protein, % DM	58.6	44.7
Lipid, % DM	25.4	22.6
Starch, % DM	< 1	17.1
Energy, kJ/g DM	24.7	23.6
Ash, % DM	11.3	9.1

0C, diet without carbohydrate; 20C, diet with carbohydrate; DM, dry matter.

[1] Sopropeche, Boulogne-sur-Mer, France

[2] Lestrem 62, Roquette, France

[3] North sea fish oil (Sopropeche, Boulogne-sur-Mer, France)

[4] Alginate GF 150 (Louis François exploitation, Saint-Maur, France)

[5] Supplied the following (kg^{-1} diet): calcium carbonate (40% Ca) 2.15 g, magnesium oxide (60% Mg) 1.24 g, ferric citrate 0.2 g, potassium iodide (75% I) 0.4 mg, zinc sulphate (36% Zn) 0.4 g, copper sulphate (25% Cu) 0.3 g, manganese sulphate (33% Mn) 0.3 g, dibasic calcium phosphate (20% Ca, 18% P) 5 g, cobalt sulphate 2 mg, sodium selenite (30% Se) 3 mg, potassium chloride 0.9 g, Sodium chloride 0.4 g.

[6] Supplied the following (kg^{-1} diet): DL-a tocopherol acetate 60 IU, sodium menadione bisulphate 5 mg, retinyl acetate 15000 IU, DLcholecalciferol 3000 IU, thiamin 15 mg, riboflavin 30 mg, pyridoxine 15 mg, vit. B_{12} 0.05 mg, nicotinic acid 175 mg, folic acid 500 mg, inositol 1000 mg, biotin 2.5 mg, calcium panthotenate 50 mg, choline chloride 2000 mg.

Table 7. *Composition of second pair of diet (Experiment 3)*

	VOC-	VOC+
Ingredients, %		
Fish meal[1]	60	60
Wheat gluten[2]	20	0
Gelatinized starch[2]	0	20
Vegetable oil mix[3]	18	18
Mineral mix[4]	1	1
Vitamin mix[5]	1	1
Analytical composition		
Dry matter, %	94.0	94.4
Protein, % DM	58.6	43.0
Lipid, % DM	25.4	25.7
Starch, % DM	1.8	17.1
Energy, kJ/g DM	24.9	24.4
Ash, % DM	9.3	8.6

VOC-, diet without carbohydrate; VOC+, diet with carbohydrate; DM, dry matter.

[1] Sopropeche, Boulogne-sur-Mer, France

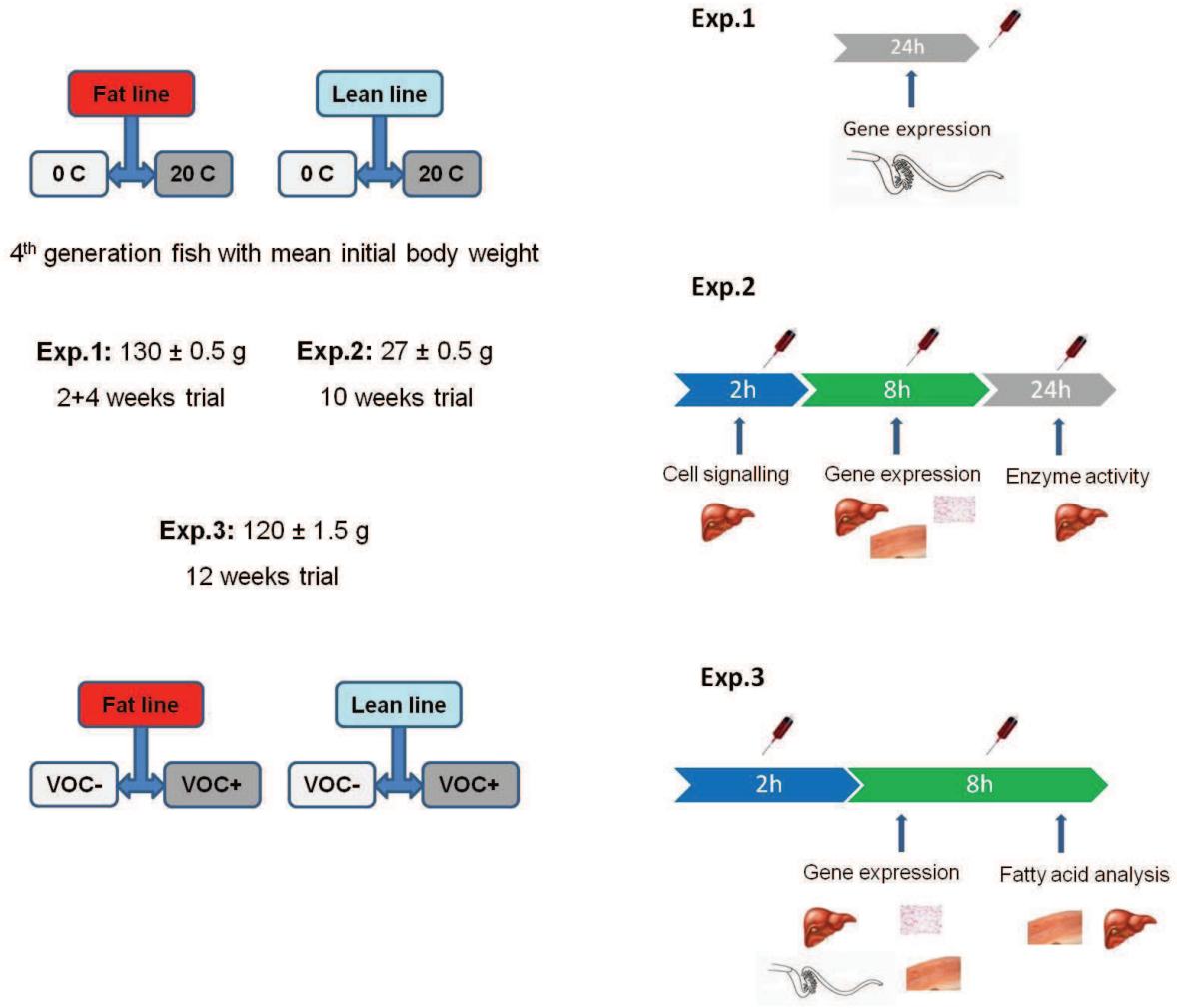
[2] Roquette, Lestrem, France

[3] Linseed/Palm/Rapeseed oil in the ratio 50:30:20 (Daudruy, Dunkerque, France)

[4] Supplied the following (kg^{-1} diet): calcium carbonate (40% Ca) 2.15 g, magnesium oxide (60% Mg) 1.24 g, ferric citrate 0.2 g, potassium iodide (75% I) 0.4 mg, zinc sulphate (36% Zn) 0.4 g, copper sulphate (25% Cu) 0.3 g, manganese sulphate (33% Mn) 0.3 g, dibasic calcium phosphate (20% Ca, 18% P) 5 g, cobalt sulphate 2 mg, sodium selenite (30% Se) 3 mg, potassium chloride 0.9 g, Sodium chloride 0.4 g.

[5] Supplied the following (kg^{-1} diet): DL-a tocopherol acetate 60 IU, sodium menadione bisulphate 5 mg, retinyl acetate 15000 IU, DLcholecalciferol 3000 IU, thiamin 15 mg, riboflavin 30 mg, pyridoxine 15 mg, vit. B_{12} 0.05 mg, nicotinic acid 175 mg, folic acid 500 mg, inositol 1000 mg, biotin 2.5 mg, calcium panthotenate 50 mg, choline chloride 2000 mg.

Figure 14. *Experimental design and sampling protocol*



This figure illustrates the 2 diets × 2 fish lines factorial design adopted in all the three studies, the size details of the experimental fish used and the sampling protocol showing the postprandial time of sampling, tissues sampled and the analyses performed.

visual satiation. Fish were allowed to adapt to the diets for 10 days, then faeces were collected for 2 weeks using an automatic, continuous sieving system as described by Choubert et al. (1982). The rapid recovery of faeces from the water (5–10 s) without manipulating the fish limits nutrient loss through leaching and also prevents stress to the fish. The faeces were collected daily over a two week period, pooled per tank and stored at −20 °C. Freeze-dried samples of the pooled feces were used for further biochemical analyses. The apparent digestibility coefficients (ADC, %) were calculated as: ADC dry matter = 100−[100∗(% of Cr2O3 in diet/% of Cr2O3 in feces)]; ADC of protein, lipid, starch or energy (%) = 100−[100∗(% of X in feces/% of X in diet)∗(% of Cr2O3 in diet/% of Cr2O3 in feces)], where X is the dietary component or energy.

2.4. Feeding trial and sampling procedure (Figure 14)

Fish of each line were distributed into six tanks. Triplicate groups of each genotype were fed either the diet with or without starch, twice a day *ad-libitum* during the experimental period. In long-term trials (Experiment 2 and 3), the fish were bulk weighed every 3 weeks and counted to calculate the mean body mass. Feed intake was recorded and feed efficiency was calculated. In the first experiment, at the end of the 4 week feeding period, 15 fish per treatment were randomly sampled at 24 h after the last meal, to analyse the adaptation of the fish to the diets (not the effect of a test meal) and to ensure that digestion was completed. In the second and third experiments, at the end of the 10 week and 12 week feeding trial, respectively, 9 fish per treatment were randomly sampled at 2 and 8 h (also 24 h for the second experiment) after the last meal. The 2 h time point allows the analysis of diet related changes in cell signalling, whereas the 8 h time point corresponds to the post-prandial peak of nutrient absorption in juvenile rainbow trout reared at 17°C. Trout were anaesthetised in diluted 2-phenoxyethanol (0.05%), individually weighed and sacrificed after collecting blood, by severing the spinal cord behind the head or by a sharp blow on the head. Blood was removed from the caudal vein into heparinised syringes and centrifuged (3000 *g*, 5 min); the recovered plasma was immediately frozen and kept at -20°C until analysis. Gut content of each fish was systematically checked to confirm that the fish sampled had effectively consumed the diet. Liver, epaxial white muscle, perivisceral white adipose tissue and intestine were dissected, weighed, immediately frozen in liquid nitrogen and kept at -80°C until analysis. The weight of liver and viscera was used to calculate the hepato/viscero-somatic index [in (%) = 100 × (X weight/body weight), where X is liver or viscera]. In the long term trials, six fish per genotype at the beginning of the feeding trial and three fish per

tank at the end were killed and then frozen for analysis of whole body composition. They were pooled per tank, ground and freeze-dried before being analysed.

2.5. Calculations of growth performance, feed utilisation and nutrient retention

Growth performance and nutrient utilisation traits were calculated for each fish tank, which represented one experimental unit. Final body weight was calculated as the final biomass divided by the number of fish in each tank at the end of the feeding trial. Daily growth coefficient was calculated as 100 × (mean final body weight$^{1/3}$ – mean initial body weight$^{1/3}$)/day. Daily feed intake was calculated as the total amount of ingested food (kg) divided by the mean biomass over the trial [(initial biomass + final biomass)/2, expressed in kg wet weight (WW)] and the number of days. Feed efficiency was estimated as the gain in total biomass [(final biomass – initial biomass) (kg WW)] divided by the amount of ingested dry matter (kg DM). Protein efficiency ratio was the gain in total biomass divided by the amount of ingested crude protein (kg). Nitrogen and fat gain were calculated as (final carcass nutrient content – initial carcass nutrient content) divided by the mean biomass over the trial [(initial biomass + final biomass)/2 (kg WW)] and the number of days, where nutrient refers to nitrogen and lipid. Protein and lipid retention were calculated as [100 × (final body mass × final carcass nutrient content) – (initial body mass × initial carcass nutrient content)]/nutrient intake, where nutrient refers to protein and lipid.

2.6. Analytical methods

The chemical composition of the diets and the freeze-dried samples of whole body and faeces was analysed using the following procedures: dry matter after drying at 105 °C for 24 h, lipid content of feed and whole body by petroleum ether extraction (Soxtherm, Gerhardt, Konigswinter, Germany), lipid content of faeces by Folch method (Folch et al., 1957), protein content (N×6.25) by the Kjeldahl method after acid digestion, gross energy in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany), ash content by incinerating the samples in a muffle furnace at 600°C for 6 h and starch content of the diet by enzymatic method (InVivo labs, France). Glycogen levels in the liver and muscle were determined using the amyloglucosidase method (Keppler and Decker, 1974). Plasma glucose (Glucose RTU, bioMérieux, Marcy l'Etoile, France), triacylglycerol (Triglycerides PAP 150, bioMérieux), free fatty acid (NEFA C kit, Wako Chemicals, Neuss, Germany) and cholesterol (Cholesterol RTU, bioMérieux) levels were determined using commercial kits adapted to a microplate format, according to the recommendations of the manufacturer. Total plasma free amino acid

Table 8. *Primer sequences*

Gene	Foward primer (5' - 3')	Reverse primer (5' - 3')	Database and Accession No.	Annealing Temperature, ^{o}C	Amplicon size, bp
Reference gene					
EF1	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	GenBank AF498320	59	159
18S	CGGAGGTTCGAAGACGATCA	TCGCTAGTTGGCATCGTTTAT	GenBank AF308735	56	62
Intestinal absorption markers					
SGLT1	TCTGGGGCTGAACATCTACC	GAAGGCATAACCCATGAGGA	GenBank AY210436	59	154
GLUT2	GTGGAGAAGGAGGCGCAAGT	GCCACCGACACCATGGTAAA	GenBank AF321816	59	227
MTP	CTCACTGACCACTCCCAGGT	ATGGCTCCCTTGTTGTTGAC	GenBank BX860503	55	152
ApoB	CCCTGTCTTCAAAGCCACAC	GTGGCGGGAGACACTCATAG	GenBank CA383905	55	196
ApoA1	CGCAGGTACCCAGGCTTTTC	AATGGACCTCTGTGCGGTCA	GenBank AF042218	59	115
ApoA4	AGCTGGGACAGGATGTCAAT	AGACGCTCTCTCAGCACCTC	GenBank CA363690	55	148
PepT1*	CCTGTCAATCAACGCTGGT	CACTGCCCATAATGAACACG	GenBank EU853718	60	161
B(0)AT	AGTCTGCAAGTCGCAGGTTT	CACATCCGTCTACGCTGCTA	Sigenae FYV3OTN01ALW29.s.om.10	60	226
Glucose metabolism markers					
GK	TGAAGGATCAGAGGTGGGTGATT	GAAGGTGAAACCCAGAGGAAGC	GenBank AF135403	59	253
HK1	CTGGGACGCTGAAGACCAGA	CGGTGCTGCATACCTCCTTG	GenBank AY864082	59	159
6PFK-liver	GGTGGAGATGCACAAGGAAT	CTTGAT GTTGTCCCCTCCAT	Sigenae tcbk0069c.k.05_s.1	59	158
6PFK-muscle	GGGA CCTCGAGATGAACGTA	GAGGGC GAAAGATGAAGTCTG	Sigenae tcad0007a.e.10_3.1.2.1	58	-
PK-liver	CCATCGTCGCGGTAACAAGA	ACATAGGAAAGGCCAGGGGC	GenBank AF246146	59	192
PK-muscle	CCTGTATCGCGGGATCTTCC	CCACACGCATGGTGTTGGTA	Sigenae tcav0004c.c.07_3.1.om.4	59	-
G6Pase1	CTCAGTGGCGACAGAAAGG	TACACAGCAGCATCCAGAGC	Sigenae tcay0019b.d.18_3.1.s.om.8	55	77
G6Pase2	TAGCCATCATGCTGACCAAG	CAGAAGAACGCCCACAGAGT	GenBank AF120150	55	82
FBPase	GCTGGACCCTTCCATCGG	CGACATAACGCCCACCATAGG	GenBank AF333188	59	182
PEPCK	GTTGGTGCTAAAGGGCACAC	CCCGTCTTCTGATAAGTCCAA	GenBank AF246149	59	149

levels were determined by ninhydrin reaction according to the method of Moore (1968) with glycine as a standard.

2.7. Total lipid and fatty acid analysis

Total lipids of the muscle and liver samples were extracted according to Folch et al. (1957), using dichloromethane instead of chloroform as the solvent and quantified gravimetrically. Fatty acid composition was determined on the total lipid extract. Fatty acid methyl esters were prepared by acid-catalysed transmethylation of total lipids using boron trifluoride (BF3) in methanol (14%) according to Shantha and Ackman (1990). They were analysed in a Varian 3800 gas chromatograph (Varian, Les Ulis, France). The chromatograph was equipped with a DB Wax fused silica capillary column (30 m x 0.25 mm internal diameter, film thickness 0.25 μm; J & W Scientific, Folsom, CA, USA). Injection was made in a split mode (ratio 1:40) with 1 μL injected. Injector and flame ionization detector temperatures were 260 and 250°C, respectively. Helium was used as carrier gas (1 ml/min) and the thermal gradient during separation was 100 to 180°C at 8°C/min, 180 to 220°C at 4°C/min and a constant temperature of 220°C during 20 min. Fatty acid methyl esters were identified by comparison with known standard mixtures (Sigma189-19, St Louis, MO, USA) and quantified using the STAR computer package (Varian).

2.8. mRNA level analysis: quantitative RT-PCR

Analyses of mRNA levels were performed on tissue samples from the liver, white muscle, intestine (pyloric caeca, midgut and hindgut) and perivisceral adipose tissue. Tissue samples from six individual fish per experimental condition, chosen based on homogeneity of plasma glucose levels were used as biological replicates. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. In case of adipose tissue and intestinal samples, phase separation step with chloroform was done twice to remove excess lipids. The extracted RNA was quantified by spectrophotometry (absorbance at 260 nm) and integrity was controlled by agarose gel electrophoresis. One microgram of the resulting total RNA was reverse transcribed into cDNA using the SuperScript III RNaseH- reverse transcriptase kit (Invitrogen) and random primers (Promega, Charbonniéres, France), according to the instruction of each manufacturer. Quantification of target gene expression levels were carried out in an iCycler iQ real-time PCR detection system (Bio-Rad) using iQ SYBR green supermix and specific primers (Table 8). PCR was performed using 5 μl of the diluted cDNA (1:50) mixed with 200 nM of each primer in a

Table 8. *Primer sequences (continued)*

GLUT4	GGCGATCGTCACAGGGATTC	AGCCTCCCAAGCCGCTCTT	GenBank AF247395	60	207
G6PD	CTCATGGTCCTCAGGTTTG	AGAGAGCATCTGGAGCAAGT	GenBank CA351434	59	176
Lipid metabolism markers					
ACLY	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	GenBank CA349411.1	60	149
ACC	TGGAGCTCTACGCAGACAGA	CTCCGGTGTACCAAGCTGTT	Sigenae tcbk0010c.b.21_5.1.om.4	55	152
FAS	TGATCTGAAGGCCCGTGTCA	GGGTGACGTTGCCGTGGTAT	Sigenae tcab0001c.e.06_5.1.s.om.8	60	161
D9D	GCCGTCCGAGGGTTCTTCTT	CTCTCCCCACAGGCACCAAG	GenBank FP323026	60	204
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	GenBank CA048941.1	60	59
D6D	AGGGTGCCTCTGCTAACTGG	TGGTGTTGGTGATGGTAGGG	Genbank AF301910	59	175
Elovl2	TGTGGTTTCCCCGTTGGATGCC	ACAGAGTGGCCATTTGGGCG	Sigenae FYV3OTN01A4WMI.s.om.10	59	146
Elovl5**	GAACAGCTTCATCCATGTCC	TGACTGCACATATCGTCTGG	Genbank AY605100	59	149
CPT1a	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACTGG	GenBank AF327058	55	166
CPT1b	CCCTAAGCAAAAAGGGTCTTCA	CATGATGTCACTCCCGACAG	GenBank AJ606076	55	149
CPT1c	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	GenBank AJ619768	59	187
CPT1d	CCGTTCCTAACAGAGGTGCT	ACACTCCGTAGCCATCGTCT	GenBank AJ620356	59	154
HOAD	GGACAAAGTGGCACCAGCAC	GGGACGGGGTTGAAGAAGTG	Sigenae tcad0001a.i.15_3.1.om.4	59	126
PPARα***	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	GenBank AY494835	54	195
PPARβ***	CTGGAGCTGGATGACAGTGA	GTCAGCCATCTTGTTGAGCA	GenBank AY356399	60	195
PPARγ***	GACGGCGGGTCAGTACTTTA	ATGCTCTTGGCGAACTCTGT	Genbank CA345564	60	171
LPL	TAATTGGCTGCAGAAAACAC	CGTCAGCAAACTCAAAGGT	GenBank AJ224693	59	164
VLDLR	GTTTTGGACAGATGGGAGA	AGCCTTCTCATTGCACCAGT	GenBank BX077158	60	160
CD36	CCACTGAAGTTGAGCCATGA	TGCTAGACTCATGCCGTGTC	GenBank BX300637	60	121

Primers reported by * Ostaszewska et al. (2010); ** Kennedy et al. (2007); *** Sánchez-Gurmaches et al. (2012)

final volume of 15 µl. The PCR protocol was initiated at 95°C for 90 s for initial denaturation of the cDNA and activation of the hot-start iTaq TM DNA polymerase, followed by a two-step amplification program (20 s at 95°C and 30 s at specific primer hybridization temperature) repeated 40 times. At the end of the last amplification cycle, melting curves (temperature gradient at 0.5°C/10 s from 55 to 94°C) were systematically monitored to confirm the specificity of the amplification reaction. Each PCR run included replicate samples (duplicate of reverse transcription and PCR amplification, respectively) and negative controls (reverse transcriptase and RNA free samples, respectively). The qPCR assay was optimized with a linear standard curve (R^2>0.985) and checked for consistency across replicates. PCR reaction efficiency for each run was estimated based on the slope of the 5 points standard curve obtained with serial dilution of pooled sample cDNAs. E values ranging from 1.85 to 2.05 were considered to be acceptable. Elongation factor-1 (EF1α) or 18S ribosomal RNA (18S) were employed as non-regulated reference genes (Olsvik et al., 2005) and they were found to be stably expressed in this study. Relative quantification of target gene expression was performed using the mathematical model described by Pfaffl (2001), after correcting for reaction efficiency (efficiency-calibrated model).

2.9. Enzyme activity analysis

To substantiate/verify the results from gene expression analysis of certain enzymes (FAS, G6PD and PK), we measured the corresponding enzyme activities. Liver samples from six individual fish per experimental condition, chosen based on homogeneity of plasma glucose levels were used as biological replicates. The tissue used to measure the lipogenic enzyme activities were homogenised by ultrasonic disruption in four volumes of ice-cold buffer (0.02 mol.l^{-1} Tris–HCl, 0.25 mol.l^{-1} sucrose, 2 mmol.l^{-1} EDTA, 0.1 mol.l^{-1} NaF, 0.5 mmol phenylmethyl sulphonyl fluoride, 0.01 mol.l^{-1} β-mercaptoethanol, pH 7.4), centrifuged at 24,000 *g* at 4°C for 20 min and the supernatant were used immediately for enzyme assays in pre-established conditions. G6PD (final substrate concentration 0.5 mmol.l^{-1} glucose-6-phosphate) and FAS (final substrate concentration 50 mmol.l^{-1} malonyl-CoA) were assessed following the method described in Figueiredo-Silva et al. (2010) adapted to trout tissues. PK enzyme activity was measured as described by Kirchner et al. (2003). Enzyme reaction rates were determined by the increase or decrease in absorbance of NADH or NADPH at 340 nm using a microplate spectrophotometer. Enzyme activity units (IU) were defined as micromoles of substrate converted to product, per minute, at 37°C. The measurements from each individual sample were performed in duplicate.

2.10. Western blot analysis

Protein extraction and western blotting were performed on liver samples of six individual fish per experimental condition, chosen based on homogeneity of plasma glucose levels. Frozen livers (200 mg) were homogenised on ice with an Ultraturrax homogeniser in 2 ml of buffer containing 150 mmol.l^{-1} NaCl, 10 mmol.l^{-1} Tris, 1 mmol.l^{-1} EGTA, 1 mmol.l^{-1} EDTA (pH 7.4), 100 mmol.l^{-1} sodium fluoride, 4 mmol.l^{-1} sodium pyrophosphate, 2 mmol.l^{-1} sodium orthovanadate, 1% Triton X-100, 0.5% Nonidet P-40-IGEPAL and a protease inhibitor cocktail (Roche, Basel, Switzerland). Homogenates were centrifuged at 1000 *g* for 15 min at 4°C and supernatants were again centrifuged at 20,000 *g* for 30 min. The resulting supernatants were aliquoted and stored at –80°C. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with bovine serum albumin as standard. Liver protein lysates (10 mg of protein for Akt and S6, and 20 mg for AMPK) were subjected to SDS-PAGE and western blotting using the appropriate antibody. Anti-phospho Akt (Ser473), anti-carboxyl terminal Akt, antiphospho-S6 (Ser235/236), anti-S6 ribosomal protein, anti-phospho-AMPK (Thr172) and anti-AMPK antibodies were used (Cell Signaling Technology, Saint Quentin Yvelines, France). These antibodies have been shown to cross-react successfully with rainbow trout proteins of interest (Skiba-Cassy et al., 2009; Polakof et al., 2011). After washing, membranes were incubated with an IRDye infrared secondary antibody (LI-COR Biosciences, Lincoln, NE, USA). Bands were visualised by infrared fluorescence using the Odyssey imaging system (LI-COR Biosciences) and quantified by Odyssey Infrared Imaging System software (version 1.2, LI-COR Biosciences).

2.11. Statistical analysis

The results are presented as means ± s.d. The effect of diet, line, and diet x line interaction on the different parameters was tested using statistical software (StatView 5.0, SAS Institute, Cary, NC) by means of a two-way analysis of variance (ANOVA) with diet and line as independent variables. *Post-hoc* comparisons were made using a Student–Newman–Keuls multiple range tests and differences were considered statistically significant at $P<0.05$. When diet x line interaction was significant, means were compared using one way ANOVA.

Chapter 3

RESULTS

The data generated from the three experiments performed under the framework of this thesis, have been the subject of three peer reviewed publications. Therefore in this section, we present the original scientific articles in the order of the objectives of the thesis.

3.1. Publication 1

Kamalam, B.S., Panserat, S., Aguirre, P., Geurden, I., Fontagné-Dicharry, S., Médale, F. 2013. Selection for high muscle fat in rainbow trout induces potentially higher chylomicron synthesis and PUFA biosynthesis in the intestine. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* 164, 417-427.

This article presents the difference between the two trout lines in macronutrient (starch) digestibility, intestinal nutrient transport (glucose transport) and fatty acid bioconversion, in relation to dietary starch intake.

3.2. Publication 2

Kamalam, B.S., Medale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S., Panserat, S. 2012. Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. *The Journal of Experimental Biology* 215, 2567-2578.

This article presents the growth, nutrient utilisation and metabolic response of the two trout lines to a diet with or without carbohydrates. It basically tested the hypothesis that the fat line has a higher ability to metabolise dietary starch than the lean line.

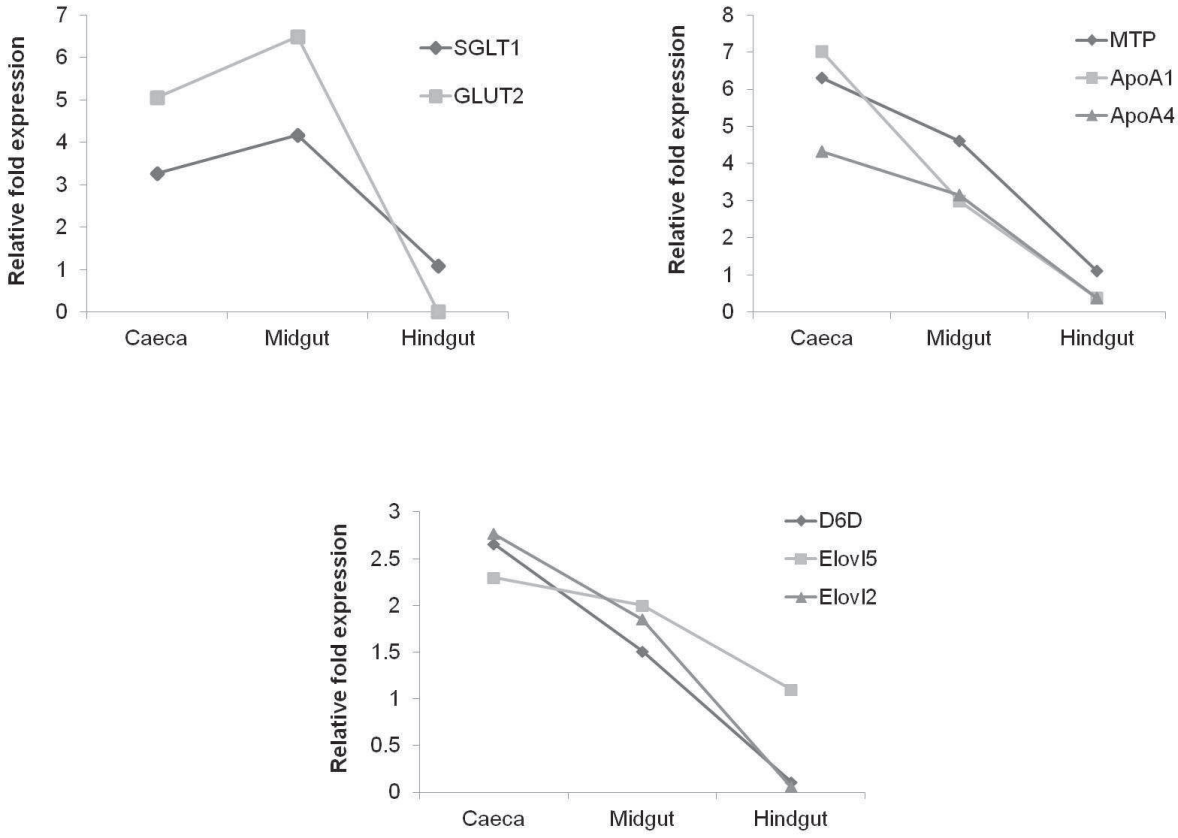
3.3. Publication 3

Kamalam, B.S., Medale, F., Larroquet, L., Corraze, G., Panserat, S. 2013. Metabolism and fatty acid profile in fat and lean rainbow trout lines fed with vegetable oil: effect of carbohydrates. (In press *PlosOne*).

This article presents the effect of dietary carbohydrates on metabolism, with special focus on fatty acid bioconversion and flesh lipid composition in the two trout lines, under a vegetable oil based dietary regime.

PUBLICATION - 1

Figure 15. *Result highlights from the first experiment – Regional abundance of intestinal transcripts*



Presentation of the article

Objective

Nutrients absorbed across the gastrointestinal epithelium are the principal source of exogenous substrates for metabolic processes and so the primary aim of this study was to investigate if there are differences between the fat (F) and lean (L) trout line in apparent digestibility of macronutrients and in the potential for intestinal transport of absorbable nutrients, especially in relation to dietary starch intake. Moreover, as fish intestine is known to be actively involved in fatty acid bioconversion, we also determined the potential genotypic differences in this metabolic pathway linked to beneficial LC-PUFA synthesis.

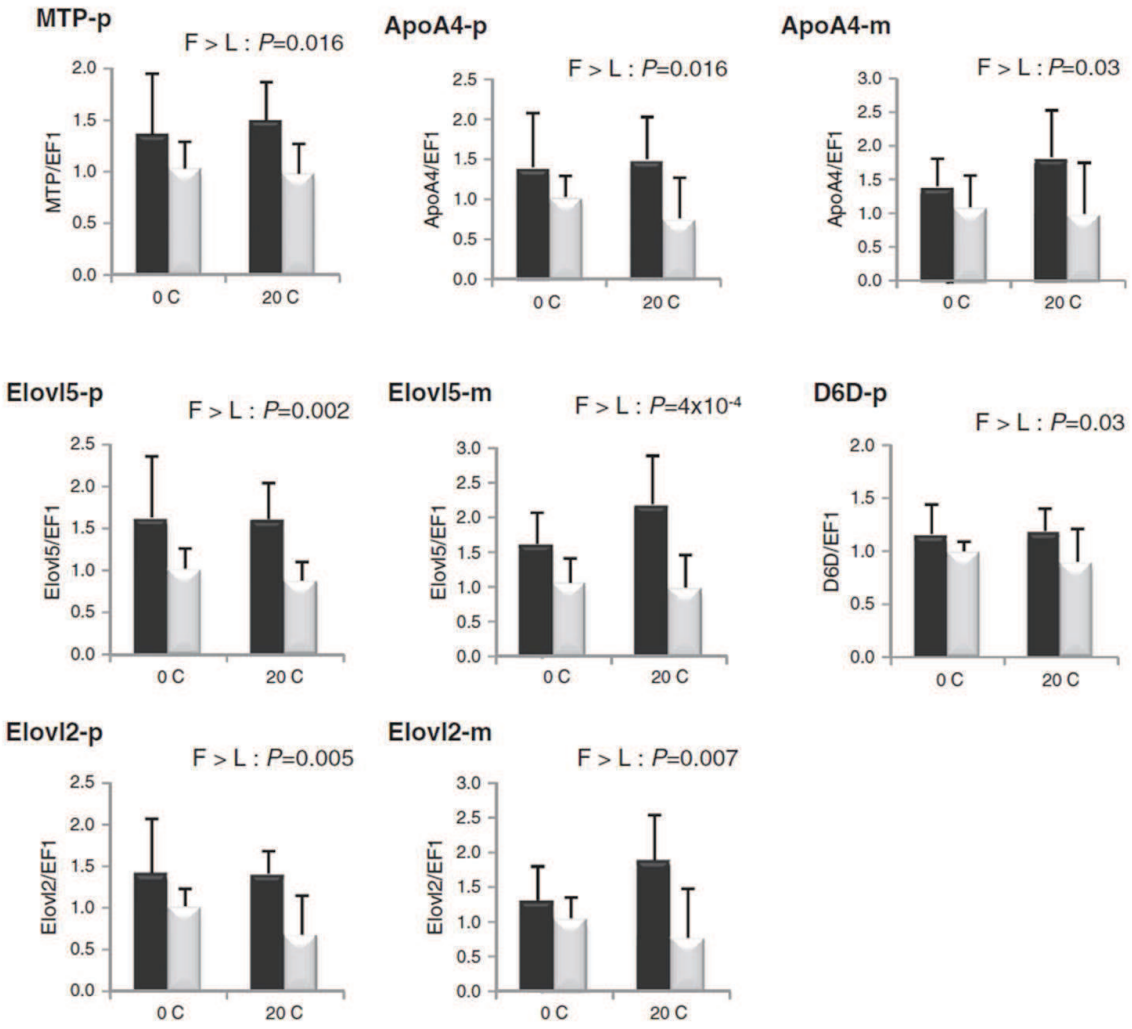
Experiment and Analyses

The study involved a digestibility trial for 2 weeks, followed by a feeding trial for 4 weeks. For the entire duration, triplicate groups of juvenile trout from the two lines divergently selected for muscle fat content were fed diets either with or without gelatinised starch. During the digestibility trial, faeces were collected by using automatic faecal collection system and at the end of the feeding trial, blood, pyloric caeca, midgut and hindgut were sampled at 24 h after the last meal, to analyse the adaptation of the fish to the diets (not the effect of a test meal). We measured (1) the apparent digestibility coefficients of macronutrients by indirect method using Cr_2O_3 as inert marker; (2) mRNA levels of intestinal protein transporters involved in glucose, peptide, amino acid transport; metabolic enzymes involved in alternate pathway of glucose export; proteins involved in chylomicron assembly and key enzymes of fatty acid bioconversion pathway in the pyloric caeca, midgut and hindgut of the two trout lines; and (3) concentration of glucose, triglycerides and free amino acids in the plasma.

Results and Conclusion

Apparent digestibility coefficients of protein (~95%), lipid (~98%) and starch (>96%) were high in both lines, but not significantly different between them. Transcripts of almost all the analysed proteins in glucose transport (SGLT1 and GLUT2), peptide/amino acid transport (PepT1 and B(0)AT), chylomicron assembly (MTP, ApoA1 and ApoA4) and enzymes involved in fatty acid bioconversion (D6D and Elovl2) were highly expressed in the pyloric caeca and midgut (proximal intestine), and relatively much less in the hindgut (distal intestine). Based on this, we do not consider the differences between genotypes or dietary groups observed in the hindgut to bear significance (Figure 15). In general, the regional distribution of transcripts correlates well with the phenotypic description of functional

Figure 16. *Result highlights from the first experiment – Gene expression of markers of chylomicron assembly and fatty acid bioconversion*



activities i.e. maximum absorption of glucose, peptides/amino acids and lipids and active LC-PUFA biosynthesis occurs in the proximal intestine.

Concerning intestinal glucose transport, excluding the slightly higher abundance of GLUT2 transcripts in the caeca of the F line, none of the other proteins involved in trans-epithelial glucose transport were found to be differentially expressed between the two trout lines. Furthermore, dietary starch intake did not stimulate a higher expression of SGLT1 and GLUT2 in both trout lines, when compared to a diet without starch. Concerning the mammalian like alternate metabolic pathway for the export of glucose to the blood independently of GLUT2, we found an enhanced expression of G6Pase in the midgut as a result of dietary starch intake, but unchanged HK expression annuls the outcome. Plasma glucose levels also do not reflect a long term improvement of glucose uptake with dietary starch intake or any difference between the two lines.

In lipid absorption, the higher expression of MTP and ApoA4 in the F line fish indicates the enhanced potential of chylomicron assembly in their enterocytes, which was however not evident in the lipid digestibility estimates or plasma triglyceride levels. In peptide/amino acid transport, we found no genotypic difference in the expression of PepT1 and B(0)AT corresponding to the protein digestibility estimates. Dietary starch intake was interestingly associated with the higher expression of ApoA1 and PepT1 in the midgut, for which the exact reason is unknown. Finally and significantly, the F line exhibited coherently higher intestinal mRNA levels of D6D, Elovl2 and Elovl5 than the L line, indicating its higher potential in fatty acid bioconversion (Figure 16).

In conclusion, we found that genetic selection for higher muscle fat content was associated with enhanced potential for chylomicron synthesis and fatty acid bioconversion in the intestine. But, no genotypic differences were found in the expression of intestinal glucose transporters as well as in any of the apparent nutrient digestibility estimates (starch, lipid and protein). Also, both trout lines did not show adaptive transcriptional response of glucose transporters when fed dietary starch, true to their carnivorous nature.

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Selection for high muscle fat in rainbow trout induces potentially higher chylomicron synthesis and PUFA biosynthesis in the intestine

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ABSTRACT

Two lines of rainbow trout divergently selected for muscle fat content, fat line (F) and lean line (L) were used to investigate the effect of genetic selection on digestion, intestinal nutrient transport and fatty acid bioconversion, in relation to dietary starch intake. This study involved a digestibility trial for 2 weeks using Cr_2O_3 as inert marker, followed by a feeding trial for 4 weeks. For the entire duration, juvenile trout from the two lines were fed diets with or without gelatinized starch. Blood, pyloric ceca, midgut and hindgut were sampled at 24 h after the last meal. Transcripts of the proteins involved in nutrient transport and fatty acid bioconversion were abundant in the proximal intestine. GLUT2 transcripts were slightly higher in the F line ceca than in the L line. Dietary starch intake did not enhance the transcription of intestinal glucose transporters, SGLT1 and GLUT2; but it was associated with the higher expression of ApoA1 and PepT1 in the midgut. Significantly, the F line exhibited higher intestinal mRNA levels of MTP, ApoA4, Elovl2, Elovl5 and D6D than the L line, linked to chylomicron assembly and fatty acid bioconversion. Apparent digestibility coefficients of protein, lipid and starch were high in both lines, but not significantly different between them. In conclusion, we found a higher potential of chylomicron synthesis and fatty acid bioconversion in the intestine of F line, but no adaptive transcriptional response of glucose transporters to dietary starch and no genotypic differences in nutrient digestibility.

1. Introduction

Rainbow trout are known to be poor users of dietary carbohydrate, characterized by a persistent post-prandial hyperglycemia and a weak utilization of glucose in the peripheral tissues after a high carbohydrate meal (Moon, 2001). This constitutes a major stumbling block to develop diets with higher levels of plant ingredients that contain starch. However, with the existing opportunities to exploit differences in the genetic potential of salmonids, an emerging prospect is to identify genotypes with improved capacity to utilize dietary carbohydrates (Kamalam et al., 2012). The gastrointestinal tract (GIT) is the first organ that has access to dietary nutrients and it is well known that post-absorptive metabolism is strongly dependent on digestion and absorption of nutrients. Considerable differences in nutrient digestibility exist even in phylogenetically related species such as Atlantic salmon and rainbow trout (Krogdahl et al., 2004). Moreover, there are few reports about intraspecific variations in digestibility of macro-nutrients in rainbow trout genotypes or families, associated with improved production traits (Medale, 1993; Valente et al., 1998; Rasmussen and Jokumsen, 2009; Rungruangsak-Torrissen et al., 2009). In this context, understanding the genetic architecture of the GIT and the impact of genetic selection on its functions is vital.

The fish GIT is a highly organized, multi-functional organ comprising several distinct regions that differ in morphology, histology and physiological functions (Wilson and Castro, 2011). The rainbow trout has an extendable stomach and a short intestine, apt for processing a highly digestible, nutrient dense diet that is high in protein and low in carbohydrate (Buddington et al., 1997). The entire length of the post-gastric gut of fish is capable of active nutrient transport, but the proximal regions of the intestine generally contribute more to nutrient absorption than the more distal regions (Ferraris and Ahearn, 1984). General mechanisms of nutrient absorption such as simple diffusion or via specialized protein transporters appear to be conserved in fish during evolution (Collie and Ferraris, 1995; Bakke et al., 2011). This is evidenced by the functional and genetic characteristics of the Na^+/glucose symporter SGLT1 and the H^+ dependent peptide transporter PepT1 that are similar to mammals (Pajor et al., 1992; Verri et al., 2003). Glucose facilitative

Abbreviations: 0C, diet without carbohydrates; 20C, diet with carbohydrates; ADC, apparent digestibility coefficient; ApoA1, apolipoprotein A1; ApoA4, apolipoprotein A4; ApoB, apolipoprotein B; B(0)AT, sodium dependent neutral amino acid transporter; D6D, Δ6 fatty acyl desaturase; D9D, Δ9 fatty acyl desaturase; Elovl2, elongation of very long chain fatty acids like-2; Elovl5, elongation of very long chain fatty acids like-5; F, fat line; G6Pase, glucose 6-phosphatase; GLUT2, glucose facilitative transporter type 2; HK, hexokinase; L, lean line; MTP, microsomal triglyceride transfer protein; PepT1, peptide transporter 1; SGLT1, sodium dependent glucose co-transporter type 1.

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transporter GLUT2 transcripts and Na^+ dependent amino acid transporters are also present in fish intestine (Collie and Ferraris, 1995; Krasnov et al., 2001). The absorption of lipid in fish is basically comparable to the mammalian process. Lipid droplets which accumulate in the supranuclear space of enterocytes are re-esterified into triacylglycerides and packed into smaller lipoprotein particles similar to chylomicrons in mammals, before being exported by exocytosis (Sire et al., 1981; Tocher, 2003). The major proteins associated with chylomicron assembly are apolipoproteins apoB, apoAIV, apoAI, and apoCs and microsomal triglyceride transfer protein, MTP (Hussain et al., 2001; Black, 2007); they have been genetically characterized in few fish species (Kondo et al., 2005; Kim et al., 2009; Fang et al., 2010; Holtta-Vuori et al., 2010). In higher vertebrates, intestinal nutrient transport proteins such as SGLT1, GLUT2, PepT1, MTP and apo-proteins are regulated at the transcriptional level, as indicated by the link between mRNA, protein and activity levels (Vayro et al., 2001; Cui et al., 2003; Daniel, 2004; Black, 2007; Hussain et al., 2011). Moreover, the nutrient transporters are regulated by dietary substrates, though the regulatory pattern varies among the transporters (Ferraris and Diamond, 1989). Dietary carbohydrates enhance the expression of SGLT1 in rainbow trout (Kirchner et al., 2008) and also influence lipid digestion and absorption (Spannhof and Plantikow, 1983; Storebakken et al., 1998).

Studies in poultry suggest that genetic selection for better performance may involve enhanced digestive and absorptive efficiency (Mitchell and Smith, 1991), whereas similar information is scanty in fish. Recently, divergent selection for muscle fat content using a non-invasive technique (Distell fish fat meter) generated two lines of rainbow trout, namely fat line (F) and lean line (L) having higher and lower muscle fat content, respectively (Quillet et al., 2005). Previous studies demonstrated that the two lines differed in growth and hepatic intermediary metabolism, under different dietary regimes (Kolditz et al., 2008a; Skiba-Cassy et al., 2009; Kamalam et al., 2012). The F line exhibited higher gene expression for the hepatic glycolytic, lipogenic and fatty acid bioconversion enzymes than the L line, with dietary starch specifically enhancing the expression of lipogenic enzymes in the F line (Kamalam et al., 2012). Since we know that the energy and substrates required for all the metabolic process are primarily provided by the nutrients absorbed across the gastrointestinal epithelium, the main objectives of the present study were to investigate 1) the differences in digestive and intestinal nutrient transport capacity between the two lines 2) the influence of dietary carbohydrates on these processes, specific to each genotype. Apart from this, the rainbow trout intestine is known to have high expression of desaturase and elongase enzymes that are involved in fatty acid bioconversion (Seiliez et al., 2001; Leaver et al., 2008). Therefore, to add to the previously observed differences in hepatic fatty acid bioconversion between the two lines (Kamalam et al., 2012), intestinal fatty acid bioconversion potential of the two lines was also determined. Thus, we measured the in vivo digestibility of macronutrients and mRNA levels of intestinal protein transporters involved in glucose, peptide, amino acid transport; metabolic enzymes involved in glucose transport; proteins involved in chylomicron assembly and key enzymes of fatty acid bioconversion pathway in the pyloric ceca, midgut and hindgut of the two trout lines.

2. Materials and methods

2.1. Fish and diets

The investigation was carried out with juvenile rainbow trout (*Oncorhynchus mykiss*, Walbaum) from two experimental lines, namely F (fat line) and L (lean line), obtained after four generations of divergent selection for high or low muscle fat content using a non-invasive method (Distell Fish Fatmeter, Fauldhouse, West Lothian, UK). The fish were maintained in tanks kept in a thermo-regulated, re-circulating water system (INRA, St-Pée-sur-Nivelle, France) at a constant water temperature of 17 °C and a controlled photoperiod (10 h L:14 h D). The experiments were carried out in accordance with the Guidelines of the National Legislation on Animal Care of the French Ministry of Research (Decree no. 2001–464, May 29, 2001) and the Ethics Committee of INRA (INRA 2002–36, April 14, 2002). Two experimental diets, namely 0C (without carbohydrate) and 20C (with carbohydrate) were prepared in our own facilities as sinking pellets. Gelatinized starch was included as the carbohydrate source. The 0C diet contained <1% starch, whereas the 20C diet contained 15.7% starch (Table 1). The increase in dietary carbohydrate content was accompanied by a decrease in the proportion of protein in the 20C diet. Cr_2O_3 was incorporated at 1% level in the diets as an indigestible marker for digestibility measurement of dietary components.

2.2. In vivo digestibility study

Six groups of 12 fish (Mean body mass 130 ± 10 g) from each trout line were placed in 60 L cylindro-conical tanks supplied with well aerated water at a regulated flow rate of 4 L/min. Triplicate groups of each genotype were hand fed either of the diets (0C or 20C) containing Cr_2O_3, twice a day to visual satiation. Fish were allowed to adapt to the diets for 10 days before the start of feces collection using an automatic, continuous sieving system as described by Choubert et al. (1982). The rapid recovery of feces from the water (5–10 s) without manipulating the fish limits nutrient loss through leaching. The feces were collected daily over a two week period, pooled per tank and stored at −20 °C. Freeze-dried samples of the pooled feces were used for further biochemical analyses. The apparent digestibility coefficients (ADC, %) were calculated as: ADC dry matter = 100 − {100 ∗ (% of Cr_2O_3 in diet / % of Cr_2O_3 in feces)}; ADC of protein, lipid, starch or energy (%) = 100 − {100 ∗ (% of X in feces / % of X in diet) ∗ (% of Cr_2O_3 in diet / % of Cr_2O_3 in feces)}, where X is the dietary component or energy.

2.3. Feeding essay and tissue sampling

Subsequent to the digestibility trial, fish were transferred to 60 L rectangular tanks kept in a different circuit of the re-circulatory system and allowed to acclimatize. The rearing conditions remained the same.

Table 1
Composition of diets.

	0C	20C
Ingredients, %		
Fish meal[a]	81	59
Gelatinized starch[b]	0	20
Fish oil[c]	16	18
Binder[d]	1	1
Mineral mix[e]	1	1
Vitamin mix[f]	1	1
Analytical composition		
Dry matter, %	96.1	95.9
Protein, % DM	62.9	45.7
Lipid, % DM	22.1	20.3
Starch, % DM	<1	15.7
Energy, kJ/g DM	23.7	23.1
Ash, % DM	12.9	10.1

0C, diet without carbohydrate; 20C, diet with carbohydrate; DM, dry matter.
[a] Fishmeal (Sopropeche, Boulogne-sur-Mer, France).
[b] Gelatinized starch (Lestrem 62, Roquette, France).
[c] Fish oil (North Sea fish oil; Sopropeche, Boulogne-sur-Mer, France).
[d] Alginate GF 150 (Louis François exploitation, Saint-Maur, France).
[e] Mineral mix — 2.15 g calcium carbonate (40% Ca), 1.24 g magnesium oxide (60% Mg), 0.2 g ferric citrate, 0.4 mg/kg diet potassium iodide (75% I), 0.4 g zinc sulfate (36% Zn), 0.3 g copper sulfate (25% Cu), 0.3 g manganese sulfate (33% Mib), 5 g dibasic calcium phosphate (20% Ca, 18% P), 2 mg/kg diet cobalt sulfate, 3 mg/kg diet sodium selenite (30% Se), 0.9 g KCl, and 0.4 g NaCl (UPAE, Jouy, Inra, France).
[f] Vitamin mix — 60 IU DL-a tocopherol acetate, 5 mg/kg diet sodium menadione bisulphate, 15,000 IU retinyl acetate, 3000 IU DL-cholecalciferol, 15 mg/kg diet thiamin, 30 mg/kg diet riboflavin, 15 mg/kg diet pyridoxine, 0.05 mg/kg diet B12, 175 mg/kg diet nicotinic acid, 500 mg/kg diet folic acid, 1000 mg/kg diet inositol, 2.5 mg/kg diet biotin, 50 mg/kg diet calcium panthotenate, and 2000 mg/kg diet choline chloride (UPAE).

Triplicate groups of each line were fed the 0C or 20C diet, twice a day to visual satiation for a period of 4 weeks. At the end of the feeding period, 15 fish per treatment were randomly sampled, 24 h after the last meal. This postprandial time was chosen to analyze the adaptation of the fish to the diets (not the effect of a test meal) and to ensure that digestion was completed. Trout were anesthetized in diluted 2-phenoxyethanol (0.05%) and sacrificed by severing the spinal cord behind the head. Blood was removed from the caudal vein into heparinized syringes and centrifuged (3000×*g*, 5 min); the recovered plasma was immediately frozen and kept at −20 °C until analysis. Gut content of each fish was systematically checked to confirm that the fish sampled had effectively consumed the diet. Three parts of the post-gastric alimentary tract, namely pyloric ceca, midgut and hindgut were dissected, immediately frozen in liquid nitrogen and kept at −80 °C until analysis. It is important to note that all the intestinal segments contained digested feed at the sampling time, which was flushed out with physiological saline. For sampling, pyloric ceca was defined as the region from pyloric sphincter to the last pyloric caecum; midgut was the region after the last pyloric caecum to the start of the distal segment of the intestine; hindgut was recognized by its larger diameter, darker color and annular rings (Buddington, 1987).

2.4. mRNA level analysis: quantitative RT-PCR

Analyses of mRNA levels were performed on tissue samples from the pyloric ceca, midgut and hindgut. Six individual fish per experimental condition were used as biological replicates. Total RNA was extracted from a representative portion of the intestinal tissue using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations; phase separation step with chloroform was done twice to remove excess lipids. The extracted RNA was quantified by spectrophotometry (absorbance at 260 nm) and integrity was controlled by agarose gel electrophoresis. One microgram of the resulting total RNA was reverse transcribed into cDNA using the SuperScript III RNaseH-reverse transcriptase kit (Invitrogen) and random primers (Promega, Charbonniéres, France), according to the instructions of each manufacturer. Quantification of target gene expression levels were carried out in an iCycler iQ real-time PCR detection system (Bio-Rad) using iQ SYBR green supermix and specific primers (Table 2). PCR was performed using 5 μL of the diluted cDNA (1:50) mixed with 200 nM of each primer in a final volume of 15 μL. The PCR protocol was initiated at 95 °C for 90 s for initial denaturation of the cDNA and activation of the hot-start iTaq TM DNA polymerase, followed by a two-step amplification program (20 s at 95 °C and 30 s at specific primer hybridization temperature) repeated 40 times. At the end of the last amplification cycle, melting curves (temperature gradient at 0.5 °C/10 s from 55 to 94 °C) were systematically monitored to confirm the specificity of the amplification reaction. Each PCR run included replicate samples (duplicate of reverse transcription and PCR amplification, respectively) and negative controls (reverse transcriptase and RNA free samples, respectively). The qPCR assay was optimized with a linear standard curve ($R^2 > 0.985$) and checked for consistency across replicates. PCR reaction efficiency for each run was estimated based on the slope of the 5 points standard curve obtained with serial dilution of pooled sample cDNAs. E values ranging from 1.85 to 2.05 were considered to be acceptable. The transcripts analyzed were SGLT1, GLUT2, HK (EC 2.7.1.1) and G6Pase (EC 3.1.3.9) for glucose transport/metabolism; PepT1 and B(0)AT for peptide/aminoacid transport; MTP, ApoB, ApoA1 and ApoA4 for chylomicron synthesis; and D9D (EC 1.14.19.1), D6D (EC 1.14.19.3), Elovl5 and Elovl2 for fatty acid bioconversion. Elongation factor-1 (EF1α) was employed as a non-regulated reference gene (Olsvik et al., 2005) and it was stably expressed in this study. Relative quantification of target gene expression was performed using the mathematical model described by Pfaffl (2001) after correcting for reaction efficiency (efficiency-calibrated model).

2.5. Analytical methods

The chemical composition of the diets and the freeze-dried samples of feces were analyzed using the following procedures: dry matter after drying at 105 °C for 24 h, lipid in feed by petroleum ether extraction (Soxtherm), lipid in feces by Folch method (Folch et al., 1957), protein content (N×6.25) by the Kjeldahl method after acid digestion, gross energy in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany), ash content by incinerating the samples in a muffle furnace at 600 °C for 6 h and starch content by enzymatic method (InVivo labs, France). Plasma glucose (Glucose RTU, bioMérieux, Marcy l'Etoile, France), triglycerides (PAP 150, bioMérieux) and free fatty acid (NEFA C kit, Wako Chemicals, Neuss, Germany) levels were determined using commercial kits adapted to a microplate format, according to the recommendations of the manufacturer. Total plasma free amino acid levels were determined by the ninhydrin reaction according to the method of Moore (1968), with glycine as a standard.

2.6. Statistical analysis

The results are presented as means ± s.d. The effect of diet, line, and diet x line interaction on the different parameters was tested using statistical software (StatView 5.0, SAS Institute, Cary, NC) by means of a two-way analysis of variance (ANOVA) with diet and line as independent variables. Post-hoc comparisons were made using a Student–Newman–Keuls multiple range tests, and differences were considered statistically significant at $P<0.05$. Significance of the relative fold variation in gene expression between the three intestinal parts was ascertained using one way ANOVA ($P<0.05$).

3. Results

3.1. Relative distribution of transcripts along the intestine

Comparative analysis of the gene expression data from the three intestinal segments provided insights on the zonal transcript abundance of the intestinal proteins (Table 3). mRNA levels of target proteins were very high in the proximal parts of the intestine compared to the distal part. More specifically, mRNA expression of proteins involved in glucose transport and associated metabolism (GLUT2 and G6Pase) was high in the midgut, followed by the ceca and very low in the hindgut. SGLT1 was highly expressed in the anterior portion, but there was no significant difference between the ceca and midgut. HK expression in the midgut was twice that of the hindgut. Transcripts of proteins involved in chylomicron assembly and maturation were generally high in the pyloric ceca. Nevertheless, ApoA4 and MTP expression levels were not significantly different between the ceca and midgut, but much higher than in the hindgut. Due to extremely high individual variation, spatial differences in ApoB expression were not tangible. Concerning protein absorption, PepT1 expression in the pyloric ceca was twofold higher than the midgut and negligible in the hindgut, whereas B(0)AT expression was highest in the midgut. Similar to the expression pattern of transport proteins, the transcripts of the fatty acid bioconversion enzymes (D6D and Elovl2) were also several folds higher in the proximal intestine than the distal part, with highest expression in the pyloric ceca. However, no region specific difference in expression was observed for Elovl5 and D9D.

3.2. mRNA levels of target proteins

We quantified the mRNA levels of proteins involved in intestinal macro-nutrient transport and fatty acid bioconversion in the pyloric ceca, midgut and hindgut to look for potential differences between genotypes, in relation to the influence of dietary carbohydrates. Dietary starch intake did not alter the mRNA levels of trans-cellular membrane glucose transporters (SGLT1 and GLUT2) and glucose oxidation enzyme

Table 2
Primer sequences.

Gene	Primer sequences (5′–3′)	Database and accession no.	Annealing temperature, °C	Amplicon size, bp
EF1α	F: TCCTCTTGGTCGTTTCGCTG R: ACCCGAGGGACATCCTGTG	GenBank AF498320	59	159
SGLT1	F: TCTGGGGCTGAACATCTACC R: GAAGGCATAACCCATGAGGA	GenBank AY210436	59	154
GLUT2	F: GTGGAGAAGGAGGCGCAAGT R: GCCACCGACACCATGGTAAA	GenBank AF321816	59	227
HK	F: CTGGGACGCTGAAGACCAGA R: CGGTGCTGCATACCTCCTTG	GenBank AY864082	59	159
G6Pase	F: CTCAGTGGCGACAGAAAGG R: TACACAGCAGCATCCAGAGC	Sigenae **CA345537.s.om.10**	55	77
MTP	F: CTCACTGACCACTCCCAGGT R: ATGGCTCCCTTGTTGTTGAC	GenBank BX860503	55	152
ApoA1	F: CGCAGGTACCCAGGCTTTTC R: AATGGACCTCTGTGCGGTCA	GenBank AF042218	59	115
ApoA4	F: AGCTGGGACAGGATGTCAAT R: AGACGCTCTCTCAGCACCTC	GenBank CA363690	55	148
ApoB	F: CCCTGTCTTCAAAGCCACAC R: GTGGCGGGAGACACTCATAG	GenBank CA383905	55	196
PepT1*	F: CCTGTCAATCAACGCTGGT R: CACTGCCCATAATGAACACG	GenBank EU853718	60	161
B(0)AT	F: AGTCTGCAAGTCGCAGGTTT R: CACATCCGTCTACGCTGCTA	Sigenae **FYV3OTN01ALW29.s.om.10**	60	226
D6D	F: AGGGTGCCTCTGCTAACTGG R: TGGTGTTGGTGATGGTAGGG	GenBank AF301910	59	175
Elovl2	F: TGTGGTTTCCCCGTTGGATGCC R: ACAGAGTGGCCATTTGGGCG	Sigenae **FYV3OTN01A4WMI.s.om.10**	59	146
Elovl5**	F: GAACAGCTTCATCCATGTCC R: TGACTGCACATATCGTCTGG	GenBank AY605100	59	149
D9D	F: GCCGTCCGAGGGTTCTTCTT R: CTCTCCCCACAGGCACCAAG	GenBank FP323026	60	204

Primers reported by: * Ostaszewska et al. (2010); ** Kennedy et al. (2007).

(HK) in all the three intestinal segments (Fig. 1). However, midgut G6Pase transcripts were found to be elevated in the 20C dietary group. In terms of genotypic variation, the F line exhibited a higher expression of GLUT2 in the pyloric ceca and SGLT1, HK, and G6Pase in the hindgut when compared to the L line. Among all the chylomicron associated proteins analyzed, only the ApoA1 transcripts in the midgut and both ApoA1 and ApoB transcripts in the hindgut were found to be enhanced by dietary starch intake (Fig. 2). In the context of an impact of genetic selection on the assembly of chylomicrons, mRNA levels of ApoA4 and MTP were found to be significantly higher in the F line than the L line, in all the three intestinal segments (except for midgut MTP, $P = 0.06$). As shown in Fig. 3, dietary starch intake surprisingly resulted in enhanced transcript levels of PepT1 in the midgut and B(0)AT in the hindgut. The only genotypic difference was a higher expression of B(0)AT in the hindgut of the F line compared to the L line. Substantial genotypic differences were observed for the key enzymes of fatty acid bioconversion pathway (Fig. 4). The F line displayed significantly higher mRNA levels of elongases (Elovl2 and Elovl5) in all three intestinal segments. Transcripts of desaturases (D9D and D6D) were enhanced in the pyloric ceca and hindgut of the F line, but not in the midgut. The 20C diet was associated with a higher expression of Elovl5 in the hindgut and lower expression of D9D in the pyloric ceca. No instance of diet x line interaction was observed for any of the analyzed transcripts.

Table 3
Relative mRNA abundance of target proteins along the post-gastric intestine.

mRNA	Pyloric ceca	Midgut	Hindgut
SGLT1	3.3 ± 2.5^{a}	4.2 ± 1.3^{a}	1.1 ± 0.4^{b}
GLUT2	506.1 ± 202.8^{b}	649.2 ± 130.6^{a}	1.9 ± 3.2^{c}
HK	1.6 ± 0.8^{ab}	2.2 ± 1.2^{a}	1.1 ± 0.4^{b}
G6Pase	21.7 ± 11.3^{b}	35.1 ± 14.8^{a}	1.3 ± 0.9^{c}
MTP	6.3 ± 4.2^{a}	4.6 ± 1.5^{a}	1.1 ± 0.4^{b}
ApoA1	21.0 ± 10.2^{a}	9.0 ± 3.6^{b}	1.2 ± 0.7^{c}
ApoA4	13.0 ± 10.6^{a}	9.4 ± 5.3^{a}	1.1 ± 0.5^{b}
ApoB	219.2 ± 371.1	46.8 ± 51.2	1.1 ± 0.4
PepT1	5054.8 ± 2580.9^{a}	2881.4 ± 754.2^{b}	4.6 ± 10.6^{c}
B(0)AT	2.2 ± 2.3^{b}	3.9 ± 1.7^{a}	1.1 ± 0.5^{b}
D6D	26.6 ± 12.8^{a}	15.1 ± 7.9^{b}	1.1 ± 0.4^{c}
Elovl 2	55.4 ± 45.6^{a}	37.0 ± 24.5^{a}	1.2 ± 0.8^{b}
Elovl 5	2.3 ± 2.0	2.0 ± 1.1	1.1 ± 0.5
D9D	0.9 ± 0.7	1.2 ± 1.3	1.3 ± 1.0

Relative fold difference in target gene expression between the intestinal sections (with hindgut as control) are presented as means ± s.d. ($N = 12$ individuals) and were analyzed using one-way ANOVA ($P<0.05$). Mean values not sharing a common superscript ^{a,b,c} (row-wise) are significantly different from each other.

3.3. In vivo nutrient digestibility

The apparent digestibility coefficient (ADC) values of the different dietary components are summarized in Table 4. Dry matter digestibility of the 20C diet was slightly higher than the 0C diet, irrespective of the lines ($P<0.01$). Digestibility of protein, lipid and starch were high in both lines (above 94%, 97% and 96%, respectively). We found no significant difference in macro-nutrient digestibility attributable to either genetic selection or diet composition.

3.4. Plasma metabolite levels

The results of the plasma metabolites concentrations measured at 24 h after the meal are presented in Fig. 5. Triglycerides level was elevated in fish fed the 20C diet, regardless of the genotype. However, it was not significantly different between the two trout lines. Free amino acid levels of the F line were slightly higher than the L line, irrespective of the diet. Plasma glucose and free fatty acid levels did not vary with diet or genotype.

4. Discussion

The basic idea behind the present study was that differences in digestion and absorption of dietary nutrients may be one of the

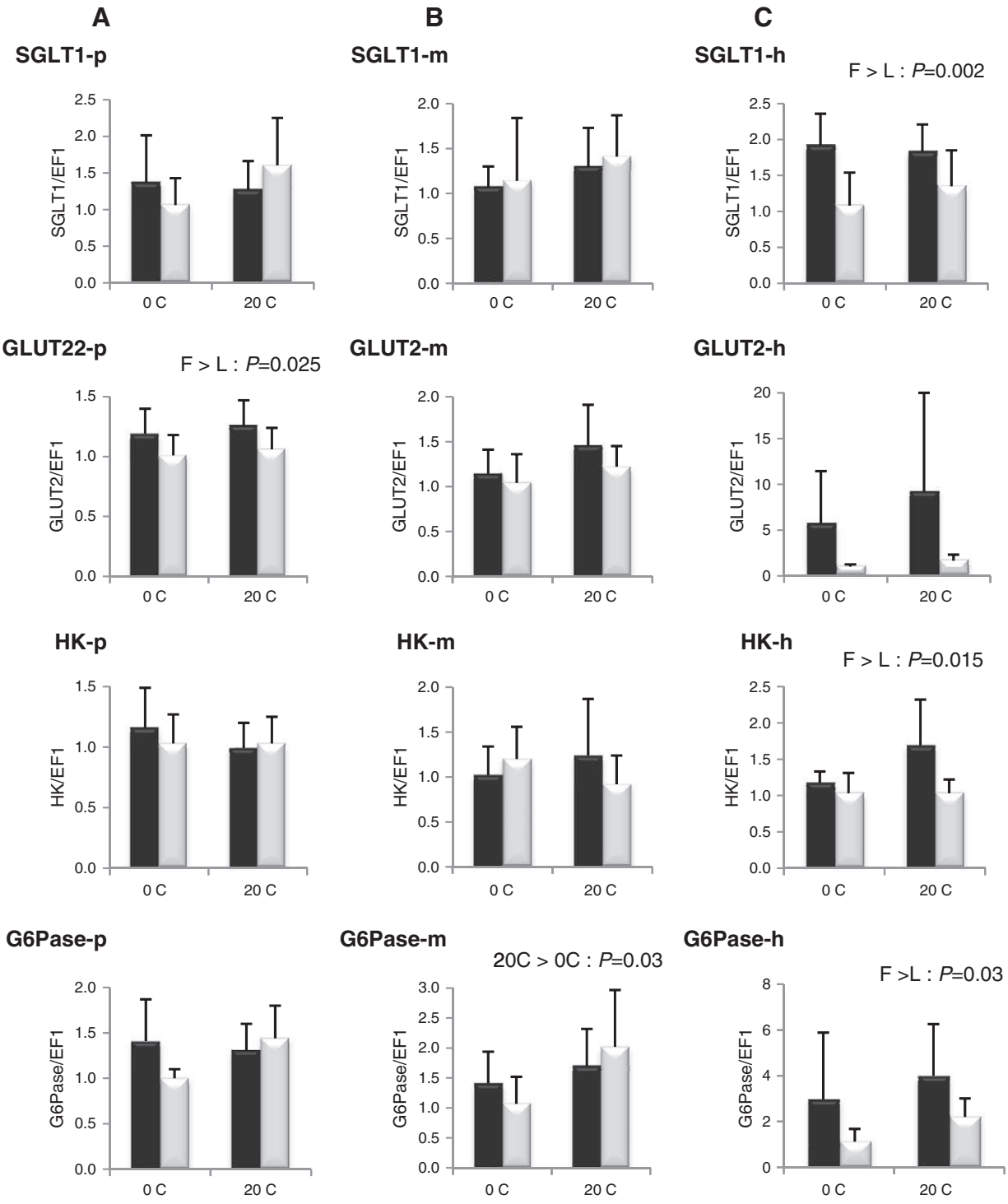

Fig. 1. Gene expression of selected transporters and enzymes involved in membrane glucose transport in the pyloric ceca (column A), midgut (column B) and hindgut (column C) of rainbow trout from a fat line (F; black bar) and a lean line (L; gray bar) fed a diet without (0C) or with (20C) carbohydrate, 24 h after the last meal. mRNA levels of sodium-dependent glucose co-transporter type 1 (SGLT1), glucose facilitative transporter type 2 (GLUT2), hexokinase (HK) and glucose6-phosphatase (G6Pase) were measured using real-time quantitative RT-PCR. Expression values are normalized by elongation factor 1-alpha (EF1α) expressed transcripts. Considering 0C-L group as control, relative fold difference between treatments are presented as means + s.d. ($N = 6$ individuals) and were analyzed using two-way ANOVA followed by Student–Newman–Keuls test for multiple comparison. Differences were considered significant at $P < 0.05$.

underlying factors influencing the differential regulation of intermediary metabolism in the two trout lines, when fed dietary carbohydrates. In addition, details of intestinal fatty acid bioconversion potential can complement the existing information on the genotypic differences observed in the liver (Kamalam et al., 2012). We will discuss subsequently our findings on the distribution of transcripts along the intestine; the influence of dietary starch on intestinal nutrient transport; genotypic differences in intestinal nutrient transport and fatty acid bioconversion capacity at the transcriptional level.

4.1. Regional distribution of intestinal transcripts is functionally relevant

An interesting characteristic of fish intestine is that nearly the entire length of the post-gastric gut is lined with absorptive epithelium

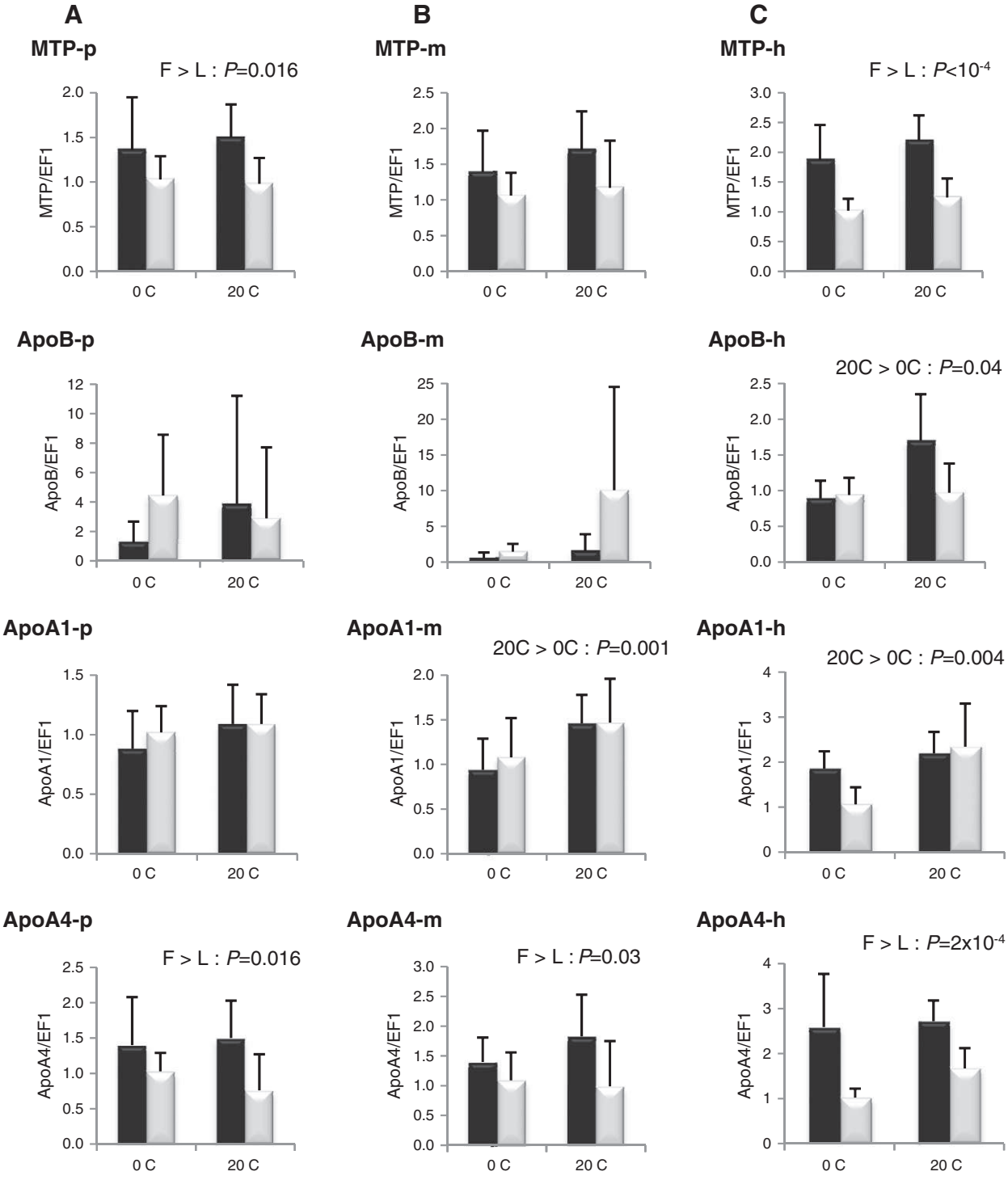

Fig. 2. Gene expression of selected proteins involved in chylomicron synthesis in the pyloric ceca (column A), midgut (column B) and hindgut (column C) of rainbow trout from a fat line (F; black bar) and a lean line (L; gray bar) fed a diet without (0C) or with (20C) carbohydrate, 24 h after the last meal. mRNA levels of microsomal triglyceride transfer protein (MTP), apolipoprotein B (ApoB), apolipoprotein A1 (ApoA1) and apolipoprotein A4 (ApoA4) were measured using real-time quantitative RT-PCR. Expression values were normalized with elongation factor 1-alpha (EF1α) expressed transcripts. Considering 0C-L group as control, relative fold difference between treatments are presented as means + s.d. ($N = 6$ individuals) and were analyzed using two-way ANOVA followed by Student–Newman–Keuls test for multiple comparison. Differences were considered significant at $P < 0.05$.

capable of active nutrient transport, which can be a physiological compensation for the relatively short gut in carnivorous fishes like rainbow trout (Ferraris and Ahearn, 1984). Hence, we found it necessary to assess the transcript abundance of nutrient transporters throughout the post-gastric gut. Our comparative analysis showed that almost all the analyzed nutrient transport proteins were highly expressed in the pyloric ceca and midgut, and relatively much less in the hindgut. This serves as a confirmation at the molecular level as to why the proximal regions contribute more to nutrient absorption than the distal part, complementing the findings of several other in vitro and in vivo nutrient uptake studies (Bakke et al., 2011). This region specific transcript abundance of nutrient transporters may also indicate the distribution pattern

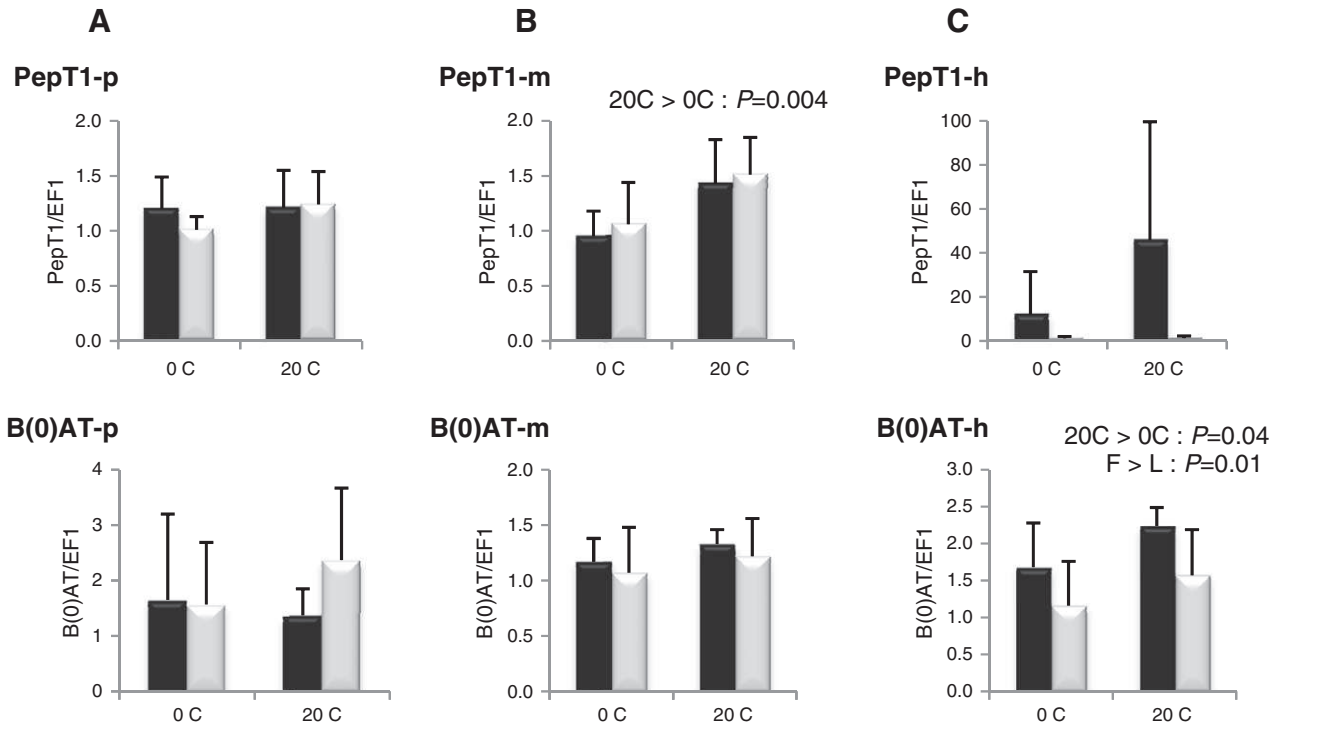

Fig. 3. Gene expression of selected transporters involved in peptide/aminoacid membrane transport in the pyloric ceca (column A), midgut (column B) and hindgut (column C) of rainbow trout from a fat line (F; black bar) and a lean line (L; gray bar) fed a diet without (0C) or with (20C) carbohydrate, 24 h after the last meal. mRNA levels of peptide transporter 1 (PepT1) and sodium dependent neutral amino acid transporter (B(0)AT) were measured using real-time quantitative RT-PCR. Expression values were normalized with elongation factor 1-alpha (EF1α) expressed transcripts. Considering 0C-L group as control, relative fold difference between treatments are presented as means + s.d. ($N = 6$ individuals) and were analyzed using 2-way ANOVA followed by Student–Newman–Keuls test for multiple comparison. Differences were considered significant at $P < 0.05$.

of these transport proteins in the trout intestine. Capacity for glucose uptake and flux rates is known to be higher in the anterior region of the salmonid intestine (Buddington and Hilton, 1987; Bakke-McKellep et al., 2000; Krogdahl et al., 2005), which is evident in the transcript abundance of the glucose transporters SGLT1 and GLUT2. The proteins involved in chylomicron assembly were highly expressed in the pyloric ceca and midgut, in accordance to the knowledge that most of the lipid absorption processes in fish occurs in those regions (Olsen et al., 1998; Denstadli et al., 2004; Hernandez-Blazquez et al., 2006). The expression of PepT1 clearly corresponds to the declining proximal to distal gradient of peptide absorption along the salmonid post-gastric intestine (Verri et al., 2009). Furthermore, we found maximal expression of D6D and Elovl2 in the ceca, which supports the finding that the pyloric ceca of rainbow trout are at least five times more active in the synthesis of polyunsaturated fatty acids than the liver and other intestinal segments (Bell et al., 2003). Overall, the regional distribution of transcripts correlates well with the phenotypic description of functional activities along the intestinal tract. Based on the relative tissue expression levels, we do not consider the differences between genotypes or diets observed in the hindgut to bear significance.

4.2. Dietary starch intake does not modify intestinal glucose transport

Traditionally, glucose uptake across the intestinal epithelium of fish is known to happen via active transport into the enterocyte by the apical SGLT1, followed by facilitated diffusion across the basolateral membrane through GLUT2 (Collie and Ferraris, 1995). In the present study, the intake of a diet with starch did not induce a higher expression of SGLT1 and GLUT2 in both trout lines, when compared to a diet without starch. The absence of enhanced SGLT1 expression contradicts previous reports in trout, whereas the unresponsiveness of intestinal GLUT2 gene expression is known (Kirchner et al., 2008; Polakof et al., 2010). In general, induction of glucose transporter activity with high dietary carbohydrate has not been demonstrated in strict carnivores such as rainbow trout, frogs and cat (Buddington and Hilton, 1987; Karasov and Diamond, 1988). It is important to note that this response is species specific, as glucose has been reported to presumably enhance the activity of both these transporters in the enterocytes of an omnivorous black bullhead catfish (Soengas and Moon, 1998). Recent studies in mammals suggest an alternate metabolic pathway for the export of glucose to the blood independently of GLUT2 but dependent on phosphorylation of glucose by HK and further dephosphorylation by G6Pase (Mithieux, 2005). An increased glycolytic potential and G6Pase activity has been reported in trout intestine, consistent with the alternative mechanism of glucose transport (Polakof et al., 2010). We found an enhanced expression of G6Pase in the midgut as a result of dietary starch intake, but unchanged HK expression annuls the outcome. The digestibility of starch in both trout lines was above 96%, similar to other studies in trout using gelatinized starch source and higher levels of incorporation (Bergot, 1993; Krogdahl et al., 2004). Therefore, the lack of regulatory response of the glycolytic enzyme and the glucose transporters at the transcriptional level cannot be attributed to poor starch quality or level of inclusion in the diet. Plasma glucose levels also do not reflect a long-term improvement of glucose uptake with dietary starch intake. Besides, it is possible that at 24 h after the last meal, the difference in plasma glucose levels does not exist anymore. Overall, the inability of rainbow trout to adjust its intestinal glucose absorption to dietary carbohydrate levels with little scope for up-regulation can be due to its evolutionarily stable natural diet with less than 1% carbohydrates, smaller amounts of absorptive tissue, lower densities of membrane glucose transporters, low basal activity levels of glucose transporters and the absence of a regulatory machinery to change the number of functional copies of the transporter per unit of tissue when fed a diet rich in carbohydrate (Karasov and Diamond, 1988; Collie and Ferraris, 1995; Krogdahl et al., 2005). In mammals, the glucose transporters are regulated also at the translational or post-translational level (Wright, 1993), so future studies in fish should focus on it.

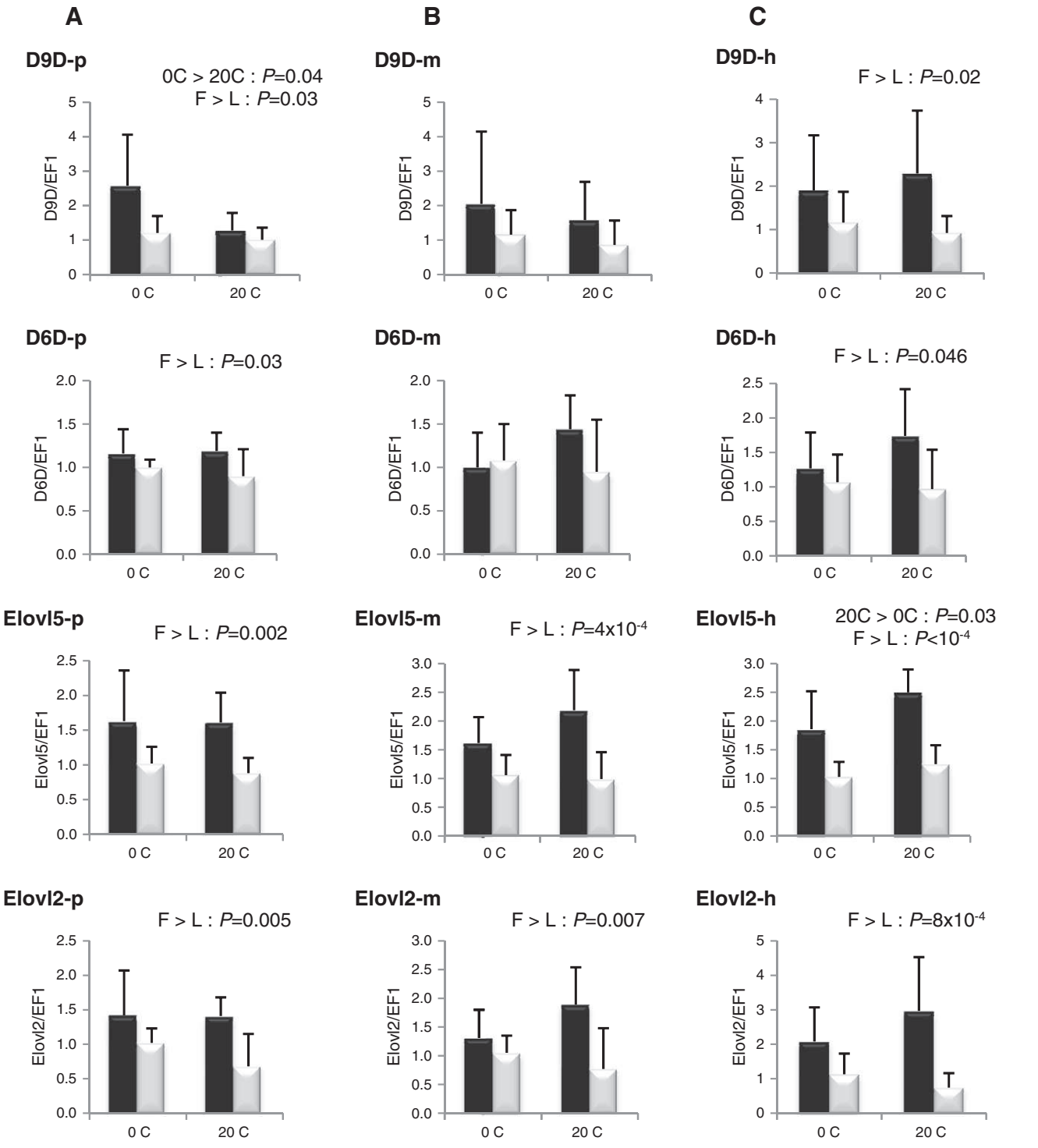

Fig. 4. Gene expression of selected enzymes involved in fatty acid bioconversion in the pyloric ceca (column A), midgut (column B) and hindgut (column C) of rainbow trout from a fat line (F; black bar) and a lean line (L; gray bar) fed a diet without (0C) or with (20C) carbohydrate, 24 h after the last meal. mRNA levels of Δ9 fatty acyl desaturase (D9D), Δ6 fatty acyl desaturase (D6D), elongation of very long chain fatty acids like-5 (Elovl5) and elongation of very long chain fatty acids like-2 (Elovl2) were measured using real-time quantitative RT-PCR. Expression values were normalized with elongation factor 1-alpha (EF1α) expressed transcripts. Considering 0C-L group as control, relative fold difference between treatments are presented as means + s.d. ($N = 6$ individuals) and were analyzed using two-way ANOVA followed by Student–Newman–Keuls test for multiple comparison. Differences were considered significant at $P<0.05$.

4.3. Dietary starch intake regulates the transcription of PepT1 and ApoA1

PepT1 is the epithelial membrane transporter responsible for absorbing short oligopeptides in the intestine of fish (Verri et al., 2009). ApoA1 is the major lipoprotein constituent of high density lipoproteins and it is involved in the assembly of chylomicrons (Black, 2007). To our knowledge, there is no existing evidence in fish for the regulation of PepT1 and ApoA1 expression by dietary carbohydrate stimuli. We found enhanced PepT1 expression in the midgut of trout fed a diet with starch and optimum level of protein compared to those of fish fed a diet without starch plus excess protein. This is surprising considering the fact that an increase in PepT1 expression is commonly coupled with high protein diet (Adibi,

Table 4
Apparent digestibility coefficients of dietary components.

Digestibility coefficient, %	F line		L line		*P* values		
	0C	20C	0C	20C	Diet	Line	Diet∗ Line
Dry matter	85.3±0.3	86.4±0.7	85.4±0.8	86.7±0.5	0.009	0.67	0.76
Protein	95.0±0.1	94.8±0.2	94.7±0.5	94.5±0.3	0.37	0.11	0.97
Lipid	97.9±0.3	98.0±0.2	97.9±0.3	97.5±0.3	0.41	0.19	0.16
Starch	–	96.3±1.0	–	98.0±1.0	–	0.10	–
Ash	41.6±0.4	38.3±0.2	41.1±1.8	37.2±0.7	2×10^{-4}	0.19	0.64
Energy	92.8±0.4	92.4±0.7	93.0±0.8	92.8±0.6	0.49	0.42	0.74

Data are presented as means±s.d. ($N=3$ tanks) and were analyzed by two-way ANOVA followed by Student–Newman–Keuls multiple comparison test. Differences were considered statistically significant at $P<0.05$.

2003). However, higher amino acid (proline) uptake associated with carbohydrate rich diet has been previously reported in trout as an adaptive response to increased digestible dietary energy (Buddington, 1987). The expression of ApoA1 was also high in the midgut of fish fed dietary starch, similar to a previous observation in mammalian liver when fed a diet rich in glucose or fructose (Mooradian et al., 1997). Though the exact reason for the upregulation of PepT1 and ApoA1 is not identified, it is tempting to speculate that it might be due to the stimulatory effect of insulin. Components of the glucose sensing pathway are known to be present in trout intestine (Polakof et al., 2010). So insulin secretion may occur in response to dietary glucose load, which in turn could influence the expression of PepT1 and ApoA1 as reported in mammals (Adibi, 2003; Mooradian et al., 2004). This hypothesis and a possible negative feedback regulation of PepT1 linked to substrate saturation needs to be investigated.

4.4. Potential genotypic differences in digestion and intestinal nutrient transport

The gastrointestinal tract is one of the most metabolically active tissues in the body. Studies examining the relationship between genotype and the intestinal tract in poultry suggested that selection influences the intestinal morphology, mass of absorptive epithelium, gut motility

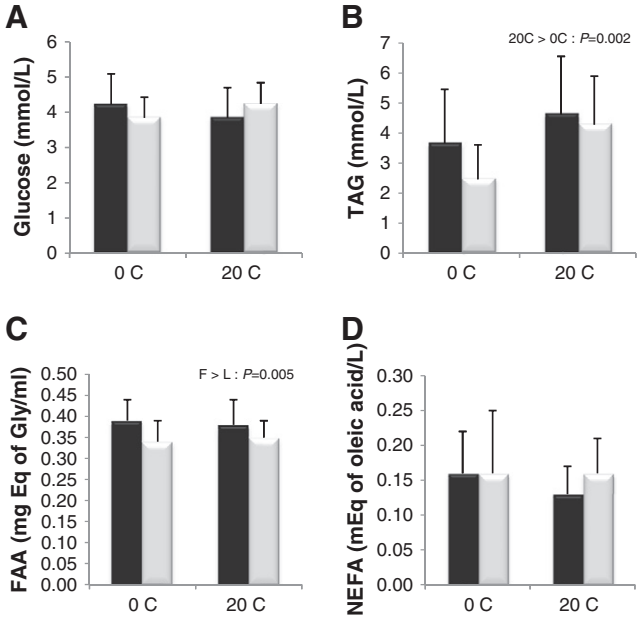

Fig. 5. Postprandial (24 h after meal) plasma metabolite levels in fish from the fat (F; black bar) and the lean (L; gray bar) trout lines fed diet without (0C) or with carbohydrates (20C) for 4 weeks. A: glucose. B: triglycerides (TAG). C: free amino acids (FAA). D: non-esterified fatty acids (NEFA). Results are presented as means + s.d. ($N=15$ individuals) and were analyzed using two-way ANOVA followed by Student–Newman–Keuls test for multiple comparison. Differences were considered significant at $P<0.05$.

and digestive capacity (Mitchell and Smith, 1991; Mignon-Grasteau et al., 2004; de Verdal et al., 2010; Rougière et al., 2012). To our knowledge, this is the first study investigating genotypic differences in transcriptional regulation of intestinal nutrient transporters in selected farm animals. Glucose absorption in the two trout lines does not seem to be modified by genetic selection. Excluding the slightly higher abundance of GLUT2 transcripts in the ceca of the F line, none of the other proteins involved in trans-epithelial glucose transport were differentially expressed between the two trout lines. Similarly, digestibility coefficient of starch and plasma glucose levels reflected the absence of genotypic variation in starch digestion and uptake. This is consistent with studies in mice and poultry that report selection for production traits may not be synchronized with beneficial changes in intestinal absorption of glucose (Fan et al., 1996; Fan et al., 1998). On the other hand, the higher expression of MTP and ApoA4 in the F line fish indicates the induction of chylomicron assembly in their enterocytes. Chylomicron assembly is a complex process designed to package dietary lipid into thermodynamically stable particles containing neutral lipid in the core and polar lipids and apolipoproteins on the surface. The critical neutral lipid transfer activity of MTP shuttling re-synthesized triglycerides to nascent ApoB molecule leads to the formation of primordial chylomicron particle, which after further lipidation receives ApoA4 and at a later stage ApoA1 (Hussain et al., 2001; Black, 2007). The genotypic differences in chylomicron synthesis, however, were not evident in the lipid digestibility estimates or plasma triglyceride levels. It might be because the genetic potential was not sufficiently amplified in the phenotype at the fourth generation. The slightly higher plasma amino acid levels found in the F line were not due to differences in protein digestibility or the expression levels of peptide/amino acid transporters. Perhaps, it may be related to higher amino acid catabolism in the liver of the F line as revealed by transcriptomic and proteomic analysis in the third generation fish (Kolditz et al., 2008b). Variations in protein digestibility do exist among rainbow trout families that have undergone selective breeding for improved growth (Medale, 1993; Valente et al., 1998; Rasmussen and Jokumsen, 2009; Rungruangsak-Torrissen et al., 2009). We found no such difference between these two trout lines selected for muscle fat content, despite known differences in growth (Kolditz et al., 2008a; Kamalam et al., 2012). As a general fact, differences in digestibilities of feed components are known to occur in genetically selected poultry and livestock animals with moderate to high heritability values ranging from 0.33 to 0.47 (Mignon-Grasteau et al., 2004; Herd and Arthur, 2009). In summary, our assumption that genetic selection may result in altered digestion and intestinal nutrient transport properties is supported only by the higher potential of the F line for chylomicron biosynthesis, which is conceivable considering the fact that muscle fat content was the criterion for the divergent selection of these two lines.

4.5. Potential genotypic differences in intestinal fatty acid bioconversion

Since the intestine is a tissue with high turnover of cells/membranes, fatty acid bioconversion may be relatively high to meet its own needs for biomembrane synthesis (Tocher et al., 2002). In liver, there is evidence to show that the activity and expression of key enzymes of this biosynthetic pathway are dependent on the genotype of the fish (Kolditz et al., 2008b; Morais et al., 2011; Kamalam et al., 2012). In salmon selected for lean or fat muscle, genotypic differences in Elovl2 and D6D expression seem to exist also in intestine, however, strongly dependant on the source of dietary oil (Morais et al., 2012). The higher intestinal D6D, Elovl2 and Elovl5 expression found in the F line compared to the L line is consistent with the genotypic difference previously observed in hepatic D6D expression (Kamalam et al., 2012). This uniform mRNA expression pattern in different tissues of the F line indicates a coherent up regulation of elongase and desaturase enzymes and eventually may result in higher levels of long chain polyunsaturated fatty acids in the muscle. As the beneficial polyunsaturated fatty acid content of the flesh is known to be a

heritable trait in salmonids (Leaver et al., 2011), the genotypic differences observed even at the transcriptional level are of interest from the point of value added production. However, it is essential to confirm the relative contributions of the intestine and liver to whole body accumulation on the basis of availability of substrate over time, the concentration of pathway enzymes, their kinetics and molecular regulation.

5. Conclusions

In the present study, we found that divergent selection of rainbow trout for muscle fat content did not cause an improvement in intestinal glucose uptake at the transcriptional level, as an adaptive response to dietary carbohydrates. The lack of any significant genotypic difference in the nutrient digestibility estimates made in vivo invalidates our assumption that differences in digestion and absorption may underlie the differential regulation of intermediary metabolism in the two trout lines. However, we found a higher genetic potential of the F line to synthesize chylomicrons, the lipoprotein particle transporting absorbed dietary fat. This possibility of genetic difference in nutrient transport between genotypes of trout should be specifically looked at in further generations. Moreover, the consistent upregulation of the fatty acid bioconversion pathway enzymes in the F line fish intestine and liver suggests a genetic predisposition of this genotype to deposit more beneficial polyunsaturated fatty acids in its flesh, which creates interest in view of human consumption.

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PUBLICATION - 2

Presentation of the article

Objective

Previous studies in two rainbow trout lines divergently selected for lean (L) or fat (F) muscle suggested that they differ in their ability to metabolize glucose, because the F line exhibited a higher glycolytic ability in the liver/muscle and apparent better control of postprandial glycemia than the L line. Based on this, we postulated the hypothesis that the F line has a higher ability to metabolize dietary carbohydrates than the L line. The aim of the present study was thus to verify the hypothesis, by investigating the metabolic response of both trout lines to diets with high level of carbohydrates (17.1%) or without carbohydrates (< 1%).

Experiment and Analyses

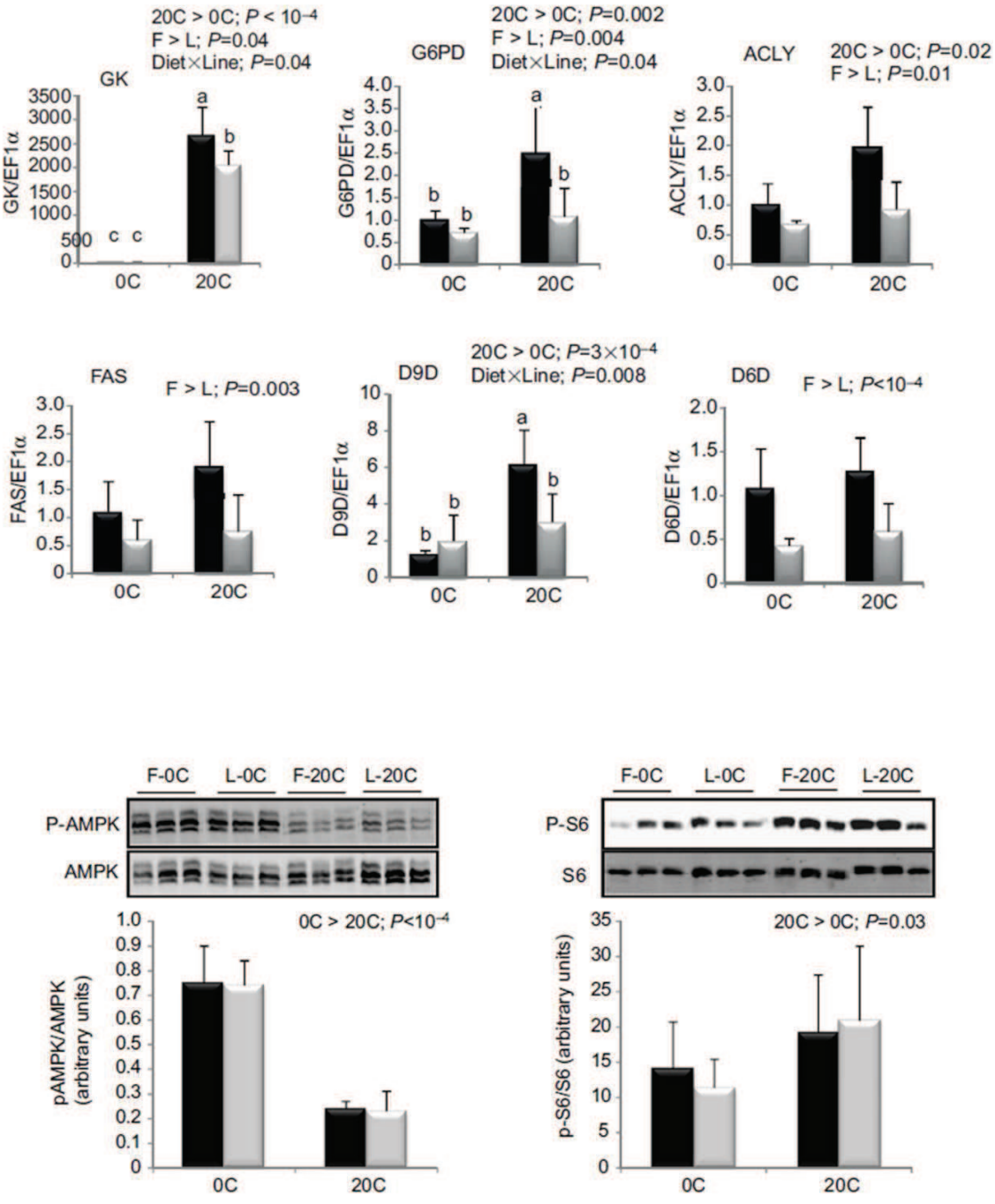
We conducted a 10 week feeding trial, in which triplicate groups of juvenile trout from the two lines were fed diets with or without gelatinised starch. At the end of the trial, blood, liver, muscle and adipose tissues were sampled at 2, 8 and 24 h after the last meal. We analysed the effects of genetic selection and dietary starch intake on growth, feed utilization, body composition and postprandial plasma metabolite levels. At tissue level, we examined the signalling of intracellular energy sensors (AMPK), activation of insulin and nutrient pathway (Akt, TOR-S6) and gene expression of key enzymes involved in glycolysis, gluconeogenesis, lipogenesis, β-oxidation and fatty acid bioconversion in liver and also gene expression of metabolic enzymes in muscle and adipose tissue.

Results and Conclusion

Intake of dietary starch had no adverse influence on growth and lead to protein sparing in both lines, as indicated by the higher protein retention value in fish fed diet with starch. By means of comparative slaughter technique, we found that the two lines did not differ in protein and lipid gain and retention values. However, growth rate and feed efficiency was lower in the F line than the L line, as documented from earlier generations.

Starch intake resulted in a moderate postprandial hyperglycaemia (6 to 7.5 mM) and a strong induction of hepatic glycogen levels, in both lines. The hypothesised better regulation of postprandial glycemia in the F line was not evident. This could be possibly explained by the concurrent higher expression of both glycolytic (GK and PK) and gluconeogenic (PEPCK and FBPase) enzymes in the liver of the F line, which suggests that the potential export of glucose synthesised de novo through unregulated gluconeogenesis may counter balance the

Figure 17. *Result highlights from the second experiment – Gene expression of markers of hepatic intermediary metabolism and phosphorylation status of cellular energy (AMPK) and nutrient sensors (S6-TOR) in the liver.*



uptake of glucose from the bloodstream for glycolytic purpose in the F line. Nevertheless, liver glycogen levels were higher in the F line than the L line, regardless of the diet.

Further concerning hepatic lipid metabolism, starch intake was associated with an increase in the expression or activity of the enzymes that are involved in *de novo* lipogenesis (FAS, ACLY, D9D) and NADPH production (G6PD), in both lines (Figure 17). While the F line fish generally exhibited a higher expression of these lipogenic enzymes (FAS, ACLY, G6PD) than the L line, some of these enzyme expression were in particular strongly stimulated in the fish fed dietary starch (G6PD and D9D). These diet × genotype interactions indicate the higher potential of the F line to convert excess glucose into lipids. In addition, D6D a key desaturase enzyme of the fatty acid bioconversion pathway was also highly expressed in the F line liver, irrespective of the starch content. On the contrary, expression of the rate limiting enzymes of fatty acid oxidation (CPT1) was lower in the liver of the F line than the L line.

The activation of Akt was paradoxically lower in the F line liver, with respect to its higher potential of glycolysis, lipogenesis and glycogen levels (insulin-dependent metabolic pathways). On the other hand, we observed that starch intake elicits a specific cellular signalling response in the liver of both lines, characterised by a decrease in the phosphorylation of energy-sensing enzyme AMPK and a corresponding increase in the phosphorylation of S6 protein (a component of the nutrient signalling TOR cascade). This indicates the contribution of dietary starch to the energy needs of the hepatocytes.

We found no effect of dietary starch or genetic selection in the expression of analysed metabolic enzymes in the muscle and adipose tissue, indicating the unresponsiveness of the two key peripheral tissues implicated in glucose use, equally in both lines.

Overall, the enhanced hepatic lipogenic potential and the higher liver glycogen content in the F line illustrate its better ability to store excess glucose than the L line. However, this does not result in an improved regulation of postprandial glycaemia in the F line possibly due to unregulated gluconeogenic enzyme transcripts. We also did not find any evidence for an improved growth related to starch intake or glucose utilization in the peripheral tissues of the F line. Thus, our hypothesis of a better dietary carbohydrate utilisation in the F line was not validated. However, the few diet × genotype interaction that we observed in metabolic markers (GK, G6PD, D9D, PK), endorses the possibility of obtaining trout genotype with an improved ability to metabolise dietary starch.

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RESEARCH ARTICLE

Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content

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SUMMARY

Previous studies in two rainbow trout lines divergently selected for lean (L) or fat (F) muscle suggested that they differ in their ability to metabolise glucose. In this context, we investigated whether genetic selection for high muscle fat content led to a better capacity to metabolise dietary carbohydrates. Juvenile trout from the two lines were fed diets with or without gelatinised starch (17.1%) for 10 weeks, after which blood, liver, muscle and adipose tissues were sampled. Growth rate, feed efficiency and protein utilisation were lower in the F line than in the L line. In both lines, intake of carbohydrates was associated with a moderate post-prandial hyperglycaemia, a protein sparing effect, an enhancement of nutrient (TOR-S6) signalling cascade and a decrease of energy-sensing enzyme (AMPK). Gene expression of hepatic glycolytic enzymes was higher in the F line fed carbohydrates compared with the L line, but concurrently transcripts for the gluconeogenic enzymes was also higher in the F line, possibly impairing glucose homeostasis. However, the F line showed a higher gene expression of hepatic enzymes involved in lipogenesis and fatty acid bioconversion, in particular with an increased dietary carbohydrate intake. Enhanced lipogenic potential coupled with higher liver glycogen content in the F line suggests better glucose storage ability than the L line. Overall, the present study demonstrates the changes in hepatic intermediary metabolism resulting from genetic selection for high muscle fat content and dietary carbohydrate intake without, however, any interaction for an improved growth or glucose utilisation in the peripheral tissues.

Key words: fish nutrition, carbohydrates, genetic selection, metabolism, gene expression.

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INTRODUCTION

The ability of fish to use dietary carbohydrates varies greatly among and within species in relation to their feeding habits. Carnivorous fishes are generally considered to be poor utilisers of dietary carbohydrates (Wilson, 1994; Moon, 2001; Hemre et al., 2002). For example, the rainbow trout (*Oncorhynchus mykiss*) exhibits persistent hyperglycaemia following ingestion of digestible carbohydrates or injection of glucose (Bergot, 1979; Wilson, 1994; Moon, 2001), without any clear increase in the provision of net energy (Hilton et al., 1987). Various assumptions were put forth to explain this poor utilisation, out of which insulin deficiency, lack of insulin-dependent glucose transporters and lack of an inducible hepatic glucokinase were proved to be false (Mommsen and Plisetskaya, 1991; Panserat et al., 2000a; Planas et al., 2000); low potency of glucose as an insulin secretagogue compared with amino acids, relatively low number of insulin receptors, lack of inhibition of endogenous glucose production and poor hepatic lipogenesis from glucose were proved to be true (Mommsen and Plisetskaya, 1991; Hemre and Kahrs, 1997; Navarro et al., 1999; Panserat et al., 2000b; Enes et al., 2009); reduced peripheral utilisation of glucose and inhibition of insulin secretion by other hormones requires more elaboration (Moon, 2001). Further, the presence of most of the key enzymes involved in carbohydrate metabolic pathways in fish indicate that the poor utilisation may be due to an aberrant hormonal and nutritional regulation caused by evolutionary adaptation (Cowey and Walton, 1989; Enes et al., 2009; Polakof et al., 2011a). For example, a carbohydrate-rich diet induces the hepatic glycolytic enzymes in rainbow trout very much as in mammals, but it does not affect the activity or mRNA levels of key enzymes of gluconeogenesis in complete contrast (Panserat et al., 2009). However, there are also reports of a better glycaemic profile in rainbow trout, which is genetically selected (Skiba-Cassy et al., 2009) or treated with anti-diabetic drug, linking enhanced *de novo* lipogenesis to better glucose homeostasis (Panserat et al., 2009; Polakof et al., 2011b).

From a practical perspective of salmonid aquaculture, the diminishing supply of fish meal and fish oil (the protein and energy source of choice in feeds, respectively) together with their escalating cost and demand emphasise the critical need to substitute them with ingredients of plant origin (naturally rich in carbohydrates) for the sustainability and expansion of production (Naylor, 2000; Tacon and Metian, 2008; Stone, 2003). Therefore, identifying and selecting new genotypes of fish with an improved capability to use the absorbed carbohydrate will be of economical advantage. Though some early studies do not indicate a promising prospect (Edwards et al., 1977; Austreng et al., 1977; Refstie and Austreng, 1981), others have revealed strains of salmonids exhibiting differences in the utilisation of dietary carbohydrates (Mazur et al., 1992; Blanc,

2002). Moreover, the large genetic variation for production traits in the farmed population of salmonids with medium to high heritabilities makes the fish family ideal for genetic selection (Gjedrem, 2000; Henryon et al., 2002).

Recently, two experimental lines of rainbow trout have been developed through divergent selection for low or high muscle fat content (Quillet et al., 2005). Metabolic studies suggest that the two lines differ in utilisation of energy sources when fed diets differing in lipid content but with a constant level (9–12%) of carbohydrates. Reduced hepatic fatty acid oxidation and enhanced glycolysis was observed in liver and muscle of the fat line (F) fish as compared with the lean line (L) fish (Kolditz et al., 2008a). The F line was also found to have lower plasma glucose levels 24 h after the last meal than the L line when fed a standard diet containing 10% starch (Skiba-Cassy et al., 2009). Based on these observations, we hypothesised that the F line has a higher ability to metabolise dietary carbohydrates than the L line. Our objective was to describe the metabolic response of both trout lines to diets with a high level of carbohydrates (17.1%) or without carbohydrates (<1%). We analysed the effects of genetic selection and dietary carbohydrate supply on growth, feed utilisation, body composition and post-prandial plasma metabolite levels. At the tissue level, we examined the signalling of intracellular energy sensors (AMPK), activation of insulin and nutrient pathway (Akt, TOR-S6) and gene expression of key enzymes involved in glycolysis, gluconeogenesis, lipogenesis, β-oxidation and fatty acid bioconversion in liver, which is the centre of intermediary metabolism. We also analysed gene expression in other tissues implicated in glucose utilisation, i.e. white adipose tissue and muscle.

MATERIALS AND METHODS

Experimental fish and diets

The study was performed with two lines of rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792), namely L (lean line) and F (fat line), obtained after four generations of divergent selection for high or low muscle fat content using a non-destructive method (Distell Fish Fatmeter, Fauldhouse, West Lothian, UK) as detailed by Quillet et al. (Quillet et al., 2005). Fish were reared in the INRA experimental facilities at Donzacq (Landes, France) at a constant water temperature of 17.5±0.5°C, under natural photoperiod during the months of July–September. They were fed a standard trout commercial diet (T-3P classic, Skretting, Fontaine-les-Vervins, France) during the acclimatisation period. Two experimental diets, namely 0C (without carbohydrate) and 20C (with carbohydrate), were prepared in our own facilities (INRA, Donzacq, Landes, France) as extruded pellets. Gelatinised starch was included as the carbohydrate source. The 0C diet contained <1% starch, whereas the 20C diet contained 17.1% starch (Table 1). The increase in dietary carbohydrate content was accompanied by a decrease in the proportion of protein in the 20C diet.

Table 1. Composition of diets

	0C	20C
Ingredient (%)		
Fish meal[a]	81	59
Gelatinised starch[b]	0	20
Fish oil[c]	16	18
Binder[d]	1	1
Mineral mix[e]	1	1
Vitamin mix[f]	1	1
Analytical composition		
Dry matter (%)	94.0	94.0
Protein (% DM)	58.6	44.7
Lipid (% DM)	25.4	22.6
Starch (% DM)	<1	17.1
Energy (kJ g^{-1} DM)	24.7	23.6
Ash (% DM)	11.3	9.1

0C, diet without carbohydrate; 20C, diet with carbohydrate; DM, dry matter.
[a]Fishmeal (Sopropeche, Boulogne-sur-Mer, France).
[b]Gelatinised starch (Lestrem 62, Roquette, France).
[c]Fish oil (North sea fish oil; Sopropeche, Boulogne-sur-Mer, France).
[d]Alginate GF 150 (Louis François exploitation, Saint-Maur, France).
[e]Mineral mix – 2.15 g calcium carbonate (40% Ca), 1.24 g magnesium oxide (60% Mg), 0.2 g ferric citrate, 0.4 mg kg^{-1} diet potassium iodide (75% I), 0.4 g zinc sulphate (36% Zn), 0.3 g copper sulphate (25% Cu), 0.3 g manganese sulphate (33% Mib), 5 g dibasic calcium phosphate (20% Ca, 18% P), 2 mg kg^{-1} diet cobalt sulphate, 3 mg kg^{-1} diet sodium selenite (30% Se), 0.9 g KCl, 0.4 g NaCl (UPAE, Jouy, Inra, France).
[f]Vitamin mix – 60 IU DL-a tocopherol acetate, 5 mg kg^{-1} diet sodium menadione bisulphate, 15,000 IU retinyl acetate, 3000 IU DL-cholecalciferol, 15 mg kg^{-1} diet thiamin, 30 mg kg^{-1} diet riboflavin, 15 mg kg^{-1} diet pyridoxine, 0.05 mg kg^{-1} diet B12, 175 mg kg^{-1} diet nicotinic acid, 500 mg kg^{-1} diet folic acid, 1000 mg kg^{-1} diet inositol, 2.5 mg kg^{-1} diet biotin, 50 mg kg^{-1} diet calcium panthotenate, 2000 mg kg^{-1} diet choline chloride (UPAE).

Feeding trial and sampling procedure

Fish of each line were distributed into six tanks of 35 animals (mean body mass 27±0.5 g). Triplicate groups of each genotype were fed either the 0C or the 20C diet for 10 weeks, two times daily *ad libitum*. The fish were bulk weighed every 3 weeks and counted to calculate the mean body mass. Feed intake was recorded and feed efficiency was calculated. At the end of the trial, nine fish per group (three per tank) were randomly sampled at 2, 8 and 24 h after the meal. Trout were killed by a sharp blow to the head and individually weighed. Blood was removed from the caudal vein into heparinised syringes and centrifuged (3000 ***g***, 5 min); the recovered plasma was immediately frozen and kept at –20°C until analysis. The gut content of each fish was systematically checked to confirm that the fish sampled had effectively consumed the diet. Liver, perivisceral white adipose tissue and a sample of dorso-ventral white muscle were dissected, weighed, immediately frozen in liquid nitrogen and kept at –80°C pending analysis. The mass of liver was used to calculate the hepatosomatic index [HSI (%)=100×(liver mass/body mass)]. Six fish per genotype at the beginning of the feeding trial and three fish per tank at the end were killed and then frozen for analysis of whole-body composition. They were pooled per tank, ground and freeze-dried before being analysed. The experiments were carried out in accordance with the Guidelines of the National Legislation on Animal Care of the French Ministry of Research (decree no. 2001-464, 29 May 2001) and were approved by the Ethics Committee of INRA (according to INRA 2002-36, 14 April 2002).

Growth performance and nutrient utilisation traits were calculated for each tank with 35 fish, which represented one experimental unit. Final body mass was calculated as the final biomass divided by the number of fish in each tank at the end of the feeding trial. Daily growth coefficient was calculated as $100\times(\text{mean final body mass}^{1/3} - \text{mean initial body mass}^{1/3})$/day. Daily feed intake was calculated as the total amount of ingested food (kg) divided by the mean biomass over the trial [(initial biomass + final biomass)/2, expressed in kg wet mass (WM)] and the number of days. Feed efficiency was estimated as the gain in total biomass [(final biomass – initial biomass) (kg WM)] divided by the amount of ingested dry matter (kg DM). Protein efficiency ratio was the gain in total biomass divided by the amount of ingested crude protein (kg). Nitrogen and fat gain were calculated

as (final carcass nutrient content – initial carcass nutrient content) divided by the mean biomass over the trial [(initial biomass + final biomass)/2 (kg WM)] and the number of days, where nutrient refers to nitrogen and fat. Protein and fat retention were calculated as [100×(final body mass × final carcass nutrient content) – (initial body mass × initial carcass nutrient content)]/nutrient intake, where nutrient refers to protein and lipid.

Analytical methods

The chemical composition of the diets and the freeze-dried samples of whole body was analysed using the following procedures: dry matter after drying at 105°C for 24 h, fat by petroleum ether extraction (Soxtherm, Gerhardt, Konigswinter, Germany), protein content (nitrogen content×6.25) by the Kjeldahl method after acid digestion, gross energy in an adiabatic bomb calorimeter (IKA, Heitersheim Grißheimer, Germany) and ash content by incinerating the samples in a muffle furnace at 600°C for 6 h. Total starch content in the diets was estimated by the enzymatic method (InVivo Labs, Vannes, France). Total lipid content in the muscle was measured according to the method of Folch et al. (Folch et al., 1957), with dichloromethane instead of chloroform as the solvent. Tissue glycogen levels were determined using the amyloglucosidase method (Keppler and Decker, 1974). Plasma glucose (Glucose RTU, bioMérieux, Marcy l'Etoile, France), triglycerides (PAP 150, bioMérieux) and free fatty acid (NEFA C kit, Wako Chemicals, Neuss, Germany) levels were determined using commercial kits adapted to a microplate format, according to the recommendations of the manufacturer. Total plasma free amino acid levels were determined by the ninhydrin reaction according to the method of Moore (Moore, 1968), with glycine as standard.

Western blot analysis

Protein extraction and western blotting were performed on liver samples of fish sampled 2 h after the meal. Six individual samples per experimental condition were used as biological replicates. Frozen livers (200 mg) were homogenised on ice with an Ultraturrax homogeniser in 2 ml of buffer containing 150 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA (pH 7.4), 100 mmol l^{-1} sodium fluoride, 4 mmol l^{-1} sodium pyrophosphate, 2 mmol l^{-1} sodium orthovanadate, 1% Triton X-100, 0.5% Nonidet P-40-IGEPAL and a protease inhibitor cocktail (Roche, Basel, Switzerland). Homogenates were centrifuged at 1000 ***g*** for 15 min at 4°C and supernatants were again centrifuged at 20,000 ***g*** for 30 min. The resulting supernatants were aliquoted and stored at –80°C. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with bovine serum albumin as standard. Liver protein lysates (10 µg of protein for Akt and S6, and 20 µg for AMPK) were subjected to SDS-PAGE and western blotting using the appropriate antibody. Anti-phospho Akt (Ser473), anti-carboxyl terminal Akt, anti-phospho-S6 (Ser235/236), anti-S6 ribosomal protein, anti-phospho-AMPK (Thr172) and anti-AMPK antibodies were used (Cell Signaling Technology, Saint Quentin Yvelines, France). These antibodies have been shown to cross-react successfully with rainbow trout proteins of interest (Skiba-Cassy et al., 2009; Polakof et al., 2011c). After washing, membranes were incubated with an IRDye infrared secondary antibody (LI-COR Biosciences, Lincoln, NE, USA). Bands were visualised by infrared fluorescence using the Odyssey imaging system (LI-COR Biosciences) and quantified by Odyssey Infrared Imaging System software (version 1.2, LI-COR Biosciences).

mRNA levels analysis: quantitative RT-PCR

Analyses of mRNA levels were performed on samples from the liver, white muscle and perivisceral white adipose tissue of fish sampled 8 h after the last meal, the time interval corresponding to the post-prandial peak of nutrient absorption in juvenile rainbow trout reared at 17°C. Six individual samples per experimental condition were used as biological replicates. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations, quantified by spectrophotometry (absorbance at 260 nm) and integrity was controlled using the Agilent 2100 bioanalyzer (Agilent Technologies, Kista, Sweden). One microgram of the resulting total RNA was reverse transcribed into cDNA using the SuperScript III RNaseH- reverse transcriptase kit (Invitrogen) and random primers (Promega, Charbonniéres, France) according to the instructions of each manufacturer. Target gene expression levels were determined by quantitative real-time PCR as previously described, using specific primers (Kolditz et al., 2008a; Skiba-Cassy et al., 2009; Panserat et al., 2009; Polakof et al., 2009), except for Δ6 desaturase (forward: AGGGTGCCTCTGCTAACTGG; reverse: TGGTGTTGG-TGATGGTAGGG; GenBank AF301910) and Δ9 desaturase (forward: GCCGTCCGAGGGTTCTTCTT; reverse: CTCTC-CCCACAGGCACCAAG; GenBank FP323026, Sigenae clone tcba0028.c.12 5). The transcripts analysed were glucokinase (GK; EC 2.7.1.2), hexokinase 1 (HK1; EC 2.7.1.1), 6-phosphofructo-1-kinase (6PFK; EC 2.7.1.11) and pyruvate kinase (PK; EC 2.7.1.40) for glycolysis; glucose-6-phosphatase (G6Pase; EC 3.1.3.9), fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11) and phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) for gluconeogenesis; glucose 6-phosphate dehydrogenase (G6PD; EC 1.1.1.49), ATP citrate lyase (ACLY; EC 2.3.3.8), fatty acid synthase (FAS; EC 2.3.1.85) and the transcription factor sterol regulatory element binding protein 1-like (SREBP-1c) for lipogenesis; Δ6 desaturase (D6D; EC 1.14.19.3) and Δ9 desaturase (D9D; EC 1.14.19.1) for fatty acid bioconversion; and carnitine palmitoyl transferase 1 (CPT1; EC 2.3.1.21) and 3-hydroxyacyl-CoA dehydrogenase (HOAD; EC 1.1.1.35) for fatty acid oxidation. When different isoforms of a gene were known in rainbow trout (as for G6Pase and CPT1), gene expression analysis was performed on each isoform. The PCR run included quadruplicates for each sample (duplicates of reverse transcription and PCR amplification, respectively) and also negative controls (reverse transcriptase and RNA free samples). Elongation factor-1 alpha (EF1α) was employed as a non-regulated reference gene (Olsvik et al., 2005) and it was found to be stably expressed in this study. Relative quantification of target gene expression was performed using the mathematical model described by Pfaffl (Pfaffl, 2001) with the Relative Expression Software Tool (REST).

Enzyme activity analysis

Enzyme activities were measured on liver samples of fish sampled 24 h after the meal. Six individual samples per experimental condition were used as biological replicates. Liver samples used to measure the lipogenic enzyme activities were homogenised by ultrasonic disruption in four volumes of ice-cold buffer (0.02 mol l^{-1} Tris–HCl, 0.25 mol l^{-1} sucrose, 2 mmol l^{-1} EDTA, 0.1 mol l^{-1} NaF, 0.5 mmol phenylmethyl sulphonyl fluoride, 0.01 mol l^{-1} b-mercaptoethanol, pH 7.4), centrifuged at 24,000 ***g*** at 4°C for 20 min and the supernatant were used immediately for enzyme assays in pre-established conditions. G6PD (final substrate concentration 0.5 mmol l^{-1} glucose-6-phosphate) and FAS (final substrate concentration 50 µmol l^{-1} malonyl-CoA) were assessed following the method described in Figueiredo-Silva et al. (Figueiredo-Silva

Table 2. Growth performance and nutrient utilization of rainbow trout from L and F lines fed 0C and 20C diets for 10 weeks

	L line		F line		*P*		
	0C	20C	0C	20C	Diet	Line	Diet × Line
Final body mass (g)	78.5±4.5	79.8±3.2	70.9±5.5	68.4±3.7	0.75	0.005	0.49
Daily growth index (%)	2.0±0.1	2.1±0.1	1.8±0.2	1.7±0.1	0.79	0.01	0.37
Feed intake (g kg^{-1} day^{-1})	16.8±0.1	16.5±0.6	16.6±0.5	16.0±0.3	0.11	0.16	0.59
Feed efficiency	0.97±0.03	1.0±0.03	0.92±0.06	0.92±0.03	0.58	0.03	0.41
Protein efficiency ratio	1.7±0.1	2.2±0.1	1.6±0.1	2.1±0.1	<10^{-4}	0.02	0.27
N gain (mg kg^{-1} ABM day^{-1})	363±9	368±30	366±18	336±13	0.29	0.22	0.14
Fat gain (g kg^{-1} ABM day^{-1})	2.3±0.3	2.3±0.3	2.4±0.1	2.2±0.1	0.86	0.89	0.54
Protein retention (%)	24.5±0.5	33.2±2.6	25.1±0.9	31.2±1.7	<10^{-4}	0.49	0.21
Lipid retention (%)	56.8±8.1	66.6±6.3	59.4±0.6	66.0±3.5	0.03	0.76	0.63

Data are means ± s.d. (*N*=3 tanks). Data were analysed by two-way ANOVA followed by a Student–Newman–Keuls multiple comparison test. Differences were considered statistically significant at *P*<0.05. ABM, mean biomass.

et al., 2010) adapted to trout tissues. PK enzyme activity was measured as described by Kirchner et al. (Kirchner et al., 2003). Enzyme reaction rates were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm using a microplate spectrophotometer. Enzyme activity units (IU), defined as micromoles of substrate converted to product, per minute, at 37°C, were expressed per gram of liver. All of the measurements for each individual sample were performed in duplicate.

Statistical analysis

The results are presented as means ± s.d. The effects of diets, lines and the diet × line interaction on the different parameters were tested using StatView (version 5; SAS Institute, Cary, NC, USA) by means of a two-way ANOVA with diet and line as independent variables. *Post hoc* comparisons were made using a Student–Newman–Keuls multiple comparison test, and differences were considered statistically significant at $P<0.05$. When interactions were significant, means were compared using one-way ANOVA ($P<0.05$).

RESULTS

Growth performance, feed efficiency and nutrient retention

Feed intake was similar irrespective of line or diet (Table 2). At the end of the feeding trial, trout from the lean line (L) had a significantly higher body mass ($P=0.005$) than fish from the fat line (F), irrespective of diet. Feed efficiency was thus better in L line than in F line fish ($P=0.03$). Dietary treatments did not affect growth rate or final body mass. But as expected, the protein efficiency ratio was higher with the 20C diet ($P<0.0001$), because of the protein-sparing effect of the carbohydrates in this diet. The L line exhibited better protein efficiency ($P=0.02$) than the F line. The 20C diet improved the protein ($P<0.0001$) and fat retention ($P=0.03$) in both the lines. Daily nitrogen and fat gains/retention were not significantly different between the two trout lines. No significant line × diet interaction was recorded for any of these parameters.

Whole-body and tissue composition

Dietary treatments did not modify the whole-body composition of fish from both lines, but an effect of genotype was found (Table 3). F line fish had higher whole-body lipid content ($P=0.046$) and reciprocally lower moisture content ($P=0.046$) than the L line fish. The protein content was similar in both lines. As expected, the target of genetic selection, the muscle lipid content was significantly higher in the F line fish than in the L line fish ($P=0.0004$), with no significant dietary influence. Both trout lines showed higher HSI when fed the 20C diet ($P<0.0001$). The measure of liver glycogen levels (Table 3) confirmed the increased storage of glucose as glycogen when fed the 20C diet ($P<0.0001$). The F line fish had higher liver glycogen levels ($P=0.03$) than the L line fish, irrespective of the diet. The glycogen levels in muscle remained unaffected by the diets or genetic selection. In perivisceral adipose tissue, glycogen content was negligible (data not shown).

Plasma metabolite levels

The results of post-prandial plasma metabolites measured at 2, 8 and 24 h after the meal are presented in Table 4. Plasma glucose levels were higher in fish of both lines fed the 20C diet at 2 and 8 h after the meal ($P<0.0001$ and $P=0.001$, respectively) compared

Table 3. Whole-body composition and tissue metabolites of rainbow trout from L and F lines fed 0C and 20C diets for 10 weeks

	L line		F line		*P*		
	0C	20C	0C	20C	Diet	Line	Diet × Line
Whole-body moisture content (%WM)	68.0±0.7	67.5±0.9	66.4±1.2	66.5±1.0	0.76	0.05	0.57
Whole-body protein content (%WM)	15.3±0.2	15.3±0.4	15.7±0.3	15.4±0.4	0.32	0.21	0.43
Whole-body lipid content (%WM)	14.5±1.1	14.6±1.1	15.7±0.7	15.7±0.5	0.95	0.05	0.91
Whole-body ash content (%WM)	2.0±0.1	2.1±0.03	2.1±0.1	2.1±0.1	0.34	0.63	0.11
Energy content (kJ g^{-1} WM)	9.2±0.3	9.3±0.3	9.7±0.4	9.7±0.4	0.97	0.04	0.75
Hepato-somatic index (%)	1.2±0.3	1.8±0.2	1.1±0.2	1.9±0.3	<10^{-4}	0.39	0.13
Liver glycogen content (μmol glycosyl units g^{-1} WM)	153±31	625±132	261±75	700±101	<10^{-4}	0.03	0.68
Muscle lipid content (% WM)	5.4±1.1	5.2±1.8	7.8±2.5	9.0±3.4	0.53	4×10^{-4}	0.40
Muscle glycogen content (μmol glycosyl units g^{-1} WM)	5.5±3.1	6.6±2.0	4.9±2.8	3.5±2.6	0.90	0.11	0.28

Data are presented as means ± s.d. (*N*=3 pools for whole-body composition analysis; *N*=6 individuals for tissue glycogen estimations; *N*=9 individuals for hepato-somatic index and muscle lipid estimation) and were analysed by two-way ANOVA followed by a Student–Newman–Keuls multiple comparison test. Differences were considered statistically significant at *P*<0.05.

Table 4. Post-prandial plasma metabolites of rainbow trout from L and F lines fed 0C and 20C diets

Plasma metabolite	Post-prandial time	L line		F line		*P*		
		0C	20C	0C	20C	Diet	Line	Diet × Line
Glucose (mmol l^{-1})	2 h	4.3±0.6^{b}	5.9±1.1^{b}	4.4±0.4^{b}	6.3±1.7	$<10^{-4}$	0.53	0.78
	8 h	5.4±1.2^{a}	7.6±2.0^{a}	5.6±1.1^{a}	6.9±1.5	0.001	0.68	0.43
	24 h	5.6±0.8^{a}	5.9±1.3^{a,b}	6.3±1.2^{a}	6.3±0.9	0.70	0.18	0.70
Triglycerides (mmol l^{-1})	2 h	5.6±2.2^{b}	6.2±1.7	9.3±3.6^{b}	9.0±4.3^{b}	0.92	0.004	0.66
	8 h	11.3±4.0^{a}	11.4±6.1	16.4±6.0^{a}	20.6±3.5^{a}	0.21	2×10^{-4}	0.23
	24 h	5.5±2.7^{b}	7.8±5.4	9.6±3.1^{b}	7.5±1.7^{b}	0.01	0.02	0.42
Free fatty acids (mequiv. oleic acid l^{-1})	2 h	0.1±0.03^{c}	0.1±0.03^{b}	0.1±0.02^{c}	0.1±0.03^{c}	0.23	0.30	0.61
	8 h	0.22±0.05^{a}	0.19±0.05^{a}	0.2±0.04^{a}	0.18±0.03^{a}	0.10	0.33	0.77
	24 h	0.15±0.02^{b}	0.17±0.04^{a}	0.15±0.03^{b}	0.14±0.03^{b}	0.40	0.08	0.33
Free amino acids (mequiv. glycine ml^{-1})	2 h	0.47±0.07^{b}	0.51±0.05^{a}	0.5±0.08^{b}	0.48±0.06^{b}	0.71	0.88	0.24
	8 h	0.68±0.15^{a}	0.51±0.1^{a}	0.62±0.09^{a}	0.59±0.08^{a}	0.02	0.81	0.06
	24 h	0.45±0.08^{b}	0.39±0.06^{b}	0.47±0.05^{b}	0.47±0.05^{b}	0.19	0.03	0.24

Data are means ± s.d. (N=9 individuals). At each post-prandial time (row wise), the effect of diet, line and interaction were analysed by two-way ANOVA (P<0.05) followed by a Student–Newman–Keuls multiple comparison test. Within each dietary treatment (column wise), significant differences in post-prandial kinetics (2, 8 and 24 h after the last meal) are represented with different superscripts (one-way ANOVA, P<0.05).

with fish fed the 0C diet. However, the level of post-prandial hyperglycaemia induced by dietary carbohydrates was not very high (<8 mmol l^{-1}) in either trout line. Particularly in the F line fish fed the 20C diet, a statistically significant post-prandial hyperglycaemia was not observed but the plasma glucose levels were relatively higher at all time intervals. No significant effect of genetic selection was found for the plasma glucose level at any of the post-prandial times analysed. Plasma free fatty acid levels were not significantly different with respect to line or diet and all the treatments exhibited a similar post-prandial pattern with a peak at 8 h. The F line fish showed a significantly higher level of plasma triglycerides than the L line fish, irrespective of diet and time. The highest plasma triglyceride levels were recorded at 8 h after the meal, and were nearly twofold higher in the F line than in the L line (P=0.0002). Fish fed the 0C diet showed significantly higher plasma free amino acid levels than fish fed the 20C diet at 8 h, the point of post-prandial peak (P=0.02). The F line fish exhibited higher plasma free amino acids than the L line at 24 h (P=0.03).

mRNA levels of enzymes involved in intermediary metabolism

Data on the mRNA levels of the glycolytic enzymes analysed are illustrated in Fig. 1. In the liver, GK mRNA levels were induced several hundred-fold in fish in both the lines when fed the 20C diet (P<0.0001); this induction was also dependent on genotype, with the F line showing a higher expression than the L line, as indicated by the significant interaction between diet and line (P=0.04). Expression levels of 6PFK mRNA remained unchanged between the lines whatever the diet. PK mRNA levels were uncharacteristically reduced in fish fed the 20C diet compared with those fed the 0C diet (P<0.0001), and F line fish showed a higher PK expression than L line fish (P=0.04). In muscle and adipose tissue, the mRNA levels of the glycolytic genes analysed were similar between lines and remained unchanged with dietary treatment, with one exception in adipose tissue. An interaction between diet and genotype was observed for PK (P=0.01), where the L line exhibited decreased mRNA levels compared with the F line when fed the 20C diet. The mRNA levels of the hepatic gluconeogenic enzymes analysed were found to be differentially regulated by dietary carbohydrates (Fig. 2). The 20C diet upregulated the expression of G6Pase-2 (P=0.04), whereas it significantly downregulated the expression of FBPase (P<0.0001). Trout from the F line exhibited significantly higher expression levels of FBPase and PEPCK mRNA than the L line (P<0.0001 for both). In the liver, mRNA expression levels of genes encoding enzymes involved in NADPH synthesis and lipogenesis (G6PD, ACLY and FAS) were significantly higher in the F line fish than in the L line fish (Fig. 3). The 20C diet enhanced the transcript levels of these lipogenic enzymes in the trout lines, particularly in the F line. A significant interaction between diet and line was observed for G6PD mRNA levels (P=0.04), with a strong induction in the F line when fed the 20C diet. No significant difference between the lines was observed for the transcription factor SREBP1c. Regardless of diet and line, no changes in relative transcript levels were observed for all the lipogenic enzymes examined in the adipose tissue except for the transcription factor SREBP1c, the expression of which was significantly higher in the F line than in the L line (P=0.01). Further concerning fatty acid bioconversion in the liver (Fig. 3), the F line demonstrated a significantly higher mRNA expression of D6D than the L line (P<0.0001) irrespective of diet. In the case of D9D, mRNA expression levels were considerably enhanced by the 20C diet in both lines (P=0.0003), but the level of induction varied significantly depending on the genotype, leading to a significant interaction (P=0.008). As shown in Fig. 4, dietary treatment induced no variation in the mRNA levels of candidate enzymes linked to fatty acid oxidation in all the three tissues analysed. But an effect of genetic selection was observed for the mRNA levels of the two hepatic β-oxidation enzymes investigated. The L line showed a significantly higher level of expression of both A and B isoforms of CPT1 than the F line (P=0.0002 and 0.0003, respectively), whereas an inverse line effect was observed for HOAD (P=0.0009).

Activities of enzymes involved in hepatic intermediary metabolism

To substantiate the results from gene expression analysis, the activities of two lipogenic enzymes, FAS and G6PD, were determined 24 h after the last meal (Fig. 5). The activity of FAS and G6PD were significantly enhanced by dietary carbohydrates in both fish lines (P=0.01 and P<0.0001, respectively), but no significant difference was observed between the lines. The PK activity was assessed because of its atypically decreased mRNA levels in both fish lines when fed the 20C diet in this study. In contrast to the mRNA levels, the activity of PK was not found to be different between the dietary treatments or the lines.

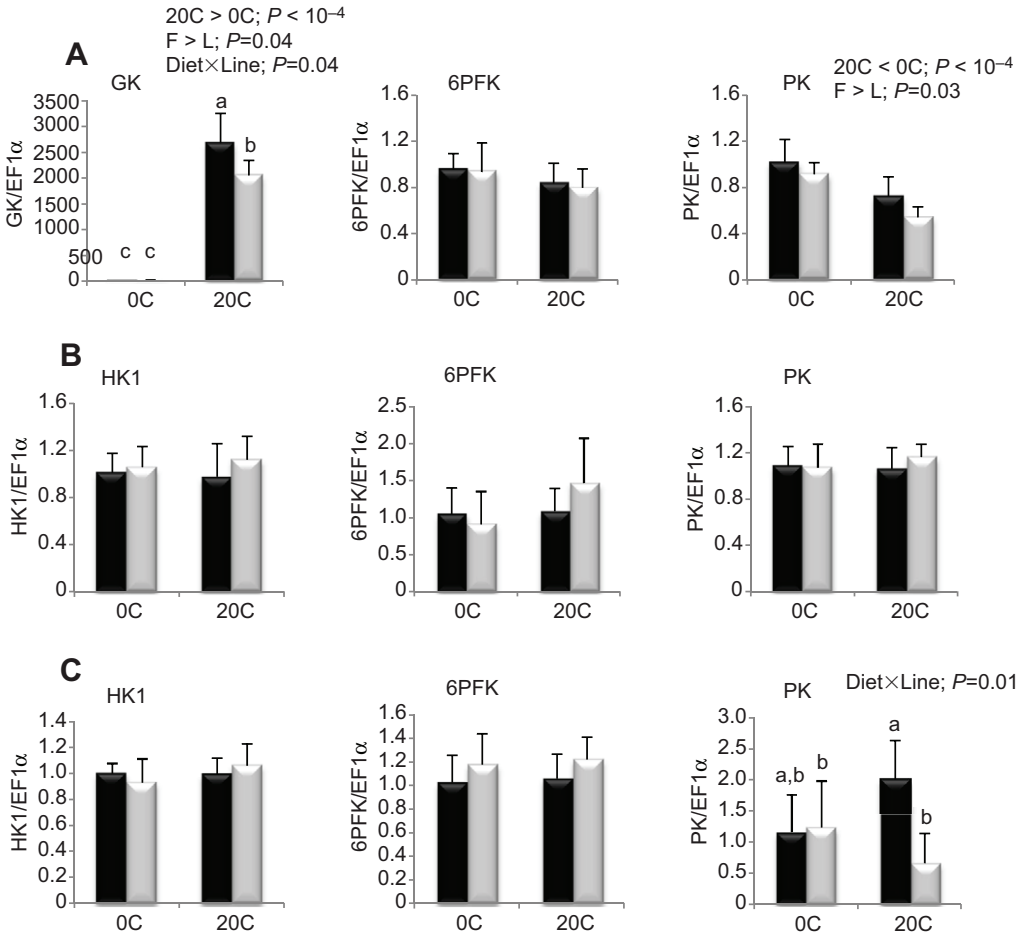

Fig. 1. Gene expression of selected glycolytic enzymes in the (A) liver, (B) muscle and (C) white adipose tissue of rainbow trout from a fat line (F; black bars) and a lean line (L; grey bars) fed a diet without (0C) or with (20C) carbohydrate, 8 h after the last meal. Glucokinase (GK), hexokinase 1 (HK1), 6-phosphofructo-1-kinase (6PFK) and pyruvate kinase (PK) mRNA levels were measured using real-time quantitative RT-PCR. Expression values are normalized by elongation factor-1 alpha (EF1α)-expressed transcripts. Relative fold differences between treatments are presented as means + s.d. (N=6 individuals) and were analysed using two-way ANOVA (P<0.05), followed by a Student–Newman–Keuls multiple comparison test. Mean values not sharing a common lowercase letter are significantly different from each other (one-way ANOVA, P<0.05).

Hepatic Akt, S6 and AMPK phosphorylation status

To better understand the gene expression regulation in liver (in the two other tissues the gene expression was almost constant), we investigated the effect of diet with or without carbohydrates on the activation of different signalling molecules involved in the insulin pathway (Akt), the nutrient sensing pathway (TOR-S6) and cellular energy homeostasis (AMPK) in the two lines. Fig. 6 illustrates the results of western blot analyses of phosphorylated (P-) and total forms of Akt, S6 and AMPK obtained in F and L lines fed either the 0C or the 20C diet. Analysis of the AMPK ratio data indicated that the phosphorylation levels were significantly reduced in fish fed the 20C diet compared with fish fed the 0C diet in both lines (P<0.0001), but were not different between the lines. In contrast, S6 ribosomal protein ratio data showed that the 20C diet increased the activation levels compared with the 0C diet (P<0.03), with no effect of genetic selection. Akt protein phosphorylation was not influenced by diet; however, it was slightly modified by genetic selection (P<0.04).

DISCUSSION

The hypothesis of the present study was that the F line has a higher ability to metabolise dietary carbohydrates than the L line, as previously suggested by Kolditz et al. (Kolditz et al., 2008a) and Skiba-Cassy et al. (Skiba-Cassy et al., 2009), who worked with these trout lines fed diets containing constant level of carbohydrates (~10%). Our objective was thus to verify this hypothesis by analysing the metabolic response of the two trout lines to diets with or without carbohydrates. The effect of diet, genotype and their interaction are discussed separately below.

Effect of diets with (20C) or without (0C) carbohydrates

The diet with carbohydrate did not adversely impact feed intake, feed efficiency or growth of the trout lines because the inclusion level of gelatinised starch was within the acceptable limits and the energy from glucose was utilised as efficiently as that of protein (Kaushik et al., 1989; Kim and Kaushik, 1992; Wilson, 1994). The higher protein efficiency ratio and protein retention associated with dietary carbohydrate intake indicate a protein-sparing effect, as was expected (Kim and Kaushik, 1992; Hemre et al., 2002). Dietary carbohydrate intake resulted in larger liver and increased glycogen content, which is also well documented (Wilson, 1994; Hemre et al., 2002). Moreover, as expected post-prandial hyperglycaemia accompanied the intake of dietary carbohydrates, even though the level of induction was relatively moderate compared with previous studies using similar levels of carbohydrate intake (Panserat et al., 2000a; Bergot, 1979; Kaushik and Oliva Teles, 1985). In the F line, long-term carbohydrate intake resulted in an overall higher glycaemic profile that was not modulated post-prandially after a single meal. We studied the regulation of metabolism by dietary carbohydrates at the signalling, molecular and enzymatic levels in target tissues involved in glucose utilisation, i.e. liver, muscle and adipose tissue. However, we caution that in the present study it was not possible to reject completely any effect of dietary protein levels, as fish fed 20C diet ingested less protein. The absolute difference in carbohydrate intake (<1% *versus* 17.1%) between the two dietary groups, but not for protein (always higher than the requirement; 59% *versus* 45%), enables us to consider that the majority of the effects on intermediary metabolism are linked to the carbohydrate intake.

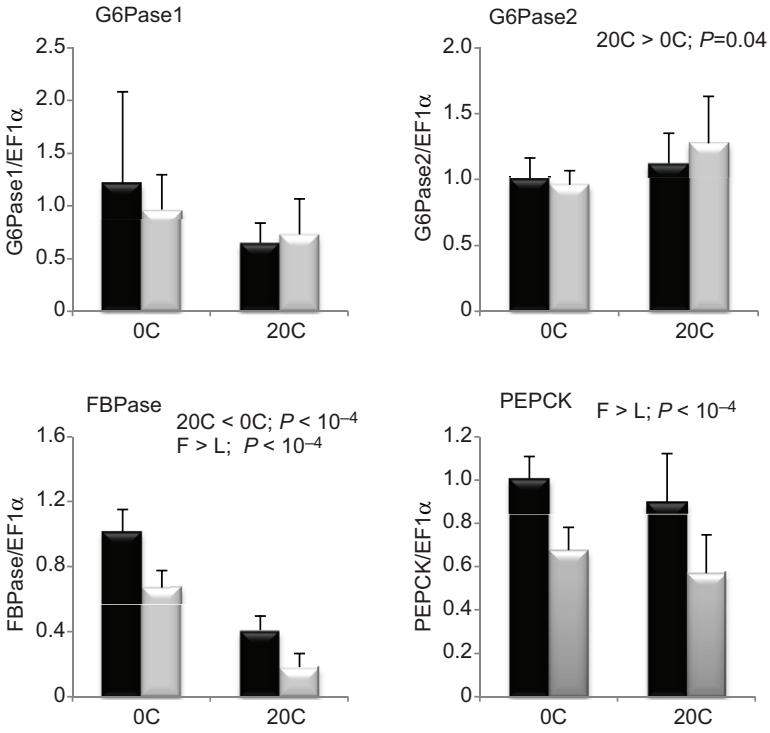

Fig. 2. Gene expression of selected gluconeogenic enzymes in the liver of rainbow trout from a fat line (F; black bars) and a lean line (L; grey bars) fed a diet without (0C) or with (20C) carbohydrate, 8 h after the last meal. Glucose-6-phosphatase isoform 1 (G6Pase1) and isoform 2 (G6Pase2), fructose 1,6-bisphosphatase (FBPase) and phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels were measured using real-time quantitative RT-PCR. Expression values are normalised by elongation factor-1 alpha (EF1α)-expressed transcripts. Relative fold differences between treatments are presented as means + s.d. (N=6 individuals) and were analysed using two-way ANOVA, followed by a Student–Newman–Keuls multiple comparison test. Differences were considered significant at P<0.05.

We analysed specific signalling pathways involved in nutrient (TOR-S6) and energy (AMPK) sensing in liver, the main tissue involved in nutrient homeostasis. We observed that carbohydrate intake decreases the phosphorylation of AMPK in rainbow trout just like in mammals (Assifi et al., 2005), which in turn enhances the activation of S6 mitochondrial protein (Wullschleger et al., 2006; Shaw, 2009). AMPK is a metabolic master switch by which cells sense and decode changes in cellular energy status, whereas S6 protein is a major indicator of the TOR signalling cascade involved in nutrient sensing and cell growth. In mammals, AMPK is known to exert a negative control over the TOR signalling (Rutter et al., 2003; Wullschleger et al., 2006; Shaw, 2009). Existence of these two proteins with a functional regulatory mechanism has already been reported in rainbow trout muscle and liver (Seiliez et al., 2008; Lansard et al., 2009; Polakof et al., 2011c). However, our results strongly suggest, for the first time in fish, that dietary carbohydrates contribute to a specific hepatic cell signalling response, as reflected by the decrease of AMPK phosphorylation as well as the increase of the S6 phosphorylation, when fish were fed with carbohydrates.

We analysed the glucose metabolism in liver, muscle and fat tissue. In liver, we reaffirm the recognised on–off regulation of GK in response to dietary carbohydrates at the transcriptional level (Panserat et al., 2000a; Panserat et al., 2009; Seiliez et al., 2011). The absence of molecular regulation of endogenous glucose production by dietary carbohydrates is also reiterated from the mRNA levels of the rate-limiting gluconeogenic enzymes, G6Pase and PEPCK (Panserat et al., 2000b; Panserat et al., 2001a; Kirchner et al., 2008). The synchronised higher expression of G6Pase 2 and GK suggests the possibility of a futile cycle following dietary carbohydrate intake; further investigations are required to validate it. Moreover, the existence of a paradoxical inhibition of hepatic PK by dietary carbohydrate at the mRNA level could also play a negative role in glucose utilisation in liver (Pilkis and Granner, 1992; Panserat et al., 2001b). This inhibition could result from an interaction between levels of dietary carbohydrates and polyunsaturated fatty acids, as reported in rats (Yamada and Noguchi, 1999), but this hypothesis requires further confirmation. However, contrary to previous reports in trout (Panserat et al., 2001b; Kirchner et al., 2008), FBPase expression was downregulated by dietary carbohydrates, similar to mammals and a few other fish species (Pilkis and Granner, 1992; Panserat et al., 2002). Though the exact reason behind this downregulation remains elusive, this could explain the moderate hyperglycaemia observed, as the rate of glucose production from substrates entering the pathway at the triose phosphate level would be limited. In the white muscle, the absence of dietary effect in the expression of glycolytic enzymes confirms the low response of this tissue to a high influx of glucose, as shown previously (West et al., 1994; Hemre et al., 2002; Panserat et al., 2009). A similar absence of differences in the scarcely studied adipose tissue reinforces the theory that glucose utilisation is poorly regulated in the peripheral tissues of carnivorous fish (Moon, 2001).

There is a strong relationship between glucose and lipid metabolism in animals, which can impact glucose use (Randle, 1998). Indeed, excess carbohydrate intake can be converted to fatty acids through *de novo* lipogenesis (Towle et al., 1997; Dentin et al., 2006). Lipogenesis from dietary carbohydrate has been previously demonstrated in rainbow trout by elevated lipogenic enzyme activities and lipid retention ratios above 100% (Kaushik et al., 1989; Brauge et al., 1995; Alvarez et al., 2000). We found that dietary carbohydrate intake enhanced mRNA levels and activity of hepatic G6PD, representing a potential higher production of NADPH, the cytosolic reducing equivalent required for lipogenesis, as shown previously in different fish (Lin et al., 1977b; Hilton and Atkinson, 1982; Hemre et al., 2002; Panserat et al., 2009). Further, even though the transcript abundance of SREBP1c, a transcription factor involved in regulation of FAS gene expression (Eberle et al., 2004; Ferre and Foufelle, 2007), did not increase with carbohydrate intake, we show evidence for increased hepatic lipogenic capacities through the enhanced activity of FAS and the elevated mRNA levels of ACLY, the main lipogenic enzyme diverting glycolytic carbon

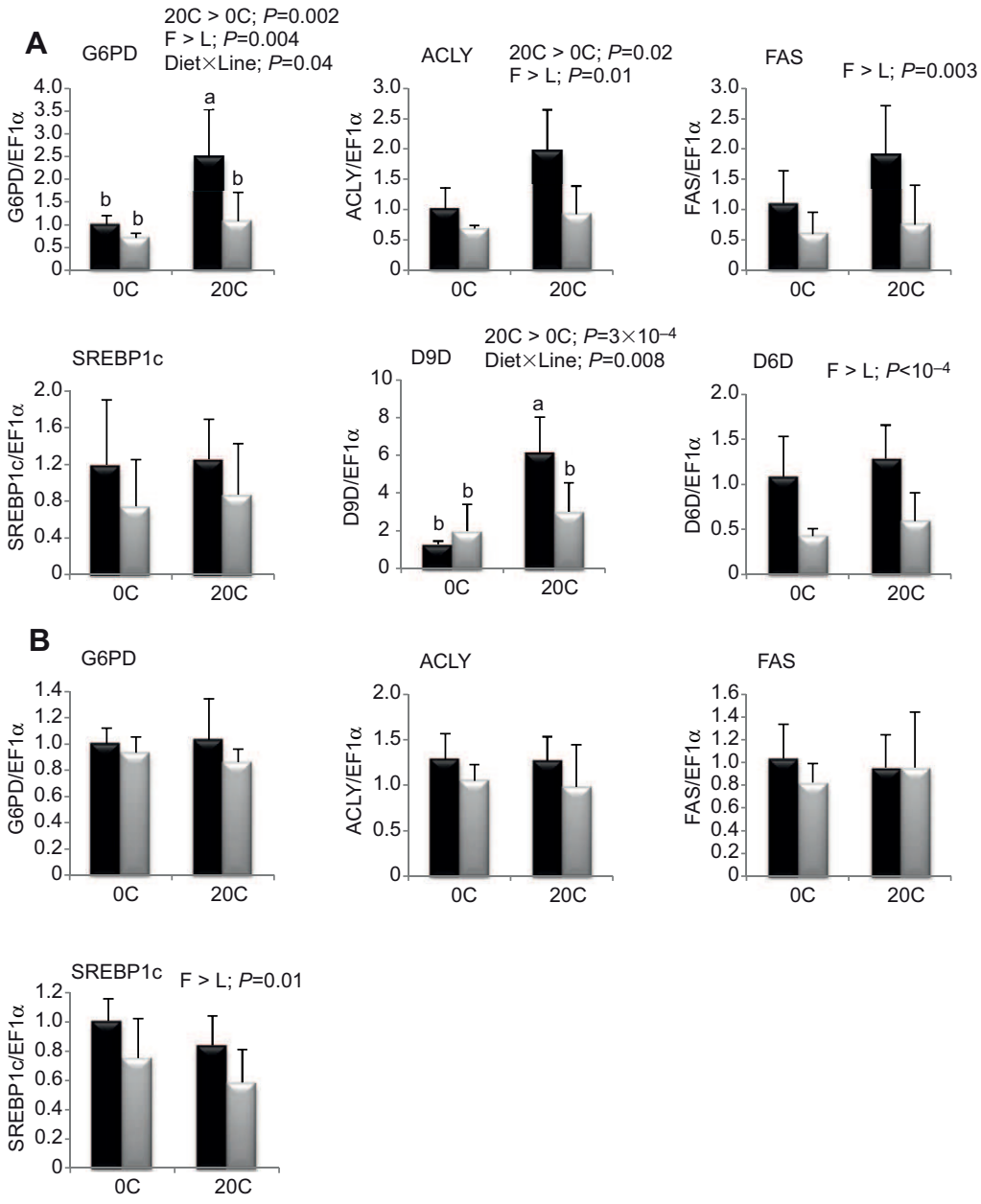

Fig. 3. Gene expression of selected enzymes and transcription factors involved in NADPH generation, lipogenesis and fatty acid bioconversion in the (A) liver and (B) white adipose tissue (excluding fatty acid bioconversion) of rainbow trout from a fat line (F; black bars) and a lean line (L; grey bars) fed a diet without (0C) or with (20C) carbohydrate, 8 h after the last meal. Glucose 6-phosphate dehydrogenase (G6PD), ATP citrate lyase (ACLY), fatty acid synthase (FAS), sterol regulatory element binding protein 1-like (SREBP-1c), Δ9 desaturase (D9D) and Δ6 desaturase (D6D) mRNA levels were measured using real-time quantitative RT-PCR. Expression values are normalised by elongation factor-1 alpha (EF1α)-expressed transcripts. Relative fold differences between treatments are presented as means + s.d. (N=6 individuals) and were analysed using two-way ANOVA (P<0.05), followed by a Student–Newman–Keuls multiple comparison test. Mean values not sharing a common lowercase letter are significantly different from each other (one-way ANOVA, P<0.05).

flux into lipid biosynthesis (Lin et al., 1977a; Lin et al., 1977b). Another interesting observation is that D9D transcript levels are also elevated by feeding dietary carbohydrates, suggesting a higher synthesis of monounsaturated fatty acids following the carbohydrate-induced lipogenesis, as in mammals (Tocher et al., 1998; Ntambi et al., 2004). Looking further along the fatty acid bioconversion pathway, we did not find any effect of dietary carbohydrate on the expression of another desaturase, the D6D, contrary to Seiliez et al. (Seiliez et al., 2001). The high level of fish oil and fish meal in the diets probably supplied enough of the essential fatty acids (Vagner and Santigosa, 2010). In the adipose tissue, we found no stimulatory effect of dietary carbohydrates on the expression of lipogenic enzymes, as has been reported in catfish (Likimani and Wilson, 1982), confirming the lack of response in salmonids to dietary manipulations (Lin et al., 1977a; Lin et al., 1977b). There was also no dietary impact on regulation of fatty acid catabolism, as reflected by the constant levels of CPT1 and HOAD mRNAs. Overall, the enhanced hepatic lipogenic pathway may play a pivotal role in storing excess glucose, ultimately leading to an improved glucose homeostasis in this glucose-intolerant species, as previously suggested using a specific anti-diabetic drug (Panserat et al., 2009; Polakof et al., 2011b).

Effect of genetic selection

We confirm the better growth potential of the L line compared with the F line, which has been reported from the third generation onwards, and it is not linked to a difference in the voluntary feed intake. Rather, it is associated with an improved feed efficiency and protein utilisation in the L line (Quillet et al., 2007; Kolditz et al., 2008a; Skiba-Cassy et al., 2009). The slightly higher whole-body lipid content in the F line can be attributed to the increased divergence in muscle lipid content with the progress of selection (Kolditz et al., 2008a; Skiba-Cassy et al., 2009). Moreover, the nutrient gain and retention estimates for protein and lipid do not reflect any preferential utilisation of energy substrates in the lines. Finally, and contrary to data from a previous study (Skiba-Cassy

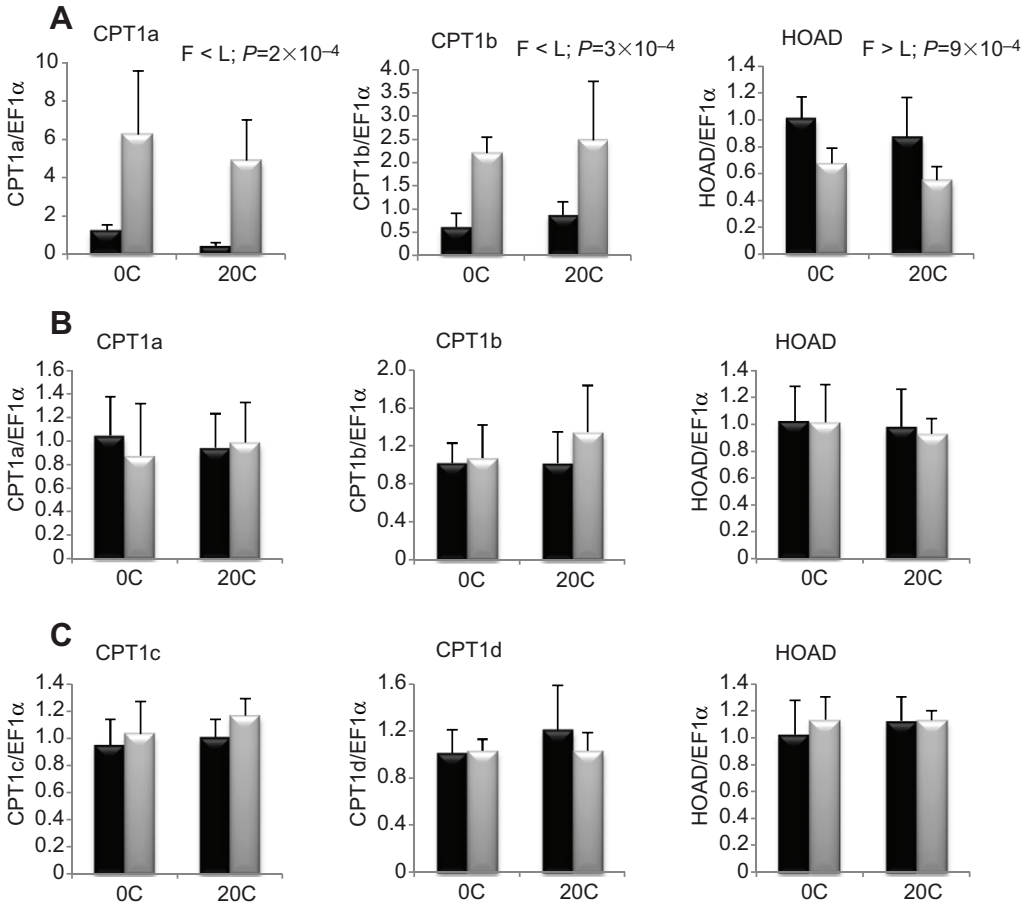

Fig. 4. Gene expression of selected fatty acid oxidation enzymes in the (A) liver, (B) muscle and (C) white adipose tissue of rainbow trout from a fat line (F; black bars) and a lean line (L; grey bars) fed a diet without (0C) or with (20C) carbohydrate, 8 h after the last meal. Carnitine palmitoyl transferase isoforms a (CPT1a), b (CPT1b), c (CPT1c) and d (CPT1d) and 3-hydroxyacyl-CoA dehydrogenase (HOAD) mRNA levels were measured using real-time quantitative RT-PCR. Expression values are normalised by elongation factor-1 alpha (EF1α)-expressed transcripts. Relative fold differences between treatments are presented as means + s.d. (*N*=6 individuals) and were analysed using two-way ANOVA, followed by a Student–Newman–Keuls multiple comparison test. Differences were considered significant at $P<0.05$.

et al., 2009), the F and L lines did not show any difference in the post-prandial regulation of glycaemia, but the measures were not made in the same preceding conditions (fasted/refed). However, the analyses of the molecular actors in glucose and lipid metabolism clearly show significant differences between the two lines.

Studies at the transcriptional level suggest a higher glycolysis in the liver of F line as indicated by the elevated mRNA levels of the enzymes GK and PK (Kolditz et al., 2008a). The higher liver glycogen content in the F line suggests better glucose storage. However, we found that control over the hepatic endogenous glucose production was weaker in the F line than in the L line irrespective of the dietary carbohydrate levels, as indicated by the higher mRNA abundance of the gluconeogenic enzymes PEPCK and FBPase. This indicates that the potential export of glucose synthesised *de novo* through unregulated gluconeogenesis may counter-balance the uptake of glucose from the bloodstream for glycolytic purposes in the F line, which may explain why we did not find any difference in the plasma glucose levels between the two lines. We must note here that the cell signalling analysis in liver cannot explain the differences in glucose metabolism between the two lines: there was a slightly lower activation of Akt phosphorylation in the liver of the F line (which show paradoxically higher insulino-dependent metabolic pathways–glycolysis and glycogen) and no difference in S6 and AMPK phosphorylation, in contrast to our previous study (Skiba-Cassy et al., 2009). In the muscle, we did not observe an enhanced glycolysis in the F line, which was suggested as a possible

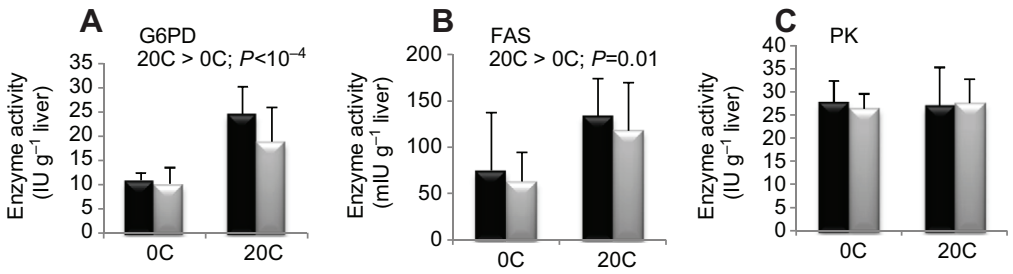

Fig. 5. Selected lipogenic and glycolytic enzyme activities in the liver of rainbow trout from a fat line (F; black bars) and a lean line (L; grey bars) fed a diet without (0C) or with (20C) carbohydrate, 24 h after the last meal. (A) Glucose-6-phosphate dehydrogenase (G6PD). (B) Fatty acid synthase (FAS). (C) Pyruvate kinase (PK). Results are means + s.d. (*N*=6 individuals) and were analysed using two-way ANOVA, followed by a Student–Newman–Keuls multiple comparison test. Differences were considered significant at $P<0.05$. Enzyme activity units (IU), defined as micromoles of substrate converted to product, per minute, at assay temperature, were expressed per gram liver tissue.

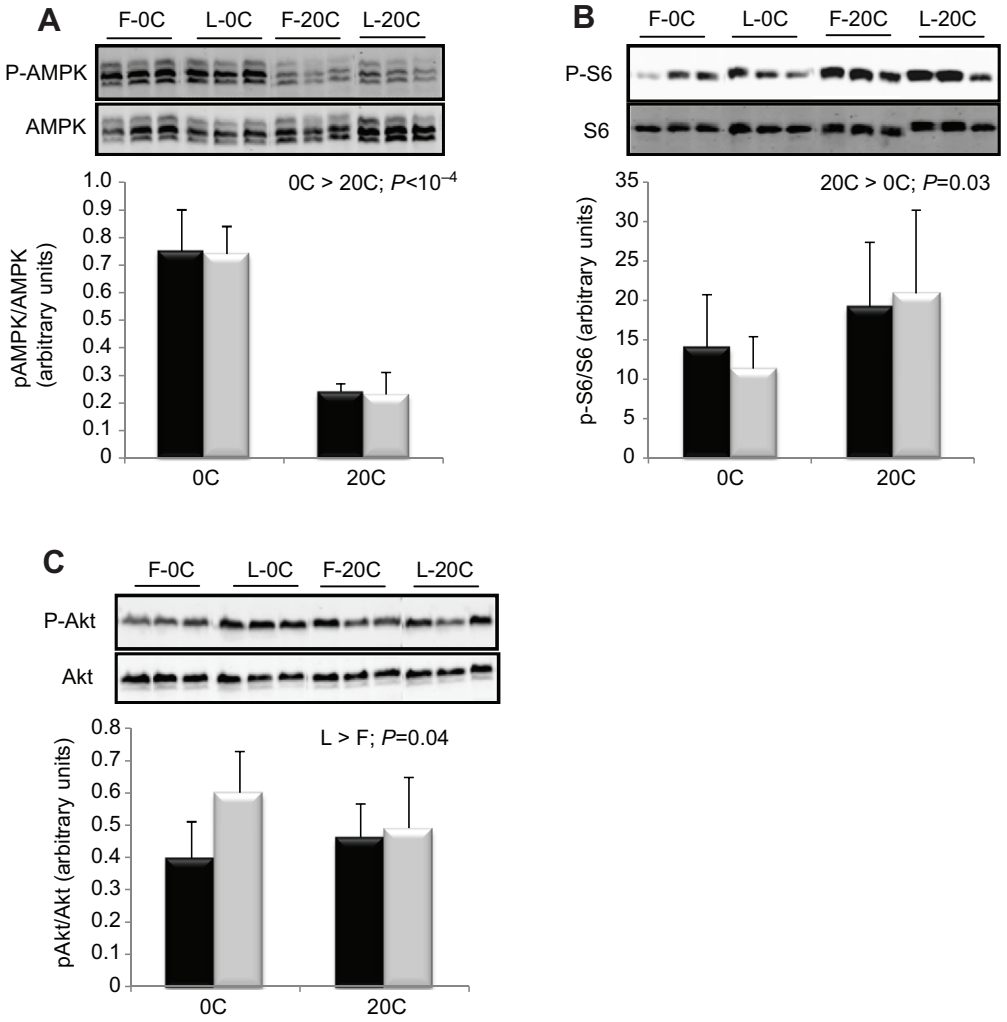

Fig. 6. Western blot analysis of (A) AMPK, (B) S6 and (C) Akt protein phosphorylation in the liver of rainbow trout from a fat line (F; black bars) and a lean line (L; grey bars) fed a diet without (0C) or with (20C) carbohydrate, 2 h after the last meal. Gels were loaded with 20 and 10 μg of total protein per lane for AMPK and S6/Akt, respectively. Western blots were performed on six individual samples per treatment and a representative blot is shown here. Graphs represent the ratio between the phosphorylated protein and the total amount of the target protein. Data are means + s.d. (N=6) and were analysed using two-way ANOVA, followed by a Student–Newman–Keuls multiple comparison test. Differences were considered significant at P<0.05.

genetic predisposition by Kolditz et al. (Kolditz et al., 2008a). This inconsistency may be due to differences in the generation of selection investigated (fourth *versus* third), rearing temperature (17°C *versus* 11°C), diet composition or post-prandial time of analysis (8 h *versus* 24 h). Our hypothesis of a better utilisation of glucose in the peripheral tissues of the F line was therefore not validated.

Regarding the lipid metabolism, we confirm the higher hepatic lipogenic potential in the F line (FAS, ACLY and G6PD) as previously shown (Skiba-Cassy et al., 2009). This is also reflected by the elevated plasma triglyceride levels in the F line. In contrast, in Atlantic salmon similarly selected for muscle adiposity, the fat genotype exhibits lower plasma triglyceride levels reportedly because of an increased lipid uptake by peripheral tissues (Morais et al., 2011b), but the lipogenic capacity of the genotypes were in agreement with our results. In the adipose tissue, though we found an abundance of SREBP1c in the F line, it was not accompanied by higher expression of lipogenic enzymes. The trout adipose tissue was recently proved to have lipogenic ability with possible involvement in glucose homeostasis (Polakof et al., 2011d). Overall, we confirm that the F line is characterised by higher lipogenic capacities than the L line, suggesting a better ability to store excess glucose as triglycerides. Moreover, the lower abundance of the hepatic transcripts of CPT1 a and b isoforms (catalysing the first limiting step for lipid catabolism) in the F line points towards a lower utilisation of fatty acids for energy supply, confirming earlier observations (Kolditz et al., 2008a). These data suggest a possibility for glucose to be used as energy source linked to a decrease in lipid oxidation in liver. Regarding fatty acid bioconversion, we found that the F line showed an enhanced ability through the higher D6D expression, although an opposite case of genotypic difference was reported in similarly selected Atlantic salmon fed a diet rich in vegetable oil (Morais et al., 2011a). The potentially decreased mitochondrial fatty acid oxidation coupled with increased lipogenesis and fatty acid bioconversion in the liver may define the fat muscle phenotype of the F line.

Interactions of diet and genotype

The F and L trout lines were recognised as unique biological material to study diet × genotype interactions focusing on the metabolic utilisation of carbohydrates, based on previous results (Kolditz et al., 2008a; Skiba-Cassy et al., 2009). However, we found only limited interactions. Growth, feed efficiency and nutrient retention did not show any interaction between diet and line, as was previously observed in different rainbow trout families (Edwards et al., 1977; Austreng et al., 1977; Refstie and Austreng, 1981). But the possible genetic determinism of adaptability to dietary carbohydrates in salmonids has been suggested (Mazur et al., 1992; Blanc, 2002). Mazur et al. (Mazur et al., 1992) reported a reduced post-prandial hyperglycaemic response in a specific strain of chinook salmon fed high levels of carbohydrate, whereas we failed to find such a response in the F line, in contrast to Skiba-Cassy et al. (Skiba-Cassy

et al., 2009). In hepatic glucose metabolism, we show a diet × line interaction for the glycolytic enzyme GK and the NADPH-producing enzyme G6PD. Dietary carbohydrates enhanced their expression more specifically in the F line. In an earlier study using the same genotypes, with diets varying in their lipid content, these two genes exhibited a diet × line interaction (Kolditz et al., 2008b). This identifies them as potentially differentiated by genetic selection, responding also to dietary manipulations. Additionally, we report here a disordinal interaction for the hepatic D9D and adipose tissue PK expression, enhanced only in the F line when fed dietary carbohydrates. Though the few interactions endorse our hypothesis that the F line has a higher capacity for using high levels of dietary carbohydrates, they are insufficient to clearly establish this to be the case.

Conclusions

We combined genetic selection and nutritional strategies to investigate the genotype-specific metabolic response to dietary carbohydrates in trout. The enhanced hepatic lipogenic potential and the higher liver glycogen content in the F line illustrate its superior ability to store excess glucose compared with the L line. However, this does not result in an improved regulation of post-prandial glycaemia in the F line, possibly because of unregulated gluconeogenic enzyme transcripts. Because all our major results were obtained in the liver and not in the supposed major glucose utilisation tissues, i.e. muscle and white adipose tissue, our study evidences their lack of response to dietary manipulations with carbohydrates. The poor uptake and utilisation of glucose in these peripheral tissues may be one of the key factors underlying the impaired glucose homeostasis in rainbow trout. Moreover, the diet and genotype interactions that we observed in glucose metabolism (even being few) suggest that it is possible to obtain a trout genotype with an improved ability for lipid biosynthesis in the presence of dietary carbohydrates.

LIST OF ABBREVIATIONS

0C	diet without carbohydrates
20C	diet with carbohydrates
6PFK	6-phosphofructo-1-kinase
ACLY	adenosine triphosphate citrate lyase
Akt	protein kinase B
AMPK	5′ adenosine monophosphate activated protein kinase
D6D	Δ6 desaturase
D9D	Δ9 desaturase
F	fat line
FAS	fatty acid synthase
FBPase	fructose 1,6-bisphosphatase
G6Pase	glucose 6-phosphatase
G6PD	glucose 6-phosphate dehydrogenase
GK	glucokinase
HK1	hexokinase 1
HSI	hepato-somatic index
L	lean line
PEPCK	phosphoenolpyruvate carboxykinase
PK	pyruvate kinase
SREBP1c	sterol regulatory element binding protein 1c
TOR	target of rapamycin

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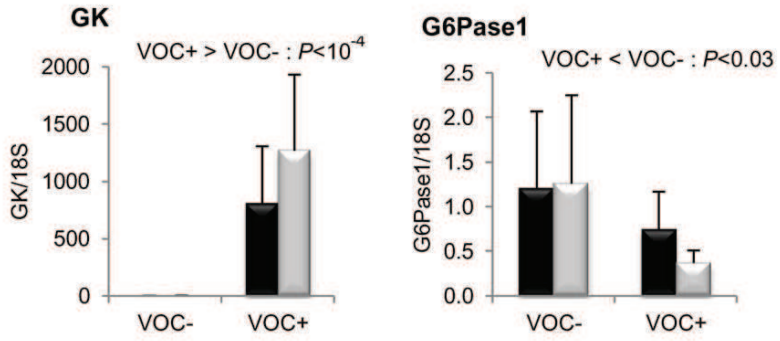
PUBLICATION - 3

Figure 18. *Result highlights from the third experiment – Gene expression of markers of hepatic glucose metabolism*



Presentation of the article

Objective

Based on pre-existing knowledge that individually vegetable oil and carbohydrates enhance the expression of a key desaturase (D6D) in rainbow trout, we hypothesized that in a low n-3 LC-PUFA (vegetable oil) dietary environment carbohydrate intake may enhance the stimulation of fatty acid bioconversion pathway leading to beneficial meat lipid composition. We tested this hypothesis in two genotypes of rainbow trout divergently selected for muscle fat content (fat-F and lean-L line) and with previously demonstrated potential differences in LC-PUFA synthesis and in their ability to metabolise or store glucose. The molecular and phenotypic (fatty acid composition) response of the two lines to a vegetable oil based diet either with or without gelatinized starch, was the subject of investigation.

Experiment and Analyses

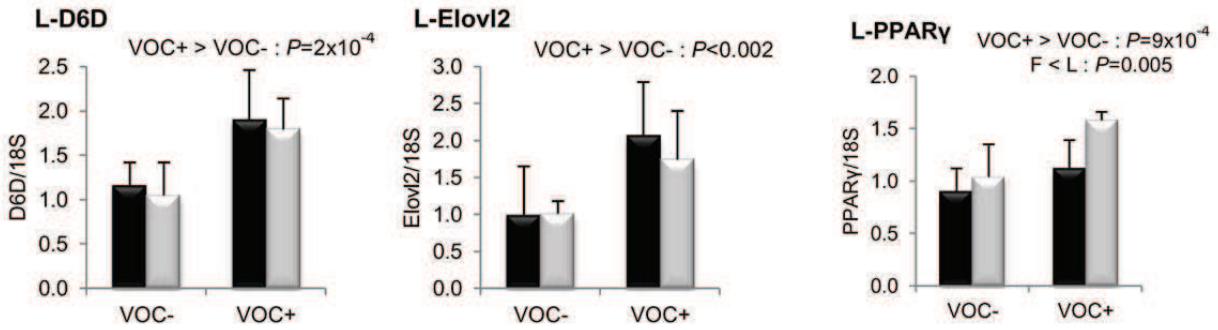
We conducted a 12 week feeding trial, applying a 2 (genotype) by 2 (diet) factorial design. Triplicate groups of juvenile trout from the two lines were fed vegetable oil based diets with or without gelatinised starch (17.1%). At the end of the feeding trial, blood, liver, muscle, intestine and adipose tissue were sampled at 2 and 8 h after the last meal. At the phenotypic level, we measured postprandial plasma metabolite levels and the fatty acid composition of the muscle and liver. At the molecular level, we analysed the gene expression of markers of: intestinal glucose and lipid absorption; carbohydrate (glycolysis and gluconeogenesis) and lipid (lipogenesis and β-oxidation) metabolism in the liver, muscle and adipose tissue; fatty acid bioconversion in the liver and intestine; and glucose and fatty acid uptake in the muscle.

Results and Conclusion

Confirming the metabolic response to starch intake in both trout lines, we found moderate postprandial hyperglycemia (6 to 7.5 mM) and a higher hepato-somatic index at the phenotypic level and further corroboration at the molecular level through the strong induction of hepatic glucokinase expression and a mammalian like down-regulation of the expression of a glucose-6-phosphatase isoform (Fig. 18). However, the level of hexokinase transcripts in the white muscle, adipose tissue and intestine remained unchanged by dietary starch intake.

In accordance with our hypothesis, dietary starch when coupled with low levels of n-3 LC-PUFA in the diet was associated with an increase in the expression of key desaturase (D6D) and elongase (Elovl5 and Elovl2) enzymes in the liver and intestine. PPARγ was identified as

Figure 19. *Result highlights from the third experiment – Gene expression of markers of hepatic fatty acid bioconversion*



the transcription factor that apparently mediates this dietary regulation of fatty acid bioconversion enzymes in the liver. Such augmentation of key fatty acid bioconversion enzymes at the transcriptional level was however not sufficient to significantly modify the fatty acid composition of muscle or liver. Therefore our hypothesis stands validated only at the molecular level and did not appear to extend to the phenotype.

Under the vegetable oil based diet regime, starch intake did not enhance the expression of all the metabolic enzymes involved in *de novo* lipogenesis (FAS, ACLY, D9D) and NADPH production (G6PD), except for ACC. But at the same time, the expression of CPT1b was paradoxically increased in fish fed dietary starch. Further, starch intake was associated with the higher expression of proteins involved in chylomicron assembly (MTP, ApoA1 and ApoA4) and elevated plasma cholesterol levels, while plasma triglyceride levels remained unaltered. All these suggest a potential disturbance in the normal link between lipid metabolism and starch intake in both lines, when fed as part of a vegetable oil based diet.

Concerning the genotype effect, feed intake and growth was higher in the L line than the F line, irrespective of the diet. The previously documented higher potential of the F line in intestinal chylomicron assembly, hepatic *de novo* lipogenesis and fatty acid bioconversion (LC-PUFA synthesis) disappeared under the vegetable oil based diet regime. Further, there was no evidence of substantial genotypic difference in the proportional fatty acid composition of the flesh, when fed diet with vegetable oils. The minor reduction in plasma glucose and triglyceride levels in the F line was linked to potentially higher glucose (GLUT4) and lipid (VLDLR and CD36) uptake in the muscle.

In summary, carbohydrate intake when coupled with low dietary content of n-3 LC-PUFA (vegetable oil) was found to stimulate the inherent LC-PUFA biosynthetic pathway at the molecular level, mediated by the transcription factor PPARγ. However, the final fatty acid profile of the flesh did not evidence a correlative augmentation of LC-PUFA content. Further, the previously documented genetic pre-disposition of higher fatty acid bioconversion in the fat genotype disappeared under the vegetable oil based diet regime. Therefore, our data emphasize the importance of deliberating dietary macro-nutrient interface, when fish nutrition strategies are developed.

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Metabolism and Fatty Acid Profile in Fat and Lean Rainbow Trout Lines Fed with Vegetable Oil: Effect of Carbohydrates

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Abstract

The present study investigated the effect of dietary carbohydrates on metabolism, with special focus on fatty acid bioconversion and flesh lipid composition in two rainbow trout lines divergently selected for muscle lipid content and fed with vegetable oils. These lines were chosen based on previously demonstrated potential differences in LC-PUFA synthesis and carbohydrate utilization. Applying a factorial study design, juvenile trout from the lean (L) and the fat (F) line were fed vegetable oil based diets with or without gelatinised starch (17.1%) for 12 weeks. Blood, liver, muscle, intestine and adipose tissue were sampled after the last meal. Feed intake and growth was higher in the L line than the F line, irrespective of the diet. Moderate postprandial hyperglycemia, strong induction of hepatic glucokinase and repressed glucose-6-phosphatase transcripts confirmed the metabolic response of both lines to carbohydrate intake. Further at the transcriptional level, dietary carbohydrate in the presence of n-3 LC-PUFA deficient vegetable oils enhanced intestinal chylomicron assembly, disturbed hepatic lipid metabolism and importantly elicited a higher response of key desaturase and elongase enzymes in the liver and intestine that endorsed our hypothesis. PPARγ was identified as the factor mediating this dietary regulation of fatty acid bioconversion enzymes in the liver. However, these molecular changes were not sufficient to modify the fatty acid composition of muscle or liver. Concerning the genotype effect, there was no evidence of substantial genotypic difference in lipid metabolism, LC-PUFA synthesis and flesh fatty acid profile when fed with vegetable oils. The minor reduction in plasma glucose and triglyceride levels in the F line was linked to potentially higher glucose and lipid uptake in the muscle. Overall, these data emphasize the importance of dietary macro-nutrient interface in evolving fish nutrition strategies.

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Introduction

Long chain polyunsaturated fatty acids (LC-PUFA) are indispensable in human nutrition due to their vital role in health, development and functionality of several organs. Compared to other food products, fish is the most salubrious source of n-3 LC-PUFA besides providing high quality protein and essential micronutrients [1,2]. Nevertheless, the fatty acid content of the fish varies depending on the species, genotype, dietary and environmental factors [3–5].

At present, an increasing proportion of fish for human consumption comes from aquaculture, where formulation of feeds with fish meal and fish oil ensures n-3 LC-PUFA rich meat production. Parallel to the expansion of aquaculture the demand for these marine ingredients also increases, but global supply has stagnated and the reduction fishery that provides it is perceived as unsustainable and over-exploitative of natural resources [6]. When addressing the imminent shortage, replacement of fish oil by vegetable oils challenges the maintenance of the recognized health benefits due to n-3 LC-PUFA content. Several studies have shown that complete or partial replacement of fish oil with single vegetable oil such as rapeseed oil, linseed oil, palm oil or with a vegetable oil blend has limited effect on growth but marked consequences on lipid metabolism, tissue lipid composition and associated factors such as digestibility, fatty acid catabolism, lipid transport and uptake, lipogenesis, fatty acid desaturation and elongation, and eicosanoid synthesis [4,7,8]. Mostly, dietary fatty acid composition is reflected in the flesh and lipid stores of the fish. However, the magnitude of the change is dependent on the species, fatness and tissues [4].

Salmonid fishes have the capacity to convert dietary essential C_{18} PUFA to physiologically essential C_{20} and C_{22} PUFA through a series of alternating desaturation and chain elongation reactions mediated by microsomal desaturase and elongase enzymes, together with the peroxisomal Sprecher shunt [9]. Molecular studies have elucidated the genetics of these processes through the cloning and characterisation of the genes, thus enabling the study of their expression, regulation and manipulation [10-15]. Vegetable oils rich in 18:3n-3 are known to enhance fatty acid elongation and desaturation processes, both at molecular and enzymatic level [15,16]. Also, one instance of an increase in $\Delta 6$ desaturase expression linked to dietary carbohydrate intake has been reported in rainbow trout [10]. Though exact mechanisms are unknown, the well known association of carbohydrate intake

and increased fatty acid synthesis through the supply of reducing equivalents and substrates [17] may extend also to fatty acid bioconversion. Thus we hypothesized that in a low n-3 LC-PUFA (vegetable oil) dietary environment; carbohydrate intake may enhance the stimulation of fatty acid bioconversion pathway leading to beneficial flesh lipid composition.

We tested this hypothesis in two genotypes of rainbow trout because genetic makeup was found to influence the lipid deposition and metabolism in fish through a highly heritable genetic component governing the capacity to synthesize and/or deposit LC-PUFA, particularly when fed diets low in these fatty acids [5,18,19]. The two experimental rainbow trout lines, namely fat line (F) and lean line (L), were developed through divergent selection for muscle fat content using a non-invasive technique [20]. Previous studies revealed that the two lines consistently differed in growth and hepatic intermediary metabolism, albeit under different dietary regimes [21–23]. Compared to the L line, the F line was found to have lower plasma glucose levels 24 h after the last meal [22]; reduced hepatic fatty acid oxidation and enhanced glycolysis in liver and muscle [21]; higher lipogenic potential coupled with higher liver glycogen content with an increase in dietary carbohydrate intake [23], all of which suggest a better ability of the F line to metabolise and store glucose. Importantly, the F line also exhibited a prospective genetic predisposition for higher LC-PUFA synthesis, both in the liver and intestine [23,24]. These features make the two lines unique models to study genotypic response to dietary manipulations focusing on sustainable feeds.

The aim of the present study was therefore to investigate the impact of dietary carbohydrate on the metabolism and tissue lipid composition of these two trout lines, when fed vegetable oil based diets containing low levels of n-3 LC-PUFA. At the phenotypic level, we measured postprandial plasma metabolite levels and the fatty acid composition of the muscle and liver. At the molecular level, we analysed the gene expression of intestinal markers of glucose and lipid absorption; markers of carbohydrate (glycolysis and gluconeogenesis) and lipid (lipogenesis and β-oxidation) metabolism in the liver, muscle and adipose tissue; transcriptional factors of fatty acid metabolism; key enzymes of the fatty acid bioconversion pathway in the liver and intestine; and markers of glucose and fatty acid uptake in the muscle.

Materials and Methods

Ethics statement

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 station is certified for animal services under the permit number A64.495.1 by the French veterinary services, which is the competent authority.

Experimental fish and diets

The study was conducted with juvenile rainbow trout (*Oncorhynchus mykiss*, Walbaum) from two experimental lines, namely fat line (F) and lean line (L), obtained after four generations of divergent selection for high or low muscle fat content using a non-invasive method (Distell Fish Fatmeter, Fauldhouse, West Lothian, UK), as detailed by Quillet et al. [20]. Fish were reared in the INRA flow-through experimental facilities at Donzacq (Landes, France) at a constant water temperature of 17°C, under natural photoperiod during the months of April-July. They were fed a standard commercial trout diet during the acclimatisation period (T-3P classic, Skretting, France). Two experimental diets, namely VOC- (vegetable oil based diet without carbohydrate) and VOC+ (vegetable oil based diet with carbohydrate) were prepared in our own facilities (INRA, Donzacq, Landes, France) as extruded pellets. The vegetable oil mix used in the diet formulation comprised of linseed oil, palm oil and rapeseed oil in the ratio 50:30:20, respectively. Gelatinized starch was included as the carbohydrate source. The VOC- diet contained 1.8% starch, whereas the VOC+ diet contained 17.1% starch (Table 1). The increase in dietary carbohydrate content was accompanied by a decrease in the proportion of protein in the VOC+ diet.

Feeding trial and sampling procedure

Fish of each line were distributed into six tanks of 30 animals (mean weight 120±1.5 g). Triplicate groups of each genotype were fed either the VOC- or the VOC+ diet, twice a day ad-libitum for a period of 12 weeks. The fish were bulk weighed every 3 wks and counted to calculate the mean body weight. Feed intake was recorded. At the end of the trial, nine fish per group (3/tank) were randomly sampled at 2 and 8 h after the last meal. Trout were anaesthetised in diluted 2-phenoxyethanol (0.05%), individually weighed and sacrificed after collecting blood, by severing the spinal cord behind the head. Blood was removed from the caudal vein into heparinised syringes and centrifuged (3,000 *g*, 5 min), the recovered plasma was immediately frozen and kept at −20°C until analysis. Gut content of each fish was systematically checked to

Table 1. Composition of diets.

	VOC-	VOC+
Ingredients, %		
Fish meal[1]	60	60
Wheat gluten[2]	20	0
Gelatinized starch[2]	0	20
Vegetable oil mix[3]	18	18
Mineral mix[4]	1	1
Vitamin mix[5]	1	1
Analytical composition		
Dry matter, %	94.0	94.4
Protein, % DM	58.6	43.0
Lipid, % DM	25.4	25.7
Starch, % DM	1.8	17.1
Energy, kJ/g DM	24.9	24.4
Ash, % DM	9.3	8.6

VOC-, diet without carbohydrate; VOC+, diet with carbohydrate; DM, dry matter.
[1]Sopropeche, Boulogne-sur-Mer, France
[2]Roquette, Lestrem, France
[3]Linseed/Palm/Rapeseed oil in the ratio 50:30:20 (Daudruy, Dunkerque, France)
[4]Supplied the following (kg^{-1} diet): calcium carbonate (40% Ca) 2.15 g, magnesium oxide (60% Mg) 1.24 g, ferric citrate 0.2 g, potassium iodide (75% I) 0.4 mg, zinc sulphate (36% Zn) 0.4 g, copper sulphate (25% Cu) 0.3 g, manganese sulphate (33% Mn) 0.3 g, dibasic calcium phosphate (20% Ca, 18% P) 5 g, cobalt sulphate 2 mg, sodium selenite (30% Se) 3 mg, potassium chloride 0.9 g, Sodium chloride 0.4 g.
[5]Supplied the following (kg^{-1} diet): DL-a tocopherol acetate 60 IU, sodium menadione bisulphate 5 mg, retinyl acetate 15000 IU, DLcholecalciferol 3000 IU, thiamin 15 mg, riboflavin 30 mg, pyridoxine 15 mg, vit. B_{12} 0.05 mg, nicotinic acid 175 mg, folic acid 500 mg, inositol 1000 mg, biotin 2.5 mg, calcium panthotenate 50 mg, choline chloride 2000 mg.
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confirm that the fish sampled had effectively consumed the diet. Liver, dorso-ventral white muscle, intestine (midgut - region after the last pyloric caecum to the start of the distal segment of the intestine) and perivisceral adipose tissue were dissected, weighed, immediately frozen in liquid nitrogen and kept at −80°C until analysis. The weight of liver and viscera was used to calculate the hepato/viscero-somatic index [in (%) = 100× (X weight/body weight), where X is liver or viscera]. Each tank with 30 fish represented one experimental unit to calculate feed intake and body weight. Daily feed intake (in dry weight basis) was calculated as the total amount of feed supplied and ingested (kg) divided by the mean biomass over the trial [(initial biomass + final biomass)/2, expressed in kg wet weight (WW)] and the number of days. Final body weight was calculated as the final biomass divided by the number of fish in each tank at the end of the feeding trial.

Analytical methods

The chemical composition of the diets was analyzed using the following procedures: dry matter after drying at 105°C for 24 h, lipid content by petroleum ether extraction (Soxtherm), protein content (N×6.25) by the Kjeldahl method after acid digestion, gross energy in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany), ash content by incinerating the samples in a muffle furnace at 600°C for 6 h and starch content by enzymatic method (InVivo labs, France). Plasma glucose (Glucose RTU, bioMérieux, Marcy l'Etoile, France), triglycerides (Triglycerides PAP 150, bioMérieux) and cholesterol (Cholesterol RTU, bioMérieux) levels were determined using commercial kits adapted to a microplate format, according to the recommendations of the manufacturer. Total plasma free amino acid levels were determined by ninhydrin reaction according to the method of Moore [25] with glycine as a standard.

mRNA levels analysis: quantitative RT-PCR

Analyses of mRNA levels were performed on samples from the liver, white muscle, intestine and perivisceral adipose tissue of fish sampled 8 h after the last meal. This time point corresponds to the post-prandial peak of nutrient absorption in juvenile rainbow trout reared at 17°C. Tissue samples from six individual fish per experimental condition (chosen based on homogeneity of plasma glucose levels) were used as biological replicates. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations, quantified by spectrophotometry (absorbance at 260 nm) and integrity was controlled by agarose gel electrophoresis. One microgram of the resulting total RNA was reverse transcribed into cDNA using the SuperScript III RNaseH- reverse transcriptase kit (Invitrogen) and random primers (Promega, Charbonniéres, France), according to the instructions of each manufacturer. Quantification of target gene expression levels were carried out in an iCycler iQ real-time PCR detection system (Bio-Rad) using iQ SYBR green supermix and specific primers (Table 2). PCR was performed using 5 µl of the diluted cDNA (1:50) mixed with 200 nM of each primer in a final volume of 15 µl. The PCR protocol was initiated at 95°C for 90 s for initial denaturation of the cDNA and activation of the hot-start iTaq TM DNA polymerase, followed by a two-step amplification program (20 s at 95°C and 30 s at specific primer hybridization temperature) repeated 40 times. At the end of the last amplification cycle, melting curves (temperature gradient at 0.5°C/10 s from 55 to 94°C) were systematically monitored to confirm the specificity of the amplification reaction. Each PCR run included replicate samples (duplicate of reverse transcription and PCR amplification, respectively) and negative controls (reverse transcriptase and RNA free samples, respectively). The qPCR assay was optimized with a linear standard curve (R^2>0.985) and checked for consistency across replicates. PCR reaction efficiency for each run was estimated based on the slope of the 5 points standard curve obtained with serial dilution of pooled sample cDNAs. E values ranging from 1.85 to 2.05 were considered to be acceptable. The transcripts analyzed were sodium dependent glucose co-transporter type 1 (SGLT1), glucose facilitative transporter type 2 and 4 (GLUT2, GLUT4) for glucose transport/uptake; microsomal triglyceride transfer protein (MTP), apolipoproteins B, A1 and A4 (ApoB, ApoA1, ApoA4) for chylomicron synthesis; lipoprotein lipase (LPL; EC 3.1.1.34), very low density lipoprotein receptor (VLDLR) and fatty acid translocase (CD36) for lipid uptake; glucokinase (GK; EC 2.7.1.2) and hexokinase (HK; EC 2.7.1.1) for glycolysis; glucose 6-phosphatase (G6Pase; EC 3.1.3.9) for gluconeogenesis; glucose 6-phosphate dehydrogenase (G6PD; EC 1.1.1.49), adenosine triphosphate citrate lyase (ACLY; EC 2.3.3.8), acetyl coA carboxylase (ACC; EC 6.4.1.2), fatty acid synthase (FAS; EC 2.3.1.85) and Δ9 fatty acyl desaturase (D9D; EC 1.14.19.1) for lipogenesis; Δ6 fatty acyl desaturase (D6D; EC 1.14.19.3), elongation of very long chain fatty acids like 5 and 2 (Elovl5, Elovl2; EC 2.3.1.199) for fatty acid bioconversion; carnitine palmitoyl transferase (CPT1; EC 2.3.1.21) for fatty acid oxidation; sterol regulatory element binding protein 1c (SREBP1c) and peroxisome proliferator-activated receptor (PPAR) for transcriptional regulatory factors. When different isoforms of a gene were known in rainbow trout (as for G6Pase, CPT1 and PPAR), gene expression analysis was performed on each isoform. 18 S ribosomal RNA (18 S) was employed as a non-regulated reference gene and it was found to be stably expressed in this study. Relative quantification of target gene expression was performed using the mathematical model described by Pfaffl [26], after correcting for reaction efficiency (efficiency-calibrated model).

Total lipids and fatty acids analyses

Total lipids of the muscle and liver samples were extracted according to Folch et al. [27], using dichloromethane instead of chloroform as the solvent and quantified gravimetrically. Fatty acid composition was determined on the total lipid extract. Fatty acid methyl esters were prepared by acid-catalysed transmethylation of total lipids using boron trifluoride (BF3) in methanol (14%) according to Shantha and Ackman [28]. They were analysed in a Varian 3800 gas chromatograph (Varian, Les Ulis, France). The chromatograph was equipped with a DB Wax fused silica capillary column (30 m×0.25 mm internal diameter, film thickness 0.25 µm; J & W Scientific, Folsom, CA, USA). Injection was made in a split mode (ratio 1:40) with 1 µL injected. Injector and flame ionization detector temperatures were 260 and 250°C, respectively. Helium was used as carrier gas (1 ml/min) and the thermal gradient during separation was 100 to 180°C at 8°C/min, 180 to 220°C at 4°C/min and a constant temperature of 220°C during 20 min. Fatty acid methyl esters were identified by comparison with known standard mixtures (Sigma189-19, St Louis, MO, USA) and quantified using the STAR computer package (Varian).

Statistical analysis

The results are presented as means ± s.d. The effect of diet, line, and diet x line interaction on the different parameters was tested using statistical software (StatView 5.0, SAS Institute, Cary, NC) by means of a two-way analysis of variance (ANOVA) with diet and line as independent variables. Post-hoc comparisons were made using a Student–Newman–Keuls multiple range test and differences were considered statistically significant at P<0.05.

Table 2. Primer sequences.

Gene	Foward primer (5′ - 3′)	Reverse primer (5′ - 3′)	Database and Accession No.	Annealing Temperature, °C	Amplicon size, bp
18 S	CGGAGGTTCGAAGACGATCA	TCGCTAGTTGGCATCGTTTAT	GenBank AF308735	56	62
SGLT1	TCTGGGGCTGAACATCTACC	GAAGGCATAACCCATGAGGA	GenBank AY210436	59	154
GLUT2	GTGGAGAAGGAGGCGCAAGT	GCCACCGACACCATGGTAAA	GenBank AF321816	59	227
MTP	CTCACTGACCACTCCCAGGT	ATGGCTCCCTTGTTGTTGAC	GenBank BX860503	55	152
ApoB	CCCTGTCTTCAAAGCCACAC	GTGGCGGGAGACACTCATAG	GenBank CA383905	55	196
ApoA1	CGCAGGTACCCAGGCTTTTC	AATGGACCTCTGTGCGGTCA	GenBank AF042218	59	115
ApoA4	AGCTGGGACAGGATGTCAAT	AGACGCTCTCTCAGCACCTC	GenBank CA363690	55	148
GK	TGAAGGATCAGAGGTGGGTGATT	GAAGGTGAAACCCAGAGGAAGC	GenBank AF135403	59	253
HK1	CTGGGACGCTGAAGACCAGA	CGGTGCTGCATACCTCCTTG	GenBank AY864082	59	159
G6Pase1	CTCAGTGGCGACAGAAAGG	TACACAGCAGCATCCAGAGC	Sigenae tcay0019b.d.18_3.1.s.om.8	55	77
G6Pase2	TAGCCATCATGCTGACCAAG	CAGAAGAACGCCCACAGAGT	GenBank AF120150	55	82
G6PD	CTCATGGTCCTCAGGTTTG	AGAGAGCATCTGGAGCAAGT	GenBank CA351434	59	176
ACLY	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	GenBank CA349411.1	60	149
ACC	TGGAGCTCTACGCAGACAGA	CTCCGGTGTACCAAGCTGTT	Sigenae tcbk0010c.b.21_5.1.om.4	55	152
FAS	TGATCTGAAGGCCCGTGTCA	GGGTGACGTTGCCGTGGTAT	Sigenae tcab0001c.e.06_5.1.s.om.8	60	161
D9D	GCCGTCCGAGGGTTCTTCTT	CTCTCCCCACAGGCACCAAG	GenBank FP323026	60	204
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	GenBank CA048941.1	60	59
D6D	AGGGTGCCTCTGCTAACTGG	TGGTGTTGGTGATGGTAGGG	Genbank AF301910	59	175
Elovl2	TGTGGTTTCCCCGTTGGATGCC	ACAGAGTGGCCATTTGGGCG	Sigenae FYV3OTN01A4WMI.s.om.10	59	146
Elovl5	GAACAGCTTCATCCATGTCC	TGACTGCACATATCGTCTGG	Genbank AY605100	59	149
CPT1a	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACTGG	GenBank AF327058	55	166
CPT1b	CCCTAAGCAAAAAGGGTCTTCA	CATGATGTCACTCCCGACAG	GenBank AJ606076	55	149
CPT1c	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	GenBank AJ619768	59	187
CPT1d	CCGTTCCTAACAGAGGTGCT	ACACTCCGTAGCCATCGTCT	GenBank AJ620356	59	154
PPARα*	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	GenBank AY494835	54	195
PPARβ*	CTGGAGCTGGATGACAGTGA	GTCAGCCATCTTGTTGAGCA	GenBank AY356399	60	195
PPARγ*	GACGGCGGGTCAGTACTTTA	ATGCTCTTGGCGAACTCTGT	Genbank CA345564	60	171
GLUT4	GGCGATCGTCACAGGGATTC	AGCCTCCCAAGCCGCTCTT	GenBank AF247395	60	207
LPL	TAATTGGCTGCAGAAAACAC	CGTCAGCAAACTCAAAGGT	GenBank AJ224693	59	164
VLDLR	GTTTTGGACAGATGGGAGA	AGCCTTCTCATTGCACCAGT	GenBank BX077158	60	160
CD36	CCACTGAAGTTGAGCCATGA	TGCTAGACTCATGCCGTGTC	GenBank BX300637	60	121

*Sánchez-Gurmaches et al. [69]

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When diet x line interaction was significant, means were compared using one way ANOVA.

Results

Feed intake was slightly higher in the L line than the F line, irrespective of the diet (Table 3). Correspondingly, the L line fish were heavier at the end of the feeding trial, than those of the F line. The VOC+ diet was associated with an increase in the weight of liver and viscera in both lines. Hepato-somatic index was higher in the F line, regardless of the diet.

Plasma metabolite levels

Plasma metabolite levels at 2 and 8 h after the meal are summarized in table 4. The intake of VOC+ diet caused a moderate but significant hyperglycemia in both lines, as compared to the VOC- diet. Temporal differences were not observed in any group. Nevertheless, the F line fish seemed to have a slightly better control of glycemia at 8 h after the carbohydrate rich meal. Triglyceride levels were found to be elevated in the plasma at 8 h in all the groups, particularly more in the L line than the F line, regardless of the diet. Dietary carbohydrate intake (VOC+) was linked to an increase in plasma cholesterol levels at 2 h after the meal.

mRNA levels of target genes

We analyzed the expression of several marker genes encoding the proteins involved in absorption, metabolism and uptake of glucose and fatty acids, to elucidate the effect of dietary carbohydrate on the molecular regulation of intermediary metabolism in the two trout lines fed a vegetable oil based diet lacking n-3 LC-PUFA. Representing intestinal nutrient absorption, the results of glucose transporters and chylomicron assembly associated proteins are shown in figure 1. Among the two glucose

Table 3. Feed intake, morphological indices and tissue lipid content.

	VOC-		VOC+		*P values*		
	Fat	**Lean**	**Fat**	**Lean**	**Diet**	**Line**	**D*L**
Feed intake, g/kg/day	12.9±0.2	13.2±0.2	12.5±0.4	13.2±0.3	0.32	0.02	0.29
Final body weight, g	216.7±28.4	268.9±6.0	210.1±12.6	269.4±3.1	0.74	3×10^{-4}	0.71
Hepato-somatic index, %	1.5±0.3	1.4±0.2	1.7±0.2	1.6±0.3	2×10^{-4}	0.03	0.58
Viscero-somatic index, %	11.5±2.0	11.8±3.0	12.3±1.8	13.4±1.1	0.006	0.15	0.35
Muscle lipid, %	7.9±1.2	4.2±0.8	8.2±1.2	4.7±0.8	0.37	$<10^{-4}$	0.83
Liver lipid, %	5.3±0.3[a]	5.2±0.3[a]	5.1±0.5[a]	4.5±0.2[b]	2×10^{-4}	0.001	0.02

The data are represented as means ± s.d. ($N=3$ tanks for feed intake and body weight estimation; $N=9$ individuals for morphological indices estimation; $N=6$ individuals for tissue lipid analysis) and were analysed by two-way ANOVA ($P<0.05$) followed by Student–Newman–Keuls multiple comparison test.
doi:10.1371/journal.pone.0076570.t003

transporters studied, GLUT2 transcripts were significantly enhanced by dietary starch intake, but such an induction was not statistically significant for SGLT1 ($P=0.09$). The mRNA levels of key proteins involved in intestinal chylomicron synthesis such as MTP, ApoA1 and ApoA4 were higher in the VOC+ dietary group. There was no difference between the two lines for these markers of nutrient absorption.

The impact of dietary starch intake on glucose metabolism is illustrated in figure 2. The glycolytic enzyme GK exhibited a strong transcriptional induction of several hundred fold in the liver of fish from both lines, when fed the VOC+ diet. However, no such response to dietary starch intake was noticed in the HK transcripts of muscle, adipose tissue and intestine. Isoforms of the gluconeogenic enzyme G6Pase showed differential dietary regulation in the liver, VOC+ diet down-regulated G6Pase1 mRNA levels, but up-regulated G6Pase2. The only genotypic variation observed in glucose metabolism, was in the liver transcript levels of the pentose pathway enzyme G6PD, which was higher in the F line than the L line (Fig. 3).

Concerning lipogenesis (Fig. 3), the mRNA levels of the key transcription factor SREBP1c was found to be significantly elevated in the liver ($P=0.008$) and adipose tissue ($P=0.005$) of the L line fish, irrespective of the diets. But this genotypic difference was not reflected in the transcript levels of FAS or the other lipogenic enzymes ACLY, ACC and D9D. In both lines, the intake of VOC+ diet was found to enhance the expression of hepatic ACC, but not the other lipogenic enzymes. Regarding fatty acid bioconversion (Fig. 4), fish fed VOC+ diet displayed a distinctly higher hepatic ($P=0.0002$) and intestinal ($P=0.001$) expression of the D6D enzyme than those fed the VOC- diet, regardless of the genotype. Likewise, mRNA levels of hepatic Elovl2 and intestinal Elovl5 were also found to be raised in the VOC+ dietary group. The expression of D6D was higher in the intestine of the L line than the F line fish, but this was not the case for the liver D6D and for the elongases. Results pertaining to fatty acid oxidation are presented in figure 5. The two lines experienced a contrasting dietary influence on their hepatic CPT1a transcript levels leading to a disordinal interaction, where VOC+ diet up-regulates CPT1a levels in the L line and vice versa. The expression of the other hepatic isoform CPT1b was generally enhanced by the VOC+ diet, but more pronounced in the L line. The mRNA levels of the transcription factor PPAR closely corresponded to the CPT1 expression (Fig. 6). Hepatic transcripts of both PPARα and PPARγ were more abundant in the L line and the VOC+ diet was found to enhance the PPARγ levels in both lines. In the other peripheral tissues, no significant differences related to either diet or genotype were observed in the CPT1 and PPAR transcripts tested. Moreover, the expression of PPARγ was not detectable in the muscle.

Table 4. Postprandial plasma metabolites.

Plasma metabolites		VOC-		VOC+		*P values*		
		Fat	**Lean**	**Fat**	**Lean**	**Diet**	**Line**	**D*L**
Glucose (mmol/L)	2 h	4.4±0.7	4.7±0.6	5.9±0.9	5.7±1.1	$<10^{-4}$	0.95	0.41
	8 h	5.1±0.8	5.3±0.8	5.7±1.0	7.5±2.4	0.006	0.05	0.09
Triglycerides (mmol/L)	2 h	1.2±0.7 [a]	1.0±0.5 [a]	1.6±0.9	1.5±0.9 [a]	0.12	0.54	0.82
	8 h	2.2±1.2 [b]	3.2±1.7 [b]	2.0±1.3	3.6±1.5 [b]	0.83	0.01	0.61
Cholesterol (mmol/L)	2 h	4.2±1.1	4.5±0.5	5.0±1.1	5.7±1.3	0.005	0.13	0.47
	8 h	4.4±1.2	4.9±1.6	5.0±1.5	5.2±1.3	0.33	0.43	0.81
Free amino acids (mg Eq. Glycine/ml)	2 h	0.5±0.1	0.5±0.06	0.5±0.08 [b]	0.44±0.06 [b]	0.08	0.24	0.74
	8 h	0.5±0.1	0.5±0.07	0.4±0.04 [a]	0.36±0.08 [a]	0.003	0.19	0.70

Data are presented as means ± s.d. ($N=9$ individuals). At each postprandial time, the effect of diet, line and interaction were analysed by two-way ANOVA ($P<0.05$) followed by Student–Newman–Keuls multiple comparison test. Within each dietary treatment (column wise), significant differences in postprandial kinetics (2 h, 8 h after the last meal) are represented with different superscripts [a,b] (one-way ANOVA, $P<0.05$).
doi:10.1371/journal.pone.0076570.t004

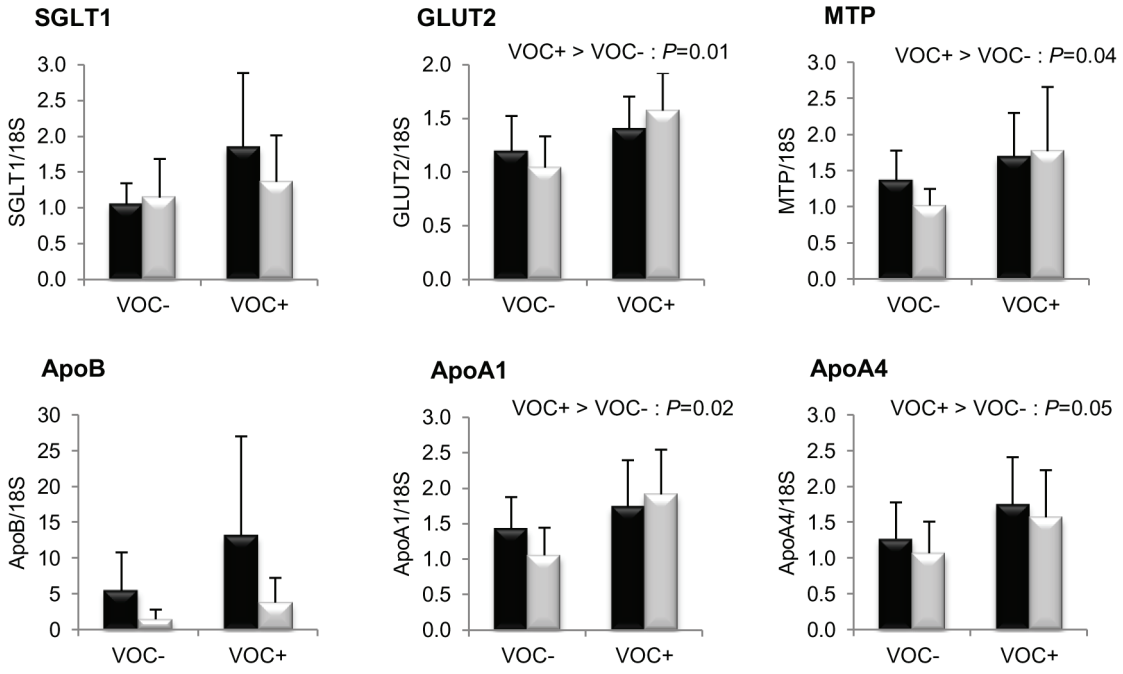

Figure 1. Gene expression of intestinal membrane glucose transporters and proteins involved in chylomicron assembly. mRNA levels of sodium-dependent glucose co-transporter type 1 (SGLT1), glucose facilitative transporter type 2 (GLUT2), microsomal triglyceride transfer protein (MTP), apolipoprotein B (ApoB), apolipoprotein A1 (ApoA1) and apolipoprotein A4 (ApoA4) were measured using real-time quantitative RT-PCR in the intestine of rainbow trout from a fat line (F; black bar) and a lean line (L; grey bar) fed a diet without (VOC-) or with (VOC+) carbohydrate, 8 h after the last meal. Expression values are normalized by 18 S ribosomal RNA (18 S) expressed transcripts. Relative fold difference between treatments are presented as means + s.d. ($N = 6$ individuals) and were analyzed using two-way ANOVA followed by Student–Newman–Keuls test for multiple comparison. Differences were considered significant at $P < 0.05$
doi:10.1371/journal.pone.0076570.g001

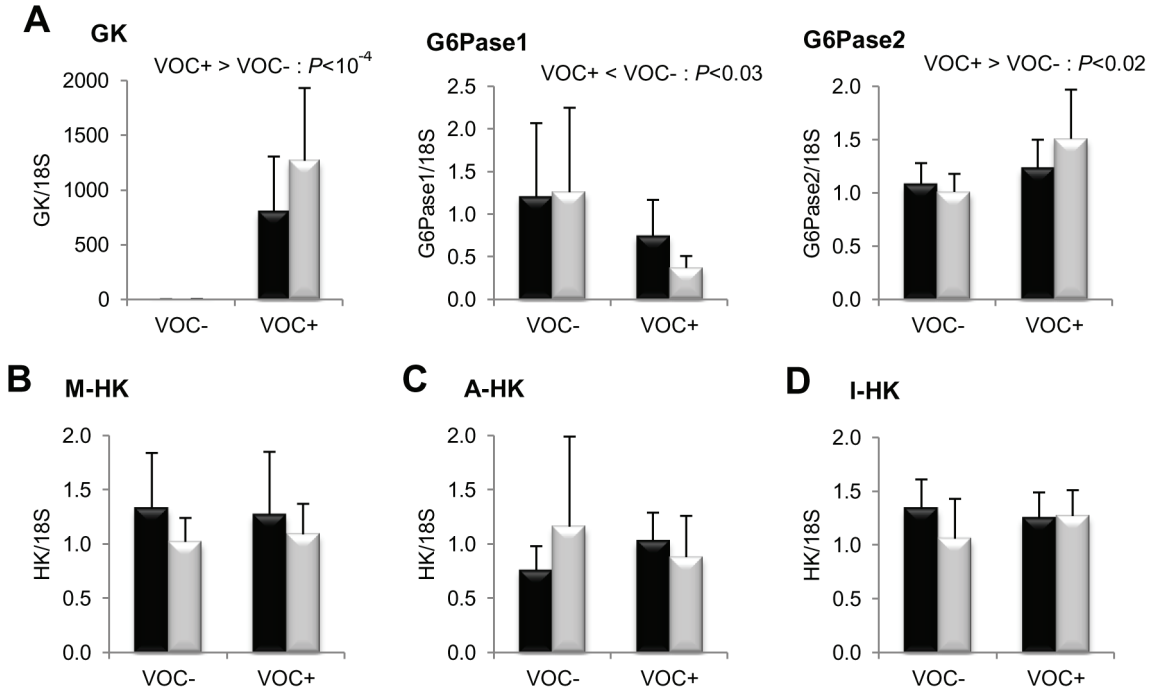

Figure 2. Gene expression of selected glycolytic and gluconeogenic enzymes. mRNA levels of glucokinase (GK), glucose-6-phosphatase isoform 1 (G6Pase1), isoform 2 (G6Pase2) and hexokinase (HK) were measured using real-time quantitative RT-PCR in the liver (row A); muscle (B), adipose tissue (C) and intestine (D) of rainbow trout from a fat line (F; black bar) and a lean line (L; grey bar) fed a diet without (VOC−) or with (VOC+) carbohydrate, 8 h after the last meal. Expression values are normalized by 18 S ribosomal RNA (18 S) expressed transcripts. Relative fold difference between treatments are presented as means + s.d. ($N = 6$ individuals) and were analyzed using two-way ANOVA followed by Student–Newman–Keuls test for multiple comparison. Differences were considered significant at $P < 0.05$
doi:10.1371/journal.pone.0076570.g002

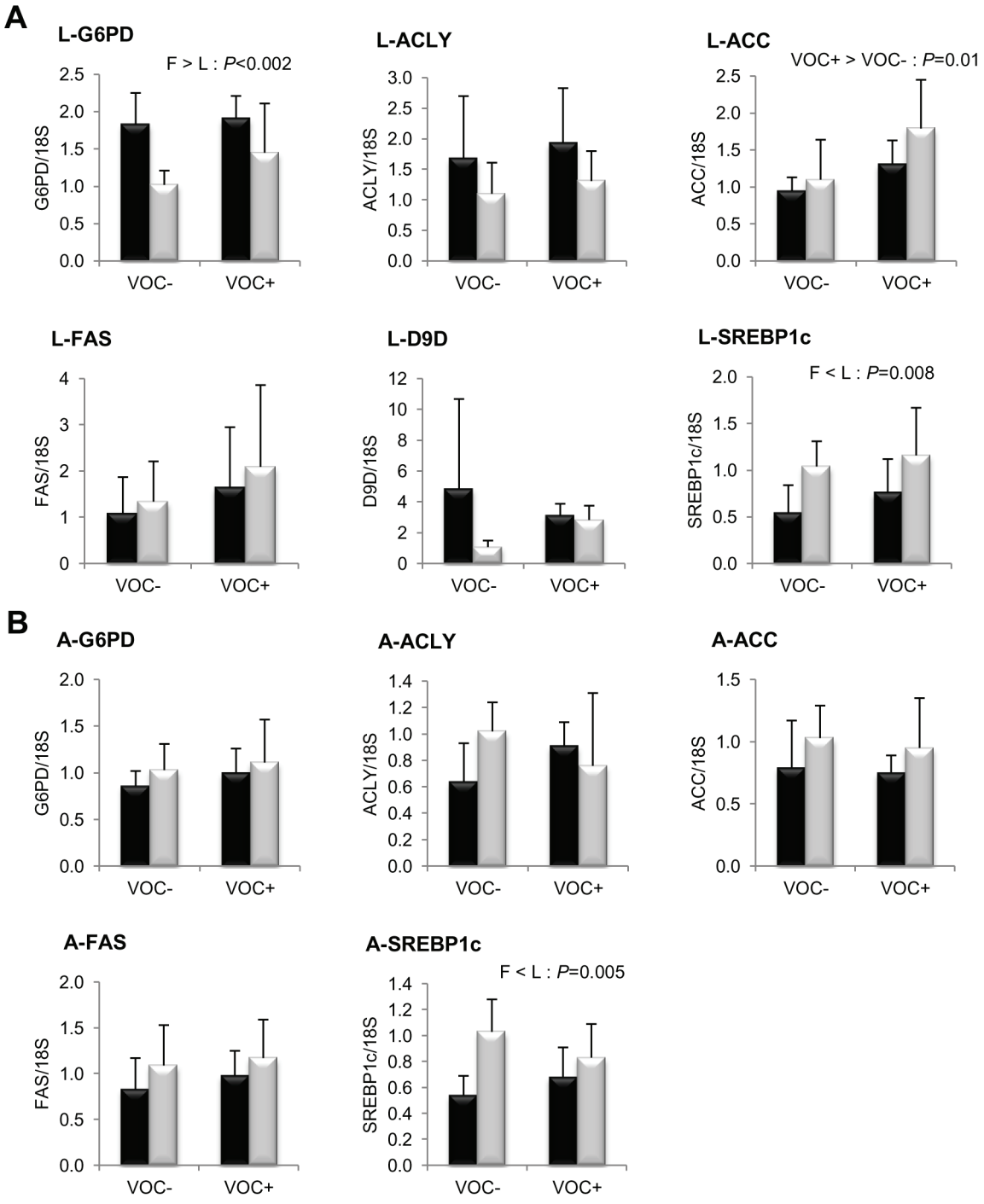

Figure 3. Gene expression of selected enzymes and transcription factor involved in NADPH generation and lipogenesis. mRNA levels of glucose 6-phosphate dehydrogenase (G6PD), ATP citrate lyase (ACLY), acetyl coA carboxylase (ACC), fatty acid synthase (FAS), Δ9 fatty acyl desaturase (D9D) and sterol regulatory element binding protein 1-like (SREBP-1c) were measured using real-time quantitative RT-PCR in the liver (A - two rows) and adipose tissue (B - two rows) of rainbow trout from a fat line (F; black bar) and a lean line (L; grey bar) fed a diet without (VOC−) or with (VOC+) carbohydrate, 8 h after the last meal. Expression values are normalized by 18 S ribosomal RNA (18 S) expressed transcripts. Relative fold difference between treatments are presented as means + s.d. ($N = 6$ individuals) and were analyzed using two-way ANOVA followed by Student–Newman–Keuls test for multiple comparison. Differences were considered significant at $P<0.05$
doi:10.1371/journal.pone.0076570.g003

Finally, mRNA levels of proteins involved in glucose and fatty acid uptake in the muscle are presented in figure 7. Transcript levels of GLUT4, VLDLR and CD36 were found to be significantly higher in the F line than the L line. However, the LPL expression was not different between the two lines. Dietary manipulation exerted no noticeable influence on the transcript abundance of these markers.

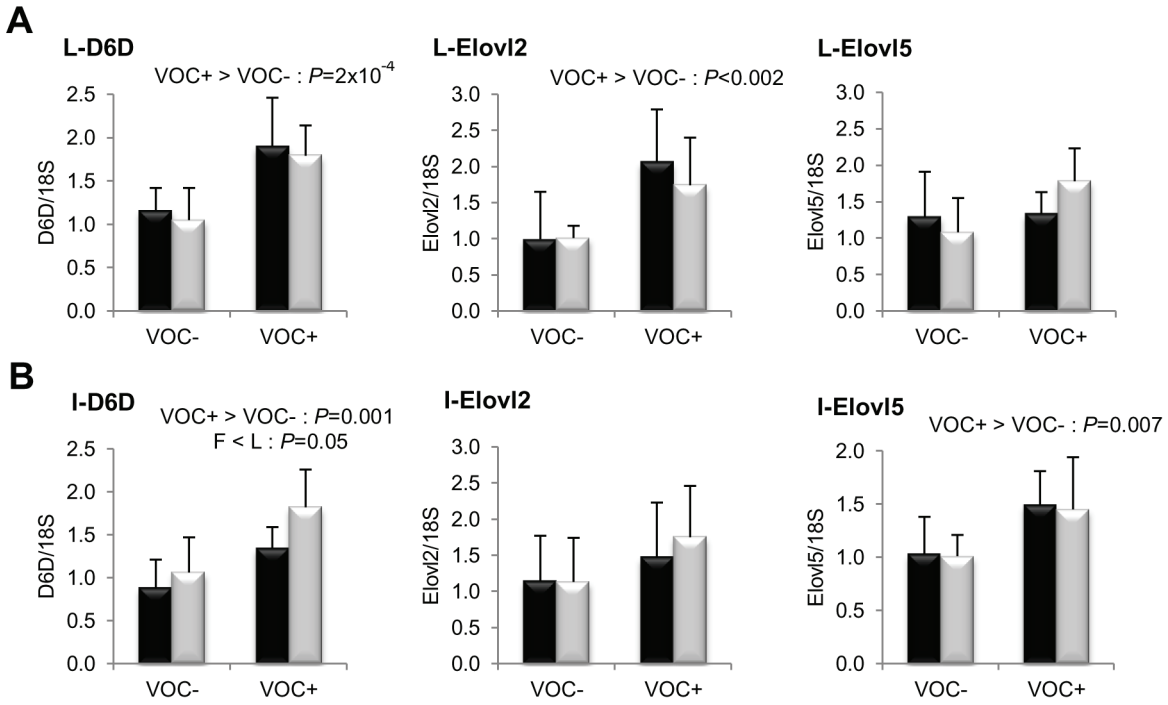

Figure 4. Gene expression of selected enzymes involved in fatty acid bioconversion. mRNA levels of Δ6 fatty acyl desaturase (D6D), elongation of very long chain fatty acids like-2 (Elovl2) and elongation of very long chain fatty acids like-5 (Elovl5) were measured using real-time quantitative RT-PCR in the liver (row A) and intestine (row B) of rainbow trout from a fat line (F; black bar) and a lean line (L; grey bar) fed a diet without (VOC−) or with (VOC+) carbohydrate, 8 h after the last meal. Expression values are normalized by 18 S ribosomal RNA (18 S) expressed transcripts. Relative fold difference between treatments are presented as means + s.d. ($N = 6$ individuals) and were analyzed using two-way ANOVA followed by Student–Newman–Keuls test for multiple comparison. Differences were considered significant at $P<0.05$
doi:10.1371/journal.pone.0076570.g004

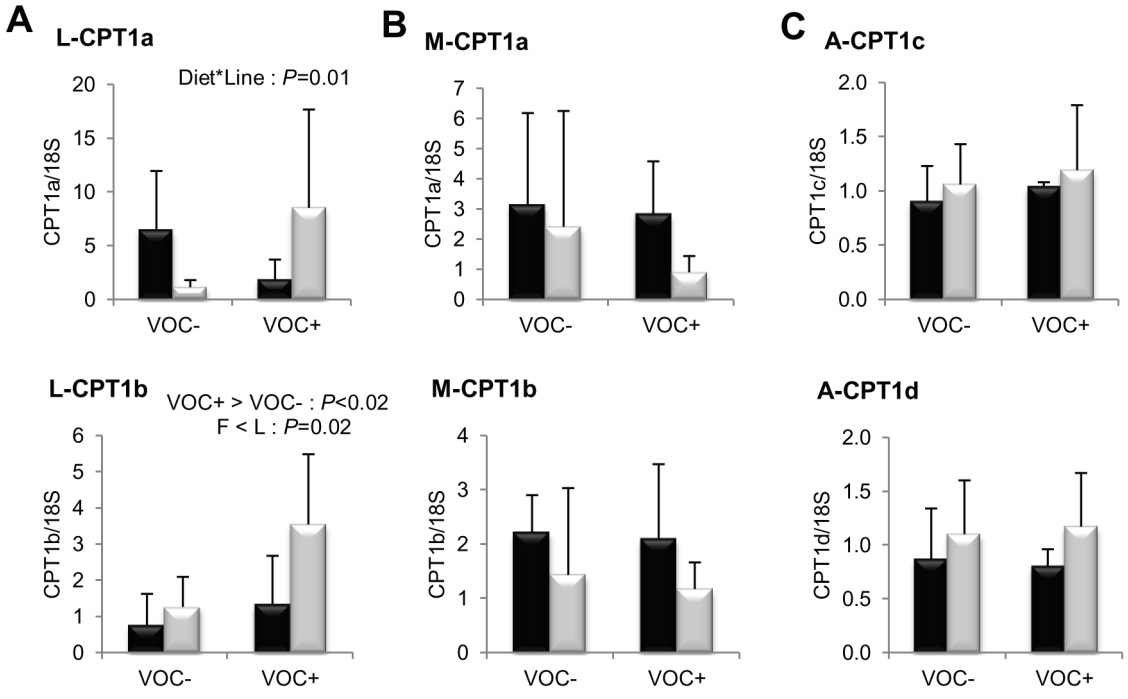

Figure 5. Gene expression of carnitine palmitoyl transferase (CPT1) isoforms involved in fatty acid oxidation. mRNA levels of CPT1a, CPT1b, CPT1c and CPT1d were measured using real-time quantitative RT-PCR in the liver (column A), muscle (column B) and adipose tissue (column C) of rainbow trout from a fat line (F; black bar) and a lean line (L; grey bar) fed a diet without (VOC−) or with (VOC+) carbohydrate, 8 h after the last meal. Expression values are normalized by 18 S ribosomal RNA (18 S) expressed transcripts. Relative fold difference between treatments are presented as means + s.d. ($N = 6$ individuals) and were analyzed using two-way ANOVA ($P<0.05$) followed by Student–Newman–Keuls test for multiple comparison. When interactions were significant, means were compared using one way ANOVA ($P<0.05$).
doi:10.1371/journal.pone.0076570.g005

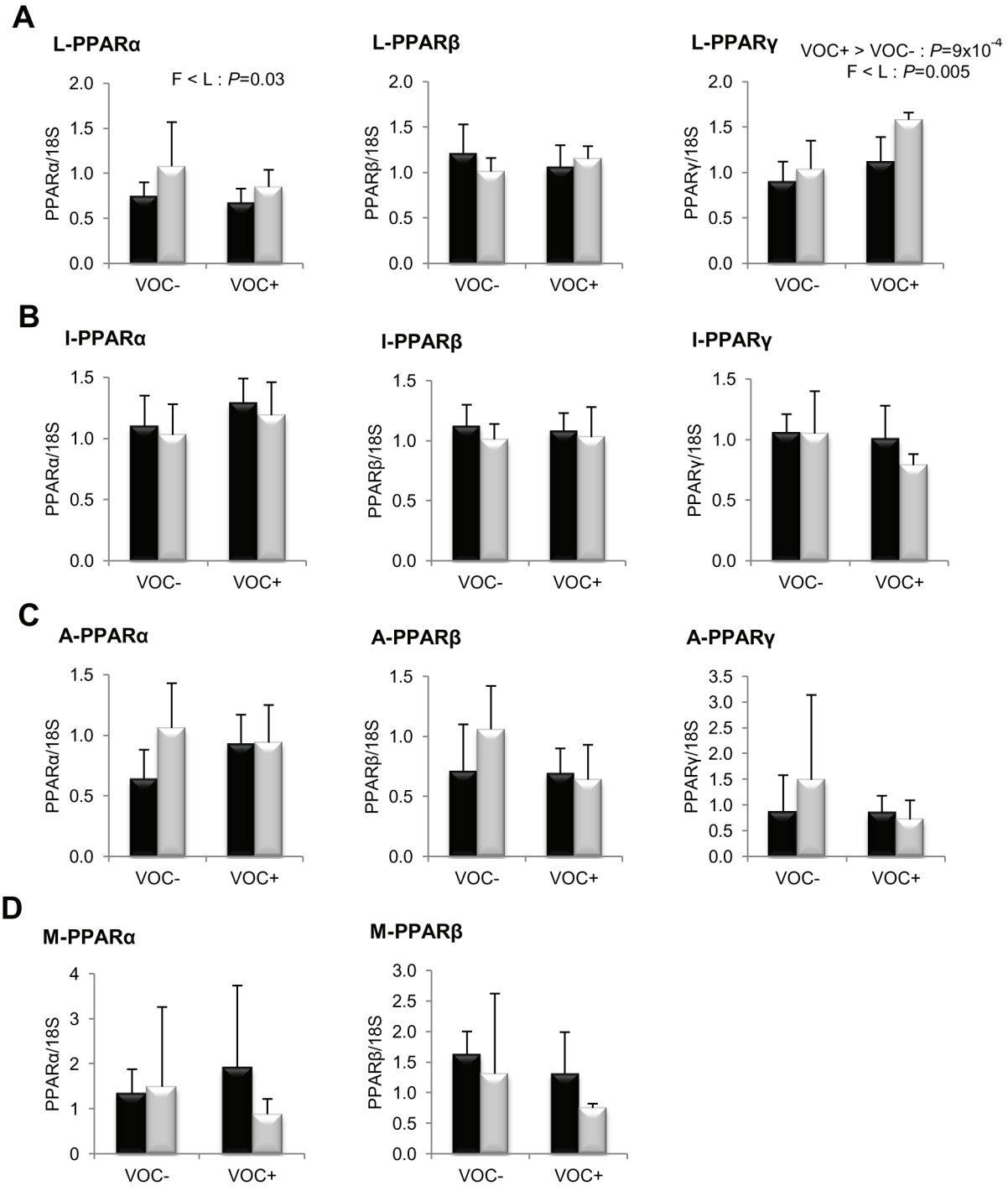

Figure 6. Gene expression of peroxisome proliferator activated receptor isoforms. mRNA levels of PPARα, PPARβ and PPARγ were measured using real-time quantitative RT-PCR in the liver (row A), intestine (row B), adipose tissue (row C) and muscle (row D) of rainbow trout from a fat line (F; black bar) and a lean line (L; grey bar) fed a diet without (VOC−) or with (VOC+) carbohydrate, 8 h after the last meal. Expression values are normalized by 18 S ribosomal RNA (18 S) expressed transcripts. Relative fold difference between treatments are presented as means + s.d. ($N=6$ individuals) and were analyzed using two-way ANOVA followed by Student–Newman–Keuls test for multiple comparison. Differences were considered significant at $P<0.05$
doi:10.1371/journal.pone.0076570.g006

Lipid content and fatty acid composition

Muscle lipid content was invariably higher in the F line, with no diet induced modification (Table 3). The liver lipid content witnessed an interaction between the dietary treatment and genotype, with a marked decrease in the L line fish fed the VOC+ diet. The fatty acid profiles of the two isolipidic diets are presented in table 5. The proportional content of the saturated and unsaturated fatty acids did not vary considerably between the two diets. However, there were minor differences such as slightly lower linoleic acid and higher linolenic acid in the VOC+ diet.

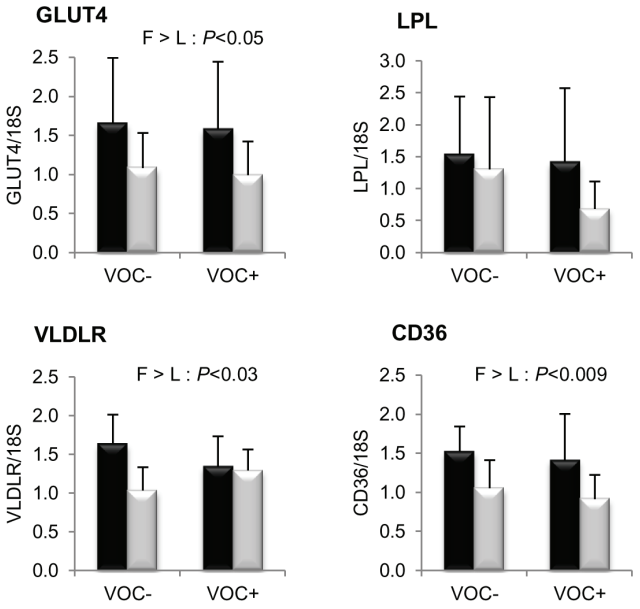

Figure 7. Gene expression of selected proteins involved in glucose and lipid uptake in the white muscle. mRNA levels of glucose facilitative transporter type 4 (GLUT4), lipoprotein lipase (LPL), very low density lipoprotein receptor (VLDLR) and fatty acid translocase (CD36) were measured using real-time quantitative RT-PCR in the muscle of rainbow trout from a fat line (F; black bar) and a lean line (L; grey bar) fed a diet without (VOC−) or with (VOC+) carbohydrate, 8 h after the last meal. Expression values are normalized by 18 S ribosomal RNA (18 S) expressed transcripts. Relative fold difference between treatments are presented as means + s.d. ($N = 6$ individuals) and were analyzed using two-way ANOVA followed by Student–Newman–Keuls test for multiple comparison. Differences were considered significant at $P<0.05$

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The muscle fatty acid composition in both lines closely reflected the profile of the respective diet consumed (Table 6). There was no evidence for a positive influence of the VOC+ diet on the muscle PUFA content of the two trout lines. In terms of genotypic difference, the L line had a slightly higher proportion of PUFA (DHA) than the F line, but exactly the opposite was observed for the MUFA proportion. Considering the divergent selection criterion applied, the F line possessed a substantially larger quantity of unsaturated and saturated fatty acids per gram of muscle (Table 7). Similar to the muscle, the fatty acid profile of the liver resembled the respective dietary profile (Table 8). Furthermore, the n-3 PUFA (DHA) content of the liver exhibited a significant diet x genotype interaction, where it was typically lower in the F line fish fed the VOC- diet. Apart from this, no other differences were significant.

Discussion

In the present study, we investigated the molecular and phenotypic response of two rainbow trout lines (fat and lean) to a vegetable oil based diet either with or without gelatinized starch.

Effect of dietary carbohydrates in fish fed with vegetable oils

The absolute difference in carbohydrate intake (1.8% *vs.* 17.1%) between the two dietary groups as compared to protein intake (always above the requirement) enables us to consider that majority of the effects are linked to carbohydrate intake. When included at an acceptable level (20%) in the diet, carbohydrate (gelatinised starch) had no adverse impact on feed intake and growth. However, feeding of dietary carbohydrate caused a relatively moderate postprandial hyperglycemia that lacked temporal induction following a single meal, probably due to long term adaptation. These findings corroborate our previous observations in the two trout lines [23]. Likewise, the intake of dietary carbohydrate resulted in a proportionately heavier liver and viscera, which can be attributed to increased glycogen and fat deposition, respectively [29]. The phenotypic observations thus suggest the influence of dietary carbohydrate on the metabolism of the fish. Endorsing this, molecular analysis revealed a well-known mammalian response in hepatic glucose metabolism that includes strong induction of glycolytic GK transcripts and reciprocal reduction of gluconeogenic G6Pase1 transcripts, the latter being unusual in trout [30–32]. Specifically in these lines, the first isoform of G6Pase is known to respond to nutritional (refeeding) regulation [22]. There were also a few ambiguities related to dietary starch intake such as the contrasting response of intestinal glucose transporters (inert SGLT1 and enhanced GLUT2) that

Table 5. Fatty acid profile of diets expressed as % of total fatty acids.

Fatty acids	VOC−	VOC+
SFA		
14:0	1.4	0.8
16:0	15.5	14.8
17:0	0.1	0.1
18:0	3.0	3.3
20:0	0.3	0.3
∑SFA	20.4	19.5
MUFA		
16:1	1.1	0.6
18:1	28.1	31.7
20:1	2.5	1.8
22:1	3.2	2.2
∑MUFA	34.9	36.4
n-6 PUFA		
18:2 n-6	13.9	13.0
20:2 n-6	0.1	0.1
20:4 n-6	0.1	0.1
∑n-6 PUFA	14.1	13.1
n-3 PUFA		
18:3 n-3	23.4	25.8
18:4 n-3	0.4	0.3
20:3 n-3	0.1	0.05
20:4 n-3	0.1	0.1
20:5 n-3	1.4	0.9
22:5 n-3	0.1	0.1
22:6 n-3	2.9	1.9
∑n-3 PUFA	28.5	29.1
Ratios		
SFA/PUFA	0.5	0.5
n3/n6	2.0	2.2

doi:10.1371/journal.pone.0076570.t005

Table 6. Fatty acid profile of muscle expressed as % of total fatty acids.

Fatty acids	VOC−		VOC+		*P* value		
	Fat	Lean	Fat	Lean	Diet	Line	Diet*Line
SFA							
14:0	1.72±0.38	1.37±0.26	1.67±0.27	1.39±0.20	0.92	0.01	0.80
16:0	12.32±1.37	12.69±1.33	11.91±0.74	12.32±0.46	0.37	0.37	0.97
17:0	0.13±0.01	0.11±0.01	0.11±0.01	0.11±0.01	0.01	0.02	0.16
18:0	2.97±0.30	3.23±0.13	2.90±0.22	3.11±0.22	0.33	0.02	0.77
20:0	0.17±0.02	0.17±0.02	0.19±0.03	0.17±0.01	0.10	0.18	0.28
∑SFA	17.49±1.61	17.74±1.59	16.96±0.82	17.26±0.53	0.33	0.59	0.96
MUFA							
16:1	2.29±0.43	1.65±0.35	2.25±0.27	1.70±0.24	0.97	3×10^{-4}	0.73
18:1	29.99±0.7 [b]	27.50±0.9 [a]	30.45±0.9 [b]	29.44±1.1 [b]	0.003	$<10^{-4}$	0.05
20:1	2.75±0.17	2.64±0.32	2.73±0.33	2.55±0.08	0.58	0.18	0.72
22:1	2.31±0.36	2.40±0.39	2.56±0.43	2.32±0.21	0.56	0.64	0.27
∑MUFA	37.33±0.65	34.19±1.41	37.99±0.62	36.01±1.35	0.01	$<10^{-4}$	0.20
n-6 PUFA							
18:2 n-6	12.49±0.61	12.01±0.39	11.19±0.60	11.05±0.52	$<10^{-4}$	0.17	0.45
18:3 n-6	0.23±0.04	0.23±0.03	0.19±0.03	0.19±0.03	0.01	0.91	0.99
20:2 n-6	0.63±0.05	0.64±0.11	0.53±0.09	0.51±0.05	0.003	0.96	0.67
20:3 n-6	0.30±0.02 [a]	0.36±0.06 [b]	0.28±0.04 [a]	0.27±0.02 [a]	9×10^{-4}	0.06	0.03
20:4 n-6	0.35±0.03	0.40±0.02	0.33±0.04	0.37±0.04	0.04	0.002	0.72
∑n-6 PUFA	14.04±0.62	13.72±0.33	12.61±0.53	12.39±0.51	$<10^{-4}$	0.21	0.81
n-3 PUFA							
18:3 n-3	13.07±0.47	14.07±0.32	14.02±1.02	15.22±0.56	8×10^{-4}	5×10^{-4}	0.72
18:4 n-3	1.40±0.13	1.45±0.11	1.44±0.24	1.45±0.22	0.81	0.67	0.80
20:3 n-3	0.79±0.11	0.89±0.17	0.85±0.16	0.82±0.11	0.91	0.51	0.29
20:4 n-3	1.00±0.16	1.15±0.27	1.13±0.18	0.92±0.08	0.49	0.75	0.03
20:5 n-3	2.43±0.32	2.34±0.18	2.30±0.41	2.39±0.15	0.73	0.97	0.45
22:5 n-3	1.05±0.25	0.89±0.07	1.12±0.24	0.97±0.06	0.31	0.04	0.99
22:6 n-3	8.47±0.92	10.94±1.33	8.75±1.13	9.94±1.47	0.48	0.002	0.22
∑n-3 PUFA	28.48±1.25	32.00±1.64	29.89±0.80	31.80±1.16	0.25	$<10^{-4}$	0.13
Ratios							
SFA/PUFA	0.41±0.05	0.39±0.04	0.39±0.02	0.39±0.01	0.72	0.33	0.63
n3/n6	2.03±0.11	2.33±0.14	2.38±0.15	2.57±0.19	10^{-4}	5×10^{-4}	0.39

The data are presented as means ± s.d. ($N=6$ individuals) and were analysed by two-way ANOVA ($P<0.05$) followed by Student–Newman–Keuls multiple comparison test. [a,b] Mean values not sharing a common letter are significantly different from each other (one way ANOVA, $P<0.05$).

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cannot validate an improvement in glucose uptake [33]. Moreover, the unaltered HK and GLUT4 expression in the muscle and adipose tissue reiterates the recognized poor ability of the peripheral tissues to adapt to high influx of glucose through the diet [34–36]. Therefore in brief, this study confirms the significant influence of dietary carbohydrate intake on glucose metabolism in the liver but not in the peripheral tissues, as evidenced before [23].

Consumption of a diet rich in carbohydrates is known to stimulate the lipogenic pathway through transcriptional mechanisms linked to enhanced glucose metabolism. This flux depends also on the availability of cofactors such as NADPH produced by the pentose phosphate pathway and lipogenic substrates [17,37]. Our previous study in these two lines clearly demonstrated the existence of the above phenomenon through an increase in the hepatic transcript abundance of GK followed by enhanced mRNA levels and activity of G6PD (involved in the production of NADPH), ACLY transcripts (involved in the transition of glycolytic carbon to lipogenic substrates) and FAS activity (key enzyme of *de novo* lipogenesis) [23]. However in the present study, despite the huge induction of GK, dietary influence was conspicuously absent in all of the above mentioned lipogenic enzyme transcripts. The main causal differences can be dietary changes related to fish oil replacement [8] or physiological changes related to the larger size of the fish [38]. The only notable exception was carbohydrate intake enhanced ACC expression, which might have prevailed due to the presence of glucose response element in their promoter region as seen in mammals [37]. The unaltered plasma triglyceride levels of the carbohydrate fed group seemed to be related to the lipogenic profile rather than the potentially elevated intestinal lipid uptake, which is indicated by the transcript abundance of key chylomicron assembly proteins (MTP, ApoA1 and ApoA4). On the contrary, we found a

Table 7. Fatty acid content of muscle expressed as mg/g of muscle (wet weight).

Fatty acids	VOC−		VOC+		*P* value		
	Fat	Lean	Fat	Lean	Diet	Line	Diet*Line
SFA	14.0±3.3	7.4±1.1	13.8±1.5	8.0±1.3	0.77	$<10^{-4}$	0.64
MUFA	29.6±4.9	14.3±2.8	31.1±4.9	16.8±3.6	0.24	$<10^{-4}$	0.77
n-6 PUFA	11.1±1.5	5.7±1.1	10.3±1.2	5.8±1.2	0.49	$<10^{-4}$	0.41
20:5 n-3	1.9±0.2	1.0±0.2	1.9±0.5	1.1±0.2	0.60	$<10^{-4}$	0.62
22:6 n-3	6.6±0.7	4.6±1.1	7.2±1.9	4.5±0.4	0.56	$<10^{-4}$	0.50
n-3 PUFA	22.4±2.5	13.4±2.7	24.5±4.2	14.7±2.1	0.17	$<10^{-4}$	0.76
n3/n6	2.03±0.11	2.33±0.14	2.38±0.15	2.57±0.19	10^{-4}	5×10^{-4}	0.39

The data are presented as means ± s.d. ($N=6$ individuals) and were analysed by two-way ANOVA followed by Student–Newman–Keuls multiple comparison test. Differences were considered statistically significant at $P<0.05$

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paradoxical increase in the plasma cholesterol levels after carbohydrate intake. Considering the isolipidic nature of the two diets and neutral triglyceride levels, it is difficult to interpret this atypical link between carbohydrate intake, chylomicron synthesis and cholesterol metabolism [39]. Another contradiction related to fatty acid oxidation is the enhanced expression of hepatic CPT1b following carbohydrate intake. Normally in higher vertebrates, fatty acid oxidation and glucose oxidation/lipogenesis are known to be reciprocally regulated, because the provision of glucose inhibits fatty acid oxidation [40]. Further, the parallel higher expression of ACC and CPT1b are unusual even if at the transcriptional level, considering the fact that malonyl-CoA (product of ACC) is an inhibitor of CPT1. But, such paradoxical imbalance in the regulation of lipogenesis and lipid oxidation pathway has been previously reported in trout after insulin infusion [41]. Overall our results in hepatic lipid metabolism disagree with well known effects of carbohydrate intake, suggesting a disturbance characterized by unexplained shift from fatty acid storage to oxidation. In the peripheral tissues, dietary manipulation yet again had no influence on lipid metabolism, reasserting their unresponsiveness to carbohydrate intake [23].

Fatty acid desaturases and elongases are key enzymes of the fatty acid bioconversion pathway, which can influence whole body lipid composition. Dietary carbohydrate intake enhanced the transcription of key desaturase (D6D) and elongase (Elovl5 and Elovl2) enzymes involved in LC-PUFA synthesis, in both these lines, in agreement with few other mammalian and fish studies [10,42–44]. The influence of carbohydrates on these enzymes can be effected through certain critical nodes such as an increased production of essential reducing power in the form of NADPH related to G6PD [45,46]; availability of substrate for elongase in the form of malonyl-CoA related to ACC [47]; insulin action mediating the effect of glucose through transcriptional factors and presence of glucose response elements in the promoter regions of the encoding genes [42,48,49]. Out of which, we observed an increase in the hepatic expression of ACC and PPARγ associated to carbohydrate intake. PPARs are a family of nuclear receptors that regulates lipid and glucose metabolism, allowing adaptation to the prevailing nutritional environment. Especially, PPAR activation is known to promote glucose utilization and D6D gene is known to contain a PPAR response element [50]. In mice, the activation of PPARγ reportedly increases glycolysis, fat storage, fatty acid desaturation, elongation and restore insulin sensitivity [51]. For the first time, we provide evidence that piscine PPARγ acts similar to their mammalian counterpart, thereby ruling out previous speculations of functional divergence [52]. Other transcriptional factors such as SREBP-1 and PPARα that play a major role in the regulation of LC-PUFA synthesis [53,54] were not influenced by carbohydrate intake. As we observed, nutritional regulation of fatty acid bioconversion enzymes independent of changes in SREBP-1 and PPARα have been previously reported in rats [55]. Based on our results, PPARγ seems to be the key transcription factor that mediates the carbohydrate induced response of desaturase and elongase enzymes in the liver, but not in the intestine, suggesting tissue specific regulation [56]. On the other hand, it is important to note that previously when the two lines were fed carbohydrates as part of fish oil based diet, transcriptional stimulation of D6D, Elovl5 and Elovl2 were absent probably as a result of higher LC-PUFA content and associated feedback inhibition [23,24]. Therefore, the induction that we find now is in fact an amplified response due to the presence of carbohydrate in conjunction with the reduced content of n-3 LC-PUFA in the diet, which indeed validates our hypothesis at the transcriptional level. Though the exact mechanisms are unknown, changes in dietary fatty acid composition (chain length and desaturation) and subsequently altered cellular metabolism as a result of energy substrate crosstalk is the most likely explanation.

Concerning fatty acid phenotype, we found no increase in the n-3 LC-PUFA content of the flesh corresponding to the molecular augmentation of desaturase/elongase expression in the liver and intestine. Instead, they closely resembled the fatty acid profile of the respective diet consumed, as commonly observed [4]. It is well known that the fatty acid composition of an animal depends not only on desaturation/elongation, but also on other interacting aspects of lipid metabolism such as b-oxidation, substrate availability, tissue uptake and hormonal status [4,42,57]. High levels of oxidation of C_{18} PUFA, which subsequently allows only small proportions for bioconversion, can be one possible explanation [58]. The elevated CPT1b expression in the carbohydrate fed group coincides with this hypothesis. The uptake and selective incorporation of fatty acids in the cellular fractions of the muscle can also have significance in the final lipid composition [59,60]. The expression of muscle lipid uptake markers, namely LPL, VLDLR and CD36 suggests no diet induced differences in fatty acid uptake. Besides, the impact of post-transcriptional modifications and related changes in enzyme activity on the final fatty acid profile is not known, as most of our data represent only the transcriptional changes. Eventually, the hypothesized additive influence of dietary carbohydrate and low n-3 LC-PUFA content

Table 8. Fatty acid profile of liver expressed as % of total fatty acids.

Fatty acids	VOC−		VOC+		*P* value		
	Fat	Lean	Fat	Lean	Diet	Line	Diet*Line
SFA							
14:0	0.62±0.16	0.41±0.10	0.61±0.08	0.56±0.08	0.14	0.01	0.08
16:0	16.39±1.54	14.31±2.22	14.83±1.89	15.15±1.70	0.64	0.25	0.12
17:0	0.13±0.02	0.13±0.01	0.11±0.02	0.12±0.01	0.21	0.44	0.35
18:0	6.67±0.92	6.63±0.31	6.54±0.84	6.52±0.47	0.67	0.91	0.96
20:0	0.15±0.01	0.19±0.03	0.16±0.04	0.17±0.02	0.51	0.04	0.17
∑SFA	24.07±1.22	21.76±2.27	22.33±1.54	22.63±1.51	0.52	0.15	0.07
MUFA							
16:1	0.82±0.17	0.60±0.15	0.80±0.28	0.74±0.13	0.47	0.08	0.31
18:1	14.36±1.50	13.17±0.66	14.25±2.10	14.19±1.18	0.45	0.31	0.36
20:1	1.78±0.80	1.68±0.45	1.79±0.66	1.74±0.35	0.89	0.76	0.91
22:1	0.56±0.32	0.60±0.18	0.70±0.18	0.77±0.63	0.31	0.73	0.93
∑MUFA	17.52±2.15	16.04±0.78	17.54±2.23	17.43±1.27	0.32	0.27	0.34
n-6 PUFA							
18:2 n-6	5.53±0.84	5.57±0.27	4.90±0.74	5.04±0.55	0.03	0.74	0.87
18:3 n-6	0.21±0.09	0.23±0.04	0.13±0.03	0.16±0.04	0.003	0.19	0.81
20:2 n-6	1.03±0.54	0.97±0.32	0.87±0.29	0.89±0.21	0.41	0.88	0.76
20:3 n-6	1.17±0.24	1.31±0.30	1.28±0.36	1.00±0.16	0.39	0.55	0.08
20:4 n-6	2.25±0.19	2.58±0.54	1.85±0.16	2.49±0.29	0.08	0.001	0.26
22:2 n-6	0.08±0.05	0.09±0.03	0.10±0.03	0.10±0.02	0.20	0.86	0.52
∑n-6 PUFA	10.26±0.46	10.74±0.26	9.14±0.77	9.67±0.27	$<10^{-4}$	0.02	0.89
n-3 PUFA							
18:3 n-3	4.00±0.61	3.85±0.53	4.56±0.87	4.47±0.85	0.06	0.68	0.93
18:4 n-3	0.93±0.69	0.78±0.24	0.74±0.31	0.80±0.33	0.61	0.78	0.56
20:3 n-3	0.88±0.41	0.82±0.26	0.92±0.23	0.85±0.22	0.74	0.58	0.93
20:4 n-3	0.89 ± 0.35^{ab}	1.0 ± 0.46^{ab}	1.41 ± 0.47^{a}	0.72 ± 0.15^{b}	0.45	0.08	0.02
20:5 n-3	4.78±1.11	4.95±0.81	3.83±0.53	4.86±0.75	0.13	0.08	0.20
22:5 n-3	0.87±0.46	1.14±0.19	1.31±0.36	1.46±0.38	0.02	0.16	0.68
22:6 n-3	28.88±1.68	33.65±3.35	32.52±4.11	32.07±2.71	0.42	0.10	0.05
∑n-3 PUFA	41.2 ± 2.4^{b}	46.2 ± 3.36^{a}	45.3 ± 3.3^{ab}	45.2 ± 2.5^{a}	0.20	<0.05	0.04
Ratios							
SFA/PUFA	0.47 ± 0.04^{a}	0.38 ± 0.06^{b}	0.4 ± 0.04^{ab}	0.4 ± 0.04^{ab}	0.48	<0.05	0.04
n3/n6	4.02±0.26	4.30±0.30	5.01±0.78	4.68±0.33	0.002	0.89	0.13

The data are presented as means ± s.d. ($N=6$ individuals) and were analysed by two-way ANOVA ($P<0.05$) followed by Student–Newman–Keuls multiple comparison test. [a,b] Mean values not sharing a common letter are significantly different from each other (one way ANOVA, $P<0.05$).

doi:10.1371/journal.pone.0076570.t008

(vegetable oil) on PUFA biosynthesis was evident at the molecular level, but did not elaborate into a beneficial fatty acid phenotype.

Effect of fat and lean genotypes in fish fed with vegetable oils

The superior growth performance of the L line as compared to the F line was known to be an outcome of improved feed efficiency and protein utilization, whereas the voluntary feed intake used to be the same under a fish oil based dietary regime [21,23,61]. But with the switch to vegetable oil based diet in the present study, the L line consumed slightly more feed than the F line irrespective of the carbohydrate content and thereby exhibited more pronounced increase in body weight. Such genotype specific preferential acceptance of vegetable oil diet adds to the basic understanding that trout can discriminate between feeds with different oil sources [62].

Concerning glucose metabolism, the F line showed a marginal but significantly lower postprandial plasma glucose levels (8 h) than the L line, following a high carbohydrate meal. There are two possible reasons, one is enhanced storage of excess glucose as glycogen as suggested by the higher hepato-somatic index of the F line fish and the other is increased glucose uptake in the white muscle of F line as evidenced by GLUT4 transcript abundance. These observations corroborate the better glycemic control and improved muscle glycolysis in the F line, reported under standard (10%) carbohydrate regime [21,22]. However in our preceding study with high level of carbohydrates given as part of fish oil based diet, contradictorily, the F line did not exhibit an improved

glycemic regulation despite having a higher ability to store excess glucose in the liver as glycogen or fat. Weaker control over hepatic endogenous glucose production in the F line was suggested to outdo its higher glycolytic ability [23]. This genotypic difference in glycolysis and gluconeogenesis disappeared under the vegetable oil based dietary regime in the present study, but it is hard to give substantial explanation for cause or consequence.

In previous studies, transcriptional analysis (after feeding fish oil based diets with or without carbohydrates to the two trout lines) demonstrated the higher potential of the F line in intestinal lipid uptake, hepatic *de novo* fatty acid synthesis (further enhanced by dietary carbohydrates) and fatty acid bioconversion in both liver and intestine [23,24]. We hypothesized that replacing fish oil with vegetable oil may augment this genetic potential of the F line because vegetable oil lacks n-3 LC-PUFA, the critical component in fish oil responsible for down regulating the genes encoding enzymes involved in lipogenesis and fatty acid bioconversion [54,57,63]. But contrary to our expectation, changing the dietary lipid source eliminated the genetic advantage of the F line in chylomicron synthesis, lipogenesis as well as fatty acid desaturation and elongation. The only exception was the consistently higher hepatic expression of the pentose pathway enzyme G6PD in the F line, irrespective of changes in dietary composition across studies [22,23,64]. So this enzyme connecting glucose and lipid metabolism may be a key marker of the fat muscle genotype.

On the other hand, we noticed few reversals in genotypic differences in the presence of dietary vegetable oil, the most prominent being the circulating triacylglycerol (TAG) phenotype. The previously reported higher plasma TAG linked to enhanced lipogenic ability in the F line [23] was inversely lowered in the F line, together with the annulation of genotypic difference in lipogenesis. A study in Atlantic salmon suggested that the differences between families in plasma TAG levels were influenced by peripheral tissue uptake rather than hepatic lipid metabolism, relating it to their lipid storage phenotype [65]. Correspondingly in the present study, the lower plasma TAG of the F line could be associated to the higher uptake of lipids in the muscle (adipocytes) as indicated by the transcript abundance of VLDLR, a receptor mediating internalisation and clearance of lipoproteins and CD36, a fatty acid translocase that determines long chain fatty acid uptake and lipid metabolism. The increased level of transcripts encoding the VLDLR and CD36 in the F line has already been reported, identifying them (and not LPL) as relevant molecular markers for fat deposition and circulating lipid uptake in the white muscle of rainbow trout [66]. A second reversal was evident in the transcriptional factor SREBP1c, which was higher in the liver and adipose tissue of the L line after fish oil replacement [23]. But, this activation was not accompanied by downstream changes in the lipogenic enzymes. Key factors regulating multiple facets of hepatic lipid metabolism such as SREBP1c, PPARα and PPARγ was uniformly up-regulated in the L line. But in target response, except for a higher expression of hepatic CPT1b in the L line related to PPARα activation, no other correlation was noticeable.

Recent data suggest that n-3 LC-PUFA content of the flesh is a highly heritable trait in salmonids [18]. Fatty acid deposition and the activity of the bioconversion pathway are also known to be dependent on the genetic background of the fish [5,67,68]. However, changes in dietary lipid source (i.e., vegetable oil) can alter the genetic potential; the magnitude and direction of response varies between family groups [19,56]. In accordance, we found that the inherently higher fatty acid bioconversion ability of the F line was eliminated or reversed (intestinal D6D) after replacing fish oil with vegetable oil [23,24,64]. But the key enzymes (desaturase and elongases) and transcriptional factors (PPARs and SREBP1c) were not coordinately regulated in both lines to portray a defined pattern of expression with biological significance. At the phenotypic level, dietary vegetable oil did not affect the higher muscle lipid content (selection criterion) of the F line and thus it retained greater quantity of saturated and unsaturated fatty acids in absolute terms, unlike in salmon where differences in muscle lipid content between the fat and lean genotype reportedly diminished in 100% vegetable oil fed group [5]. Nevertheless in relative terms, the n-3 LC-PUFA content of the F line flesh was slightly lower than the L line. This suggests that the L line may have higher responsiveness to low dietary n-3 LC-PUFA, up-regulating the biosynthetic pathway when fed diets with vegetable oil. Overall, our results emphasize that the vegetable oil based dietary regime alters lipid metabolism depending on the genetic background and the LC-PUFA biosynthesis pathway showed no pertinent genotypic difference when assessed by gene expression and fatty acid composition.

Conclusion

This study demonstrates that carbohydrate intake when coupled with lower dietary content of n-3 LC-PUFA (vegetable oil) promotes the inherent LC-PUFA biosynthetic pathway, regardless of the genetic background of the fish. At the molecular level, this was confirmed by the enhanced transcriptional response of key desaturase and elongase enzymes, mediated through the PPARγ regulatory factor in the liver of both lines; however, the final fatty acid profile of the flesh did not evidence a correlative augmentation of LC-PUFA content. Moreover, the documented genetic pre-disposition of higher fatty acid bioconversion in the fat genotype disappeared under the vegetable oil based diet regime. Dietary macro-nutrient interface is thus a critical aspect to be deliberated during the progressive shift towards plant based feeds and while analyzing nutrient × genotype interactions.

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Author Contributions

Conceived and designed the experiments: SP FM GC. Performed the experiments: BSK LL. Analyzed the data: BSK SP FM GC. Wrote the paper: BSK SP.

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Chapter 4

GENERAL DISCUSSION

The experiments undertaken in the study were a concerted attempt to elucidate the physiological and metabolic aspects of starch utilisation in two rainbow trout genotypes, namely the fat (F) and the lean (L) lines, divergently selected for muscle fat content (Quillet et al., 2005). The specific choice of these two trout genotypes and the postulated hypothesis that the F line has a higher ability to metabolise dietary starch than the L line was based on two previous observations: (1) The activity and expression of key glycolytic enzymes was found to be higher in the muscle and liver of the F line than the L line, when fed diets with a constant level of starch (9-12%) and varying lipid contents (Kolditz et al., 2008a). (2) In a later study, the F line was coherently found to have lower plasma glucose values than the L line, 24 hours after re-feeding a diet with 10% crude starch (Skiba-Cassy et al., 2009). Overall, these findings suggested a better regulation of postprandial glycemia and glucose metabolism in the F line.

The first part of this doctoral study included a pair of experiments that aimed to verify the above hypothesis by characterising the response of the two trout genotypes in terms of nutrient digestibility, intestinal transport, metabolism, growth and nutrient retention, to a diet with (16-17%) or without (<1%) gelatinised starch (namely, 20C and 0C). The second part of the study aimed to describe the effect of gelatinised starch on the metabolism and tissue fatty acid composition of the two trout genotypes when starch was fed as part of a vegetable oil based diet (namely, VOC+ and VOC-). The idea behind the second study has never been tested before and is very relevant to the recent dynamic changes in fish feed formulation.

Concerning study design, all the experiments adopted a 2 by 2 factorial approach to delineate the effect of diets, genotypes and possible interaction between them. Concerning diets, the absolute difference in starch intake (<1% *vs.* 16-17%) between the two dietary groups as compared to protein intake [59% *vs.* 45%, always above the requirement level of 38% (NRC, 2011)] enables us to consider that majority of the effects are linked to starch intake. However, we caution that it is not possible to reject completely any effect of dietary protein levels. Concerning analysis, most of the metabolic markers were analysed at the transcriptional level to reveal the differences in the genetic makeup of the two trout lines and was further related to phenotypic estimates such as nutrient digestibility, plasma metabolites and fatty acid composition, along with some targeted metabolic enzyme activity measurements. The results presented in the previous chapters are developed into a comprehensive discussion as follows:

Fat and lean lines: Are they different in utilising high level of gelatinised starch when fed as part of conventional fish oil based diet?

No apparent genotypic difference in starch digestibility and glucose absorption

In the primary limiting step i.e. digestion and absorption, there was no major difference between the two lines. The digestibility of starch in both trout lines was above 96%, similar to other studies in rainbow trout using gelatinised starch source at a tolerable level (Bergot, 1993; Krogdahl et al., 2004). In trans-epithelial glucose transport, except for a slightly higher abundance of GLUT2 transcripts in the pyloric caeca of the F line, none of the other proteins were differentially expressed between the two trout lines and this was in agreement with studies in mice and poultry that report selection for production traits may not be matched with beneficial changes in intestinal absorption of glucose (Fan et al., 1996; Fan et al., 1998). Importantly, starch intake (20C diet) did not stimulate an increase in the expression of apical membrane glucose co-transporter SGLT1, in contrast to previous studies (Kirchner et al., 2008; Polakof, S. et al., 2010) and a similar inertness was observed also for the basolateral membrane facilitative glucose transporter GLUT2 in both trout lines, when compared to the groups fed diet without starch (0C). On the contrary, in omnivorous fish such as the black bullhead catfish, glucose treatment is known to presumably enhance the activity of both these transporters in the enterocytes (Soengas and Moon, 1998). Even, we investigated the alternate metabolic pathway proposed in mammals for the export of glucose to the blood independently of GLUT2 but dependent on phosphorylation of glucose by HK and further dephosphorylation by G6Pase (Mithieux, 2005), and found no coherent response to starch intake in both trout lines. On the whole our data suggests that regardless of the genetic background carnivorous fish such as rainbow trout lacks the ability to adapt its intestinal glucose uptake capacity to a high starch diet. However, the apparent digestibility of technologically treated starch is high in both lines (>96% at 20% dietary inclusion).

No genotypic difference in the regulation of postprandial glycemia

In both lines, as expected, starch intake (20C diet) resulted in post-prandial hyperglycemia, which was moderate (6 to 7.5 mM) compared with other studies using similar dietary levels of starch (Bergot, 1979; Kaushik and Oliva Teles, 1985; Panserat et al., 2000c). But contradicting the relatively rapid clearance of blood glucose in the F line which was documented previously (Skiba-Cassy et al., 2009), no genotypic difference was observed in the regulation of postprandial glycemia at the end of the 10 week feeding trial in the present

study. A possible reason for this discrepancy maybe the different preceding conditions of measurement in the two studies (long-term feeding *vs.* fasting/re-feeding) or the dietary starch content (17% *vs.* 10%). Nevertheless, genotypic differences were found in the subsequent analysis of key enzymatic markers of hepatic intermediary metabolism.

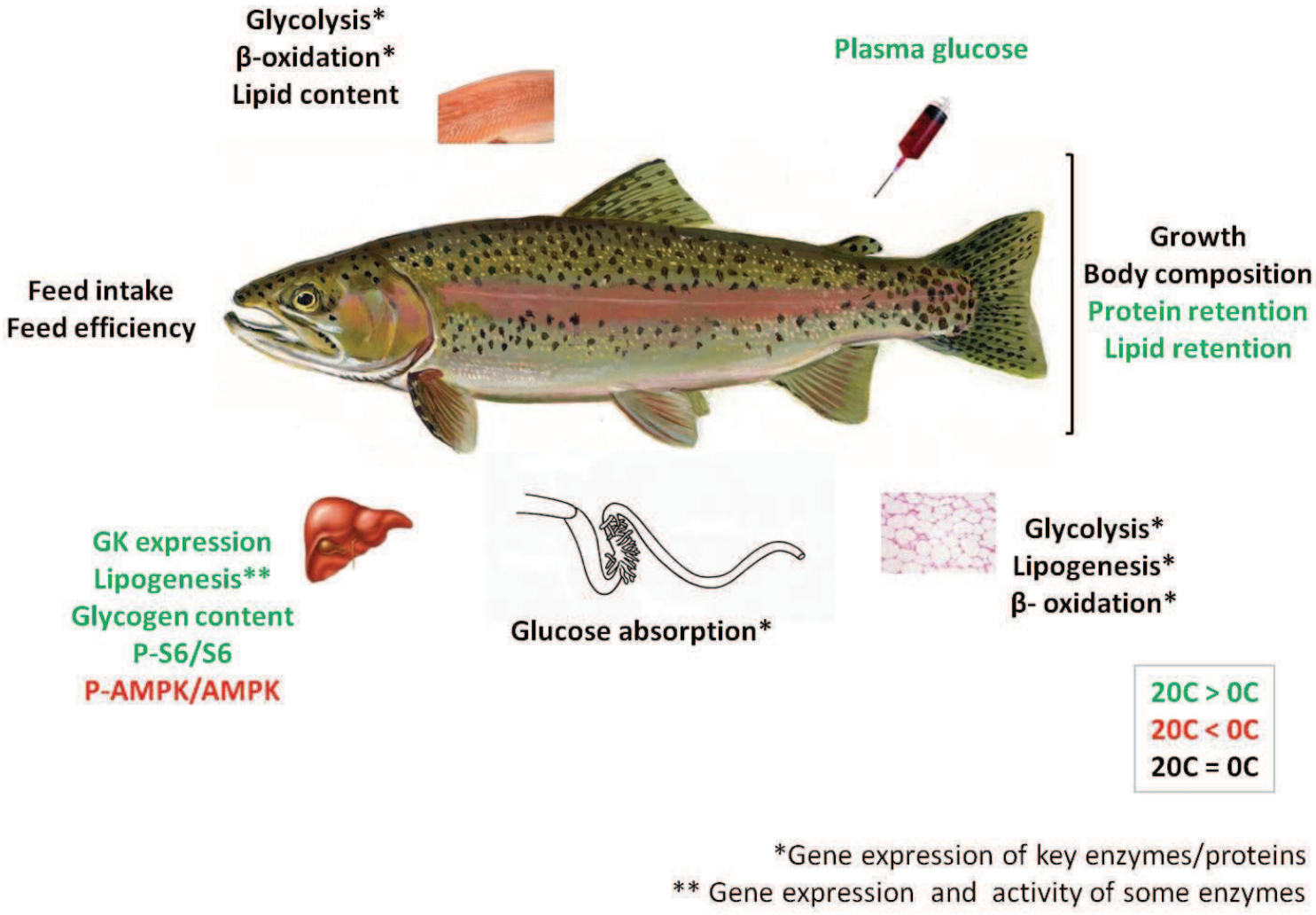
Existence of genotypic difference in hepatic intermediary metabolism

Conflicting glucose metabolism and higher glycogen storage in the F line

The transcripts of the first and last glycolytic enzymes i.e. GK and PK were more abundant in the F line as compared to the L line, suggesting a higher glycolytic potential in the F line liver, as previously observed (Kolditz et al., 2008a). Especially, dietary starch intake resulted in a more pronounced GK on-off regulation (previously well documented in trout - Panserat et al., 2000c) in the F line, as indicated by the significant diet × genotype interaction. But concurrently, the expression of gluconeogenic enzymes PEPCK and FBPase were also higher in the F line as compared to the L line, suggesting that the potential export of glucose synthesised *de novo* through unregulated gluconeogenesis may counter-balance the uptake of glucose from the bloodstream for glycolytic purposes in the F line. This may possibly explain why we could not find any difference in plasma glucose levels between the two lines. In terms of glucose storage, as expected (Hemre et al., 2002; Enes et al., 2009), the presence of starch in the diet resulted in increased hepatic glycogen content in both lines. In particular, the F line had higher glycogen content than the L line when fed both diets, suggesting better hepatic glucose storage capacity.

On the other hand, some contradictions related to dietary starch intake were observed in the glucose metabolism of both lines, such as the paradoxical inhibition of PK expression similar to few other studies in trout (Panserat et al., 2009; Seiliez et al., 2011) and the differential regulation of gluconeogenic enzyme transcripts i.e., starch intake down-regulated the FBPase expression but conversely G6Pase2 expression was up-regulated (G6Pase is also the last enzyme of the glycogenolytic pathway). Though the exact reason for the contradictory findings are unknown, the atypical PK down-regulation may have a negative effect in glucose oxidative flux in both lines and since PK is at the junction of glycolytic output and gluconeogenic inflow, it is important to clarify this enigma. Also, the concomitant high expression of the components of the GK-G6Pase substrate cycle suggests a futile cycle (Pilkis and Granner, 1992), where energy from glucose maybe lost, but this hypothesis needs

Figure 20. *Summary of the effect of dietary starch content on the various analysed parameters in both lines*



to be verified. On the positive side, the mammalian like down-regulation of FBPase by dietary starch intake, contrary to previous reports in trout (Panserat et al., 2001a; Kirchner et al., 2008), could be one of the possible explanations for the moderate hyperglycaemia observed in both lines, as the rate of glucose production from substrates entering the gluconeogenic pathway at the triose phosphate level would be limited.

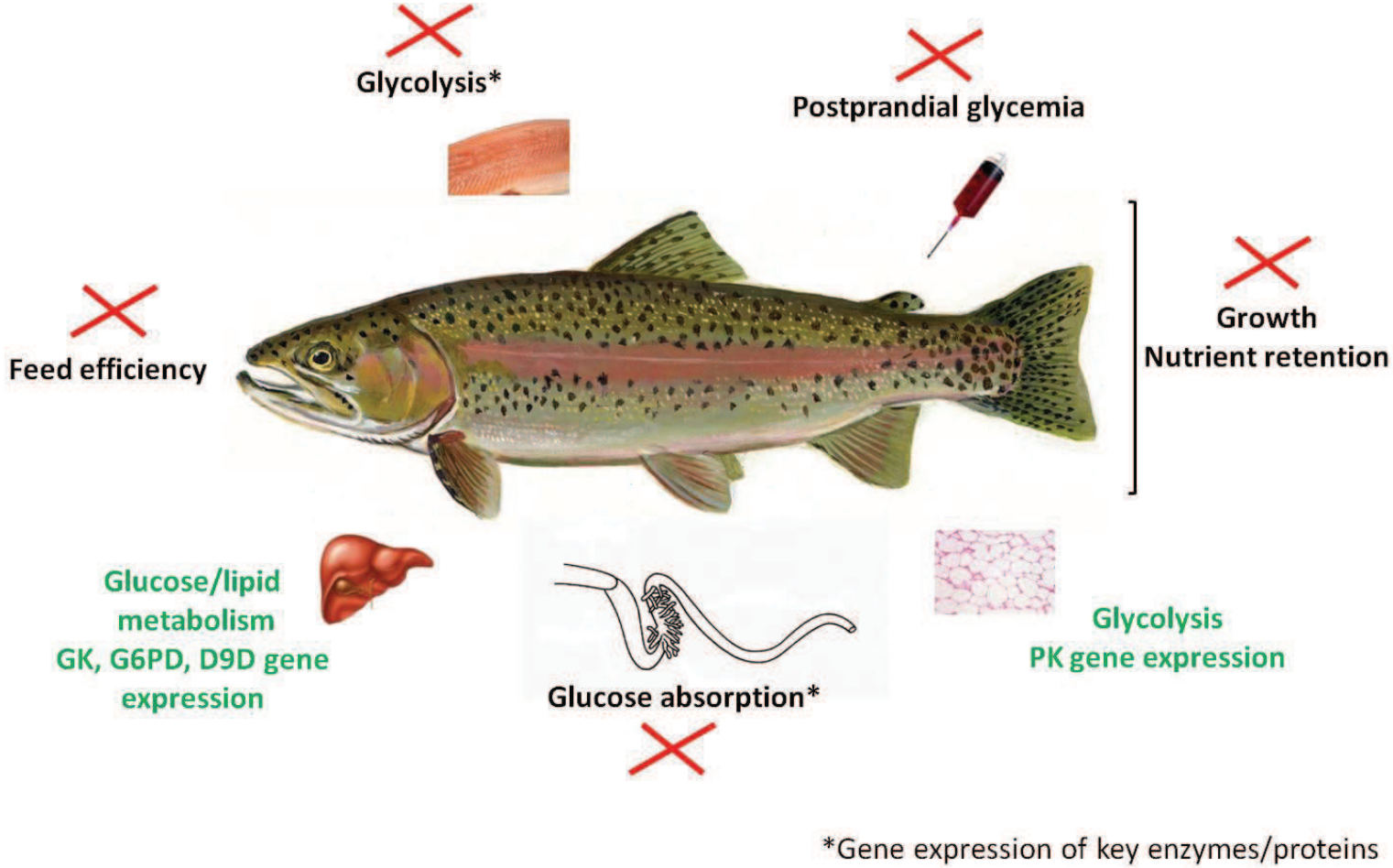
Higher potential of the F line to convert excess glucose into lipids

The relationship between glucose and lipid metabolism is another major factor that can impact glucose use (Randle, 1998; Towle et al., 1998). In this milieu, recent studies in rainbow trout have demonstrated a link between the stimulation of *de novo* lipogenesis (DNL) and improvement in glucose homeostasis (Panserat et al., 2009; Skiba-Cassy et al., 2009; Polakof et al., 2011c). Correspondingly, we observed that dietary starch intake enhanced the activity (FAS, G6PD) and expression (G6PD, ACLY, D9D) of enzymes related to DNL in both lines (Fig. 20). This dietary regulation does not seem to be mediated by the transcription factor SREBP1c, contradicting mammalian models (Strable and Ntambi, 2010) and hormonal regulation (insulin effects) in rainbow trout (Lansard et al., 2010; Polakof et al., 2010). The possible involvement of other transcription factors such as ChREBP should be investigated in future studies. With respect to the genotypes, we found that the F line has a higher potential of DNL than the L line, as indicated by the elevated expression of lipogenic and NADPH yielding enzymes such as FAS, ACLY and G6PD. Parallel to this, the expression of CPT1 isoforms (the rate limiting enzyme of β-oxidation pathway) was lower in the F line as compared to the L line, suggesting lower use of fatty acids for energy supply. Altogether, these molecular differences were reflected in the elevated plasma triglyceride levels of the F line and unambiguously confirmed previous observations (Kolditz et al., 2008a; Skiba-Cassy et al., 2009). Above and beyond all other considerations, dietary intake of starch enhanced the expression of G6PD (a key enzyme of the pentose phosphate pathway that generates NADPH) and D9D (stearoyl CoA or Δ9 desaturase, key enzyme in the synthesis of monounsaturated fatty acids) specifically to a greater extent in the F line than the L line, as indicated by the significant diet × genotype interactions. This clearly suggests that the F line may have a higher potential to convert excess glucose into lipids, than the L line.

Distinct signalling of cellular AMPK and S6 linked to starch intake

It is not possible to explain the differences in hepatic intermediary metabolism between the two lines, from the data of cell signalling analysis. The slightly lower activation of Akt

Figure 21. *Summary of the diet × genotype interactions observed in the various analysed parameters*



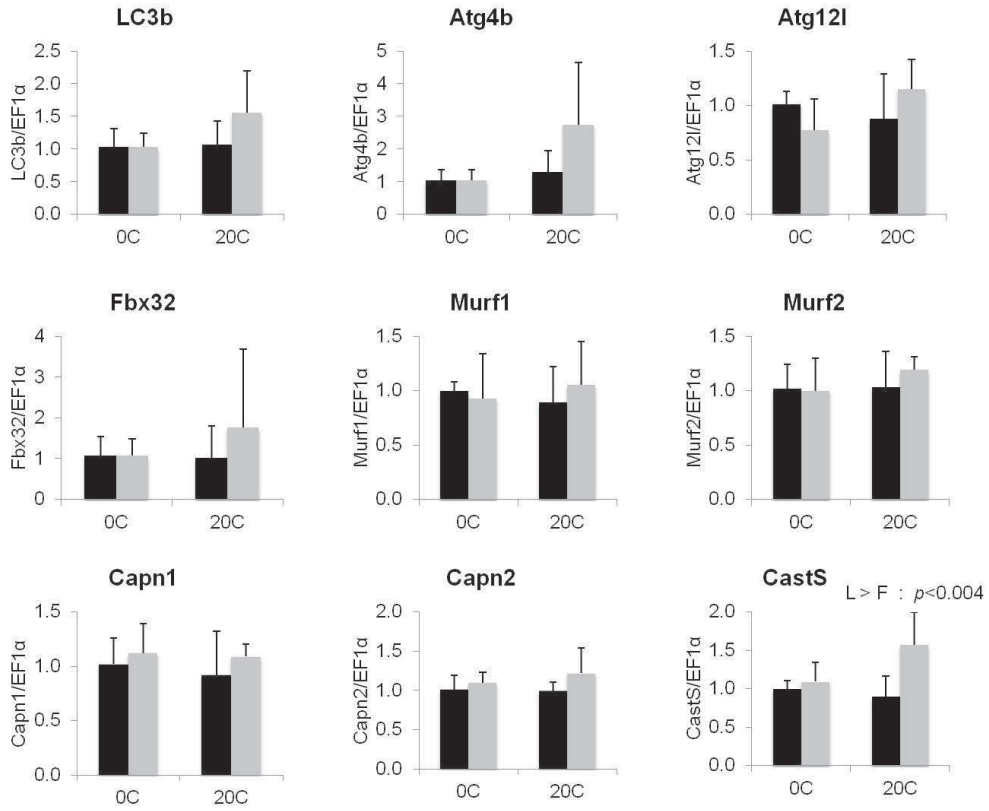
(phosphorylation status) that we found in the F line, paradoxically contradicts its higher glycolysis, lipogenesis and glycogen levels (insulin-dependent metabolic pathways). Nevertheless in both lines, our results strongly suggest for the first time in fish that dietary starch intake contribute to a specific hepatic cell signalling response, characterised by a decrease in AMPK phosphorylation and reciprocally an increase in S6 phosphorylation. As AMPK and S6 (TOR cascade) are vital regulators of intracellular energy homeostasis and cell growth, respectively (Rutter et al., 2003; Wullschleger et al., 2006; Hardie et al., 2012), the above response potentially indicates that exogenous glucose obtained from starch satisfies the cellular energy needs in the liver of these trout lines and it will be interesting to investigate whether such signalling is common (in normal trout) or if it is specific to these selected lines.

Poor glucose utilisation in the white muscle and adipose tissue: common in both lines

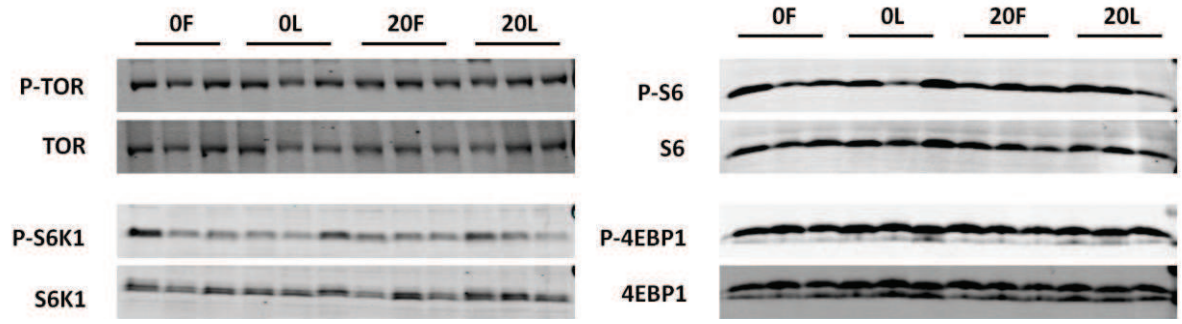
Unlike in the liver, dietary starch had no impact on the expression of glycolytic enzymes or glycogen content in the white muscle of both lines, which confirms the poor response of this tissue to a high influx of glucose (West et al., 1994; Hemre et al., 2002; Panserat et al., 2009). Importantly, we did not find any genotypic difference in the expression of genes encoding the rate-limiting enzymes of glycolysis, in absolute contrast to previous observations of enhanced expression and activity of glycolytic enzymes in the F line (Kolditz et al., 2008a). This inconsistency might have occurred due to differences in diet composition, temperature of the rearing water (17°C *vs.* 11°C), generation of selection investigated (fourth *vs.* third) and post-prandial time of analysis (8 h *vs.* 24 h) or a combination of these factors.

Likewise in the white adipose tissue, except for a higher abundance of SREBP1c transcripts in the F line and a significant diet × genotype interaction in the expression of PK, no other differences were observed. The lack of change in the expression of the analysed lipogenic enzymes despite the induction of the transcription factor SREBP1c (in contrast to the liver) can be at least partly explained by the inherent lower activities of lipogenic enzymes in the adipose tissue (Lin et al. 1977a, b). Overall, our results reinforces the theory that glucose utilisation is poorly regulated in the peripheral tissues of carnivorous fish. Concerning diet × genotype interaction, the disordinal interaction in adipose tissue PK, where expression was enhanced only in the F line fed dietary starch group is coherent with other interactions observed in the liver (GK, G6PD and D9D). Though these few interactions (Figure 21) endorse our hypothesis that the F line has a higher capacity to metabolise dietary starch, they are not sufficient to establish it.

Figure 22. *Data from analysis of proteolytic and protein synthesis markers in white muscle, to explain the mechanisms underlying the growth difference between the two lines (L > F)*



Gene expression of markers of the autophagy-lysosomal, ubiquitin-proteosomal and calpain-calpastatin pathways in the white muscle of rainbow trout from a fat line (dark bar) and a lean line (light bar) fed a diet without (0C) or with (20C) carbohydrate, 8 h after the last meal.



Western blot analysis of the TOR signalling cascade (TOR, S6K1, S6 and 4EBP1) protein phosphorylation in the white muscle of rainbow trout from a fat line (F) and a lean line (L) fed a diet without (0C) or with (20C) carbohydrate, 2 h after the last meal. No significant difference was observed in the ratio of phosphorylated to total protein between the two lines.

No genotypic difference in nutrient retention and growth related to starch intake

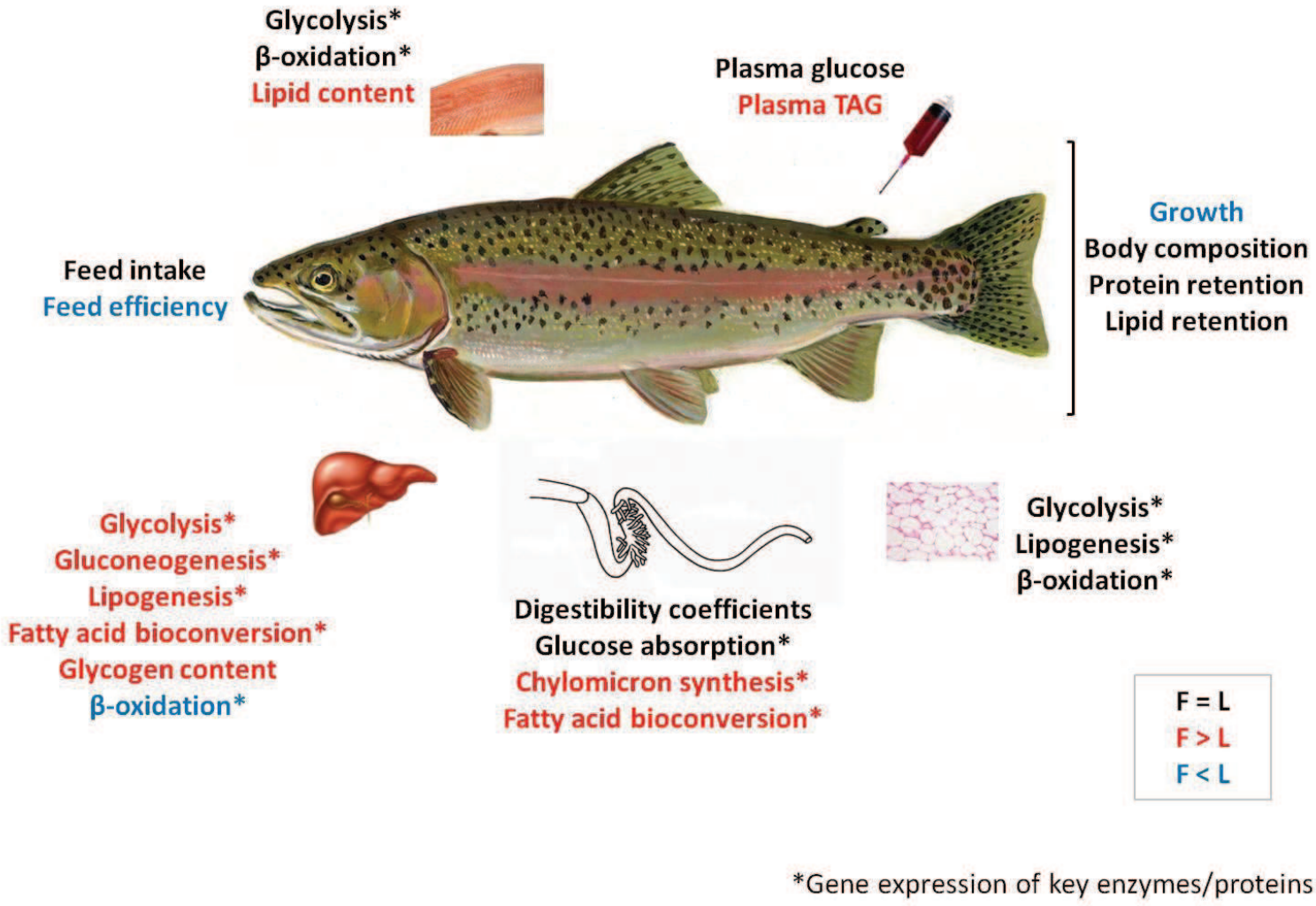
In both lines, dietary intake of starch (at 20% inclusion level) was associated with higher protein retention, indicating a protein-sparing effect, as expected (Kim and Kaushik, 1992; Hemre et al., 2002). However, the nutrient gain and retention estimates for protein and lipid do not reflect any preferential utilisation of energy substrates between the two lines. Starch intake had no adverse impact on the growth of the two lines, as the inclusion level (20%) was within the acceptable limits for this species and the energy from glucose was utilised as efficiently as that of protein (Kaushik et al., 1989; Kim and Kaushik, 1992). At the same time, the documented higher growth potential of the L line than the F line was observed also in this fourth generation of selection, linked to better feed efficiency (Quillet et al., 2007; Kolditz et al., 2008a; Skiba-Cassy et al., 2009). The absence of diet × genotype interaction in growth and nutrient retention corresponds to the outcome of few early studies that investigated familial variation in carbohydrate utilisation (Edwards et al., 1977; Austreng et al., 1977; Refstie and Austreng, 1981).

As additional information, we investigated the possible mechanisms underlying the consistent difference in growth between the two lines (L > F). In the white muscle, we could not find any substantial explanation due to lack of difference between the lines in the expression of analysed markers of the major pathways of proteolysis (Ubiquitin-proteosomal, autophagy-lysosomal and calpain-calpastatin), in phosphorylation status of the components of TOR signalling cascade involved in protein synthesis (mRNA translation) and in the expression of myogenic regulatory/inhibitory factors, at the fed state (Figure 22).

Validity of the hypothesis

On the whole, except for an enhanced metabolic potential of the F line to store excess glucose from dietary starch as lipids and glycogen, our hypothesis concerning the higher ability of the F line to use dietary starch is not validated. Regardless of the genetic background, the inability to adapt intestinal glucose uptake, poor regulation of endogenous glucose production in the liver and poor oxidation/use of glucose in the peripheral tissues (proportionally the largest part of the whole body) was found to be a common challenge for effective use of dietary starch in rainbow trout. On the other hand, the positive signalling status of intracellular energy (AMPK) and nutrient (S6) sensors indicates that energy supplied by dietary starch is efficiently used by the hepatocytes, irrespective of the genotype.

Figure 23. *Summary of the effect of genotypes on the various analysed parameters*



The physiological and metabolic differences that distinguish the two trout lines: Comparison with other genetically selected livestock

Genetic selection is essentially done based on one or more phenotypic selection criterion, but with not much information about the underlying physiological or metabolic differences and with the possibility to find several correlated differences. For instance, the two rainbow trout lines under study were two-way (divergently) mass selected using a non-invasive technique (fat-meter) for muscle lipid content, the only selection criterion (Quillet et al., 2005). Later on, the two lines were found to differ in terms of growth (L>F), allocation of fat to different body compartments (more to muscle and less to viscera in F line and vice versa in the L line), potential for fatty acid synthesis (F>L) and fatty acid oxidation (L>F) in the liver, plasma triglycerides (F>L), potential for glycolysis in the liver and muscle as well as regulation of postprandial glycemia (F>L), potential for lipid uptake in the muscle (F>L) and potential for protein/amino acid catabolism (F>L) in the liver (Quillet et al., 2007; Kolditz et al., 2008a, b; Skiba-Cassy et al., 2009; Kolditz et al., 2010). The results of the present study corroborate the previously noted genotypic differences in growth and hepatic lipid metabolism, but are not consistent with other observations such as the differences in glycolytic potential in the muscle and regulation of postprandial glycemia (Figure 23).

In other terrestrial livestock, differences in intra-muscular fat content is similarly related to the activity and expression levels of lipogenic and NADPH generating enzymes, uptake of fatty acids by muscle and a balance between fatty acid synthesis and oxidation (Hocquette et al., 2010). But the difference in glucose metabolism is disputable. The better control of plasma glucose and higher activity of glycolytic enzymes in the muscle of the F line (Kolditz et al., 2008a; Skiba-Cassy et al., 2009) is consistent with findings in chicken, where the fat line broilers has lower plasma levels of glucose and also show a better glucose tolerance and higher insulin concentrations in the plasma than the lean broilers (Leclercq et al., 1988; Saadoun et al., 1988), but contradicts findings in cattle, where genotype with reduced muscle fat content and high muscularity are known to have high glycolytic activity and low plasma glucose levels (Hocquette et al., 2010). In sheep, selected line with higher fat was found to have elevated plasma insulin concentrations than the lean line, but glucose levels did not vary (Francis et al., 1999). However, the predominant variation between animals selected for muscle or whole body fat content is in lipid metabolism, as we observed.

Studies examining the relationship between genotype and the intestinal tract in poultry suggested that selection influences the intestinal morphology, mass of absorptive epithelium, gut motility and digestive capacity (Mitchell and Smith, 1991; Mignon-Grasteau et al., 2004; De Verdal et al., 2010; Rougière et al., 2012). In that order, we found that divergent selection for high muscle fat content was associated with a higher potential to synthesize chylomicrons in the intestine, i.e., the process of lipid absorption in fish (Sire et al., 1981; Tocher, 2003). Enhanced expression of MTP (the protein that performs the critical neutral lipid transfer activity, leading to the formation of primordial chylomicron particles) and ApoA4, (the apolipoprotein involved in lipidation and maturation of chylomicrons) underlined the higher potential of the F line to assemble chylomicrons. However, the apparent lipid digestibility estimates did not reflect the molecular differences and was not significantly different between the two lines. Given the fact that both MTP and ApoA4 are known to be regulated at the transcriptional level in mammals (Hussain et al., 2001; Black, 2007), this discrepancy maybe because genetic potential of the F line was not sufficiently amplified in the phenotype at the fourth generation or regulation is distinct (post-transcriptional) in fish.

Further in lipid metabolism, fatty acid deposition and the activity of the bioconversion pathway are known to be dependent on the genetic background of the fish (Rollin et al., 2003; Bell et al., 2010; Goetz et al., 2010). Accordingly, we found a consistently higher expression of D6D, Elovl2 and Elovl5 in the liver and intestine of the F line when compared to the L line. This coherent up regulation of key desaturase and elongase enzymes involved in fatty acid bioconversion in the F line suggests the possibility of an eventual genetic predisposition to deposit more beneficial long chain polyunsaturated fatty acids (n-3 LC-PUFA) in its flesh. The interest that these results create in terms of human consumption is further amplified because recent data suggest that n-3 LC-PUFA content of the flesh is a highly heritable trait in fish (Leaver et al., 2011). Retrospectively, it is important to note here that we did not find any stimulating effect of dietary starch on the expression of any of the desaturase and elongase enzymes, contrary to Seiliez et al. (2001). This could be due to the high level of fish oil in the diet, which probably satisfied all the n-3 LC-PUFA requirements and inhibited the bioconversion pathway. So, the effect could have been different in a vegetable oil based diet.

Overall in the present study, the high fat muscle phenotype of the F line is potentially characterised (at the molecular level) by an increase in chylomicron synthesis in the intestine, lipogenesis in the liver, fatty acid bioconversion in the liver and intestine, level of triacylglycerol in the plasma and a decrease in fatty acid oxidation in the liver.

Effect of gelatinised starch on metabolism and fatty acid profile of the fat and lean trout line in a low n-3 LC-PUFA (vegetable oil) dietary environment

With the gradual inclusion of more plant ingredients in commercial fish feeds, there is growing concern over the decrease in the n-3 LC-PUFA content of the final product. Combining the putative genetic pre-disposition of the two trout lines in fatty acid bioconversion with the pre-existing knowledge that individually vegetable oil and carbohydrates enhance key Δ6 desaturase (D6D) gene expression in rainbow trout (Seiliez et al., 2001), we hypothesized that digestible carbohydrate when given as part of a low n-3 LC-PUFA diet, may exert a stimulatory effect on fatty acid bioconversion and n-3 LC-PUFA content of the flesh, particularly in the F line. To verify this hypothesis, we investigated the molecular and phenotypic response of the two lines to a vegetable oil based diet either with or without gelatinized starch. This experiment further provided a unique opportunity to compare (Table 9 and 10) and understand the metabolic response of the two lines to dietary starch, when dietary fatty acid composition is changed (from fish oil to a vegetable oil blend).

Conforming to our hypothesis, intake of starch when coupled with low n-3 LC-PUFA dietary environment (unlike our previous experiment), enhanced the transcription of key desaturase (D6D) and elongase (Elovl5 and Elovl2) enzymes involved in LC-PUFA synthesis, in both lines. Though the exact mechanism remains to be elucidated, dietary regulation of these enzymes was apparently mediated by the transcription factor PPARγ in the liver. The vertebrate D6D gene is known to contain a PPAR response element (Ferré, 2004) and the activation of PPARγ has been reported to increase fatty acid desaturation and elongation in mice (Roberts et al., 2011). We provide evidence for the first time that piscine PPARγ plays a role similar to their mammalian counterpart. Nevertheless at the phenotype, we found no increase in the n-3 LC-PUFA content of the flesh corresponding to the molecular augmentation of desaturase/elongase expression in the liver and intestine of the starch fed group. Instead, they closely resembled the fatty acid profile of the respective diet consumed, as commonly observed (Turchini et al., 2009). This discrepancy may be due to the other interacting aspects of lipid metabolism such as the higher oxidation of the 18C fatty acids that reduces substrate availability for bioconversion, preferential tissue uptake (Tocher et al., 1998; Stubhaug et al., 2005) and the regulation at the post-transcriptional level.

Concerning the difference between the lines, we had hypothesised a further augmentation of the fatty acid bioconversion potential in F line under the vegetable oil based dietary regime as

Table 9. *Comparison of the effect of dietary starch content based on dietary lipid source*

Parameter	Effect of dietary starch content	
	Fish oil based diet regime	**Vegetable oil based diet regime**
Intestine		
Glucose transport	-	GLUT2 ↑
Chylomicron synthesis	ApoA1 ↑	MTP ↑, ApoA1 ↑, ApoA4 ↑
Fatty acid bioconversion	-	D6D ↑, Elovl5 ↑
Plasma		
Metabolites	Glucose ↑	Glucose ↑, Cholesterol ↑
Liver		
Glycolysis	*GK ↑, PK ↓	GK ↑
Gluconeogenesis	G6Pase2 ↑, FBPase ↓	G6Pase1 ↓, G6Pase2 ↑
Lipogenesis	FAS ↑, ACLY ↑, *G6PD ↑, *D9D ↑	ACC ↑
Fatty acid bioconversion	-	D6D ↑, Elovl2 ↑, PPARγ ↑
Fatty acid oxidation	-	*CPT1a, CPT1b ↑
Muscle		
Glycolysis	-	-
Fatty acid oxidation	-	-
Glucose uptake	-	-
Lipid uptake	---	-
Adipose tissue		
Glycolysis	*PK	-
Lipogenesis	-	-
Fatty acid oxidation	-	-
Overall performance		
Growth	-	-
Nutrient retention	Protein ↑, Lipid ↑	---

↑ Increase due to starch content, ↓ Decrease due to starch content, - no effect of starch

* Diet × Line interaction, --- not analysed

Caution: The comparison is based on the results of two different experiments with fish from the same generation of selection (4th generation) and similar rearing conditions (flow through system, at 17^{o}C), but of different sizes (final mean body weight 70-80 g *vs.* 210-270 g).

it lacks n-3 LC-PUFA, the critical component in fish oil responsible for down regulating the genes encoding enzymes involved in fatty acid bioconversion and lipogenesis (Jump, 2002; Nakamura et al., 2004; Torstensen and Tocher, 2010). But in total contrast, we found that the previously observed higher fatty acid bioconversion potential of the F line disappeared under the vegetable oil based dietary regime. This shows that dietary fatty acid composition is a major determinant of the magnitude and direction of genetic response in PUFA biosynthesis, as previously observed in Atlantic salmon (Bell et al., 2001; Morais et al., 2011a, 2012). Nevertheless, as muscle lipid content (the selection criterion) remained unaffected by the vegetable oil based dietary regime, the F line retained greater quantity of saturated and unsaturated fatty acids in absolute terms (mg/g of muscle). However in relative terms (% of total fatty acids), the n-3 LC-PUFA content of the F line flesh was slightly lower than the L line, suggesting that the L line may have higher responsiveness to low dietary n-3 LC-PUFA.

In short, under the vegetable oil based dietary regime, genotypic difference in fatty acid bioconversion looses pertinence and the stimulatory effect of dietary starch observed at the molecular level does not elaborate into a beneficial n-3 LC-PUFA phenotype.

Change in dietary lipid source does not modify the overall effect of starch intake in glucose metabolism but influences the genotypic response to starch intake

The general effect of starch intake on glucose metabolism was mostly unaffected by the change in dietary lipid source to vegetable oils. Similar to our previous experiment (Kamalam et al., 2012), starch intake resulted in a moderate hyperglycemia (6 to 7.5 mM) and a strong induction of hepatic GK expression. However, there were some exceptions such as the mammalian like down-regulation of G6Pase1 expression that is unusual in trout (Pilkis and Granner, 1992; Panserat et al., 2000a) and the increase in intestinal GLUT2 transcript levels without any change in SGLT1, which is opposite to previous observations in trout (Kirchner et al., 2008; Polakof et al., 2010). The implications of the above response remain ambiguous. Concerning the genotypes, the higher expression of certain glycolytic and gluconeogenic enzymes in the F line that we had previously observed was not evident under the vegetable oil based regime, but the F line exhibited a marginally lower postprandial plasma glucose levels (at 8 h) than the L line, as reported by Skiba-Cassy et al. (2009). This could be linked to an increase in the expression of muscle GLUT4, suggesting higher glucose uptake in the white muscle. Nevertheless, no changes were noted in the metabolic response of muscle and adipose tissue, reinforcing the theory of poor utilisation of glucose in the peripheral tissues.

Table 10. *Comparison of the effect of genotype based on dietary lipid source*

Parameter	Effect of genotype (F>L, L>F)	
	Fish oil based diet regime	Vegetable oil based diet regime
Intestine		
Glucose transport	GLUT2	-
Chylomicron synthesis	MTP, ApoA4	-
Fatty acid bioconversion	D6D, Elovl5, Elovl2	D6D
Plasma		
Metabolites	Triglycerides	Glucose, Triglycerides
Liver		
Glycolysis	GK, PK	-
Gluconeogenesis	PEPCK, FBPase	-
Lipogenesis	FAS, ACLY, G6PD	G6PD, SREBP1c
Fatty acid bioconversion	D6D	PPARγ
Fatty acid oxidation	CPT1a, CPT1b, HOAD	CPT1b, PPARα
Muscle		
Glycolysis	-	-
Fatty acid oxidation	-	-
Glucose uptake	-	GLUT4
Lipid uptake	---	VLDLR, CD36/FAT
Adipose tissue		
Glycolysis	-	-
Lipogenesis	SREBP1c	SREBP1c
Fatty acid oxidation	-	-
Overall performance		
Growth	L > F	L > F
Nutrient retention	-	---

L: lean line, F: fat line, - no difference between the two lines, --- not analysed

Caution: The comparison is based on the results of two different experiments with fish from the same generation of selection (4th generation) and similar rearing conditions (flow through system, at 17^{o}C), but of different sizes (final mean body weight 70-80 g *vs.* 210-270 g).

Change in dietary lipid source impairs the starch intake induced de novo lipogenesis and alters or eliminates the genotypic differences in lipid metabolism

In remarkable disagreement with our previous study, consumption of starch under the vegetable oil based diet regime did not stimulate the expression of almost all the analysed enzymes involved in *de novo* lipogenesis (except ACC) and NADPH production, notwithstanding the huge induction of GK. Apart from this, we found a paradoxically enhanced expression of CPT1b (the rate limiting enzyme of the mitochondrial fatty oxidation pathway) in the starch fed group, which contradicts the common understanding in higher vertebrates that provision of glucose inhibits fatty acid oxidation (Randle, 1998) and an enigmatic *triumvirate* of unaltered plasma triglyceride levels, but elevated cholesterol levels and an increase in the expression of key proteins involved in chylomicron assembly following starch intake, which disagrees with the documented relation between these parameters and carbohydrate intake (Parks and Hellerstein, 2000). These changes suggest a disturbed link between glucose and lipid metabolism, when fed the vegetable oil based diet.

Concerning the genotypes, the change in dietary lipid source to vegetable oils was associated with the suppression of the higher potential of the F line in intestinal chylomicron assembly and hepatic *de novo* lipogenesis, as observed with fatty acid bioconversion (Kamalam et al., 2012; Kamalam et al., 2013). The only exception was the higher hepatic expression of the NADPH generating pentose pathway enzyme G6PD in the F line, which was consistent irrespective of changes in dietary composition across studies (Kolditz et al., 2008b; Skiba-Cassy et al., 2009; Kamalam et al., 2012). Furthermore, we also noticed that the circulating triacylglycerol (TAG) levels were lower in the F line than the L line, which marks a prominent reversal of genotypic differences due to change in dietary lipid source. This response could be related to a possible higher uptake of lipids in the muscle, indicated by the higher expression of VLDLR (a receptor mediating internalisation and clearance of lipoproteins) and CD36 (a fatty acid translocase that determines long chain fatty acid uptake and lipid metabolism) in the F line, in agreement with a previous postulation in Atlantic salmon fat and lean families (Morais et al., 2011b). It is important to note here that the higher expression of VLDLR and CD36 has already been reported in the white muscle of the F line, identifying them as relevant molecular markers for lipid uptake and fat deposition (Kolditz et al., 2010). On the whole, all the coherent suppressions/alterations in lipid and glucose metabolism clearly indicate that the genotypic response of these trout lines is strongly dependent on the fatty acid composition of the diet.

Limitations of the thesis

1. We analysed the regulation of all the target enzymes/proteins only at the transcriptional level. Although regulation of transcription initiation is known to be the most important form of gene control in eukaryotes, a growing body of evidence suggests that there can be a substantial role for regulatory processes after the mRNA has been made (Adeli, 2011; Vogel and Marcotte, 2012). We understand that the expression of biologically active proteins is controlled at multiple points such as the changes in chromatin structure, initiation, processing and modification of mRNA transcripts, transport of mRNA into cytoplasm, stability/decay of mRNA transcripts, initiation and elongation of mRNA translation, co/post-translational modification, and intracellular transport and degradation of the expressed protein. Therefore, we admit that the difference in mRNA levels as proxies for the concentrations/activities of the corresponding proteins may not always reflect the functional biological process. However, the mechanism by which animals adapt to their nutritional environment is mostly through regulation of gene expression by nutrients (Girard et al., 1997). Further, our results facilitate the understanding of inherent genetic difference between the two trout lines and in most case, the gene expression data was systematically associated to phenotypic data.

2. We lack a complete picture of starch use in both lines. Because: (a) We did not measure the plasma levels of insulin and glucagon, density of their receptors in the peripheral tissues and also their signalling pathway (except for Akt), which could have provided a detailed insight into possible genotypic difference in the hormonal response to starch intake and the downstream regulatory pattern (b) We did not analyse the changes in the glucosensing components of peripheral Brockmann bodies (pancreatic islets) and central hypothalamus (brain), which plays a critical role in glucose homeostasis (Polakof et al., 2011a). Nevertheless, we maximised the scale of investigation with digestion and intestinal nutrient transport, intermediary metabolism in the liver, glucose metabolism in the white muscle and perivisceral adipose tissue, plasma metabolites and overall growth and nutrient retention. Thus the study involves all the main tissues involved in nutrient use.

3. Protein intake was not the same between the two dietary groups. The fish group that was fed the diet with starch ate less protein than those fed the diet without starch. The two alternate options that we had to keep protein intake constant when the starch intake varied was: (a) adopting a pair feeding strategy (b) incorporation of bulk filler agents such as cellulose in the diet without starch. However, opting for pair feeding by manipulating the

feed ration size will not be conducive for the comparison of the ultimate phenotypes such as growth, nutrient retention and tissue fatty acid content between the different dietary groups. On the other hand, filler agents (cellulose) even at 10% inclusion level are shown to depress growth in long term salmonid feeding trials (Hilton et al., 1983). Therefore, the plausible option for us was to consider that the effects of absolute difference in dietary starch inclusion (0 and 20%) outweigh the effects of the relative difference in dietary protein levels, always provided above the optimum protein requirement of rainbow trout (38% digestible protein). Our results have been affirmative to this effect.

4. Finally, we did not have a comparative control group with non-selected fish, which could have highlighted the uniqueness of the two lines. However the aim of the thesis, which was to identify the putative differences in the utilisation of dietary carbohydrates between the selected fish lines (fat and lean), was fulfilled.

Chapter 5

Conclusions and Perspectives

The present study permits the following conclusions:

1. In both lines, dietary intake of starch did not lead to an enhanced expression of intestinal glucose transporters. Nevertheless, the apparent digestibility coefficients of starch and other macronutrients were high in both lines, but not significantly different between them.

2. Despite the higher potential of the F line to convert excess glucose from dietary starch into lipids and glycogen in the liver, there was no evidence for a better regulation of postprandial glucose homeostasis. The possible reasons could be (a) the concurrent higher expression of hepatic glycolytic and gluconeogenic enzymes in the F line fed dietary starch as compared to the L line and (b) the absence of adaptive metabolic response to dietary starch in muscle and adipose tissue, suggesting poor use/disposal of glucose in the peripheral tissues.

3. The few diet × genotype interactions observed at the metabolic level (expression of GK, G6PD, D9D in the liver and PK in adipose tissue) endorses our hypothesis that the F line has a higher ability to metabolise dietary carbohydrates than the L line, but are insufficient to completely validate it (because no such interactions were found at the phenotypic level).

4. In both lines, we demonstrate for the first time a unique cellular response linked to dietary starch intake characterised by a decrease in the phosphorylation of AMPK and an increase in the phosphorylation of S6, suggesting its contribution to cellular energy needs in the liver.

5. Selection for high muscle fat content was apparently correlated to a higher disposition of chylomicron synthesis in the intestine and fatty acid bioconversion (n-3 LC-PUFA synthesis) in the liver and intestine, as shown by the expression of key proteins and enzymes involved, when fed a diet that contains fish oil.

6. Regardless of the genetic background, starch intake when coupled with low dietary content of n-3 LC-PUFA (vegetable oil) stimulated the expression of key desaturase and elongase enzymes of the fish, apparently mediated by the transcription factor PPARγ. However, this did not result in a corresponding augmentation of the final n-3 LC-PUFA content of the flesh.

7. Under the vegetable oil based dietary regime, the starch intake induced *de novo* lipogenesis was impaired and was further linked to a disturbed lipid metabolism (at the molecular level).

8. Under the vegetable oil based dietary regime, the genotypic differences that we previously observed in glucose (glycolysis and gluconeogenesis) and lipid metabolism (fatty acid synthesis/bioconversion and chylomicron assembly) either got altered or disappeared.

Perspectives to improve dietary carbohydrate use in carnivorous fish

Selection and propagation of carnivorous fish genotypes that can adapt better to dietary changes involving digestible carbohydrates and plant based ingredients, by exploiting the large genetic variability and phenotypic plasticity found in fish populations, could be the way forward in the evolving aquaculture scenario. For instance, selection for the ability to adapt to a totally plant-based diet has been proven to be successful in rainbow trout, at the first generation (Le Boucher et al., 2012). In this context, the major questions that arise from the present study are (1) The feasibility of selecting carnivorous fish based on their ability to metabolise dietary carbohydrates (2) If yes, what could be the selection criterion. The few diet × genotype interactions that we observed in hepatic intermediary metabolism and the genotypic differences in converting excess glucose to lipids and glycogen indicate that there are possibilities of genotypic variation in metabolising dietary starch in rainbow trout. In case of direct selection, blood glucose clearance after a high carbohydrate meal can be used as an invasive but non-destructive selection criterion, given that all the tested fish are made to consume equal amount of feed. Similar selection has already been done in sheep (Francis et al., 1994), using blood glucose clearance (fast or slow) after an intravenous glucose tolerance test (GTT) as the selection criterion. The progeny differed significantly in glucose clearance and average heritability estimate was 0.10. The 'fast' clearing line had higher plasma insulin concentrations during GTT and more sub-cutaneous fat than the 'slow' line, indicating differential partitioning of nutrients into adipose tissue. It is worthy to note here again that selectively bred fast growing families of rainbow trout was found to have higher plasma insulin levels than their slow growing counterparts (Sundby et al., 1991). This could be possibly due to a better use of carbohydrates as energy source and subsequent protein sparing, but unfortunately it was not tested. In this promising milieu, when the objective is to genetically select carnivorous fish for better utilisation of dietary carbohydrates, **we propose that blood glucose clearance response after consuming high starch diets would be a more reliable selection criterion rather than after glucose injection (GTT)**. If any such selection attempt turns out positive and heritable, it could ultimately benefit aquaculture. Another possibility is indirect selection, based on better protein sparing and growth when fed high starch diets. Though this approach was previously reported not to be very promising on a family based response (Austreng et al., 1977; Edwards et al., 1977), tagging individuals as Le Boucher et al. (2013) did and using individual traits to select could be possibly an effective strategy to overcome the previous constraints.

In the present study, changing the dietary lipid source from fish oil to vegetable oil had a major influence on the effect of carbohydrates on hepatic intermediary metabolism as well as the direction of genotypic response, which emphasizes the **critical need to consider the significance of macro-nutrient interface on carbohydrate utilisation**, especially while studying nutrient × genotype interactions. Finding a fine balance between the different macro-nutrients thus probably holds the key for better use of carbohydrates for protein sparing in carnivorous fish, without causing any disturbance to the animal's physiology. For this purpose, it is important to improve and integrate our understanding on the diet related biological network properties such as metabolic changes in the tissues implicated in glucose use (liver, muscle, intestine and adipose tissue), alterations in hormonal response, nutrient signalling pathways and glucose sensing in the central and peripheral tissues (brain and pancreas). In the future, creating genome scale metabolic network reconstructions maybe will help to improve diet formulations with substantial carbohydrate inclusion.

Nutritional or metabolic programming is another interesting application prospect to improve carbohydrate utilisation in carnivorous fish. The principle is that, dietary influences exerted at critical developmental stages in early life may have long-term consequences on physiological functions in later life (Lucas, 1998; Patel and Srinivasan, 2002). This has been previously tested in rainbow trout by applying a short, strong hyperglucidic stimulus in early life (Geurden et al., 2007). The programming concept was successful for digestive enzymes (induced higher expression of amylase and maltase during a challenge test in juveniles), but not for glucose transport or metabolism. The duration, source and intensity/level of the hyperglucidic stimulus and the point of application i.e. the critical transition window in which the stimulus is applied are key factors that possibly determines the success of the concept. Recently, micro-injection of glucose into fertilised zebra fish eggs during specific stages of embryonic development was carried out to investigate the programming effect (Rocha et al. unpublished). Glucose metabolism was altered in early larval stages of the injected embryos, however it was not prominent. Alternatively, modifying broodstock diets using quasi-vitamins (myoinositol) and trace elements (chromium) that are insulin sensitizers may produce a vertical carryover effect of better adaptation/modified glucose metabolism in offsprings. Apart from the stimuli and stage of application, it is important to understand the possible biological mechanisms for storing the nutritional programming event until adulthood such as adaptive changes in gene expression (e.g. epigenetic phenomenon).

Throughout the history of life, eukaryotes have acquired novel metabolic capacities by establishing symbiotic relationships with prokaryotes. For instance, a number of metabolic capacities encoded in the human gut metagenome are absent in hosts (Karasov et al., 2011). This makes us curious about the possibility to enhance carbohydrate utilisation in carnivorous fish by **altering the quantity and characteristic of gastro-intestinal microbiome**. The basic trial could include the use of prebiotics (indigestible oligosaccharides) to re-orientate the intestinal microbiota, thereby creating eubiosis. Following which, the metagenome can be analysed for prospective higher carbohydrate metabolic capacity. Later on, given the fact that the intestinal tract of carnivorous fish is devoid of amylolytic microbiota (Ray et al., 2012), it could be interesting to transplant the inherent gut microbiota of a herbivorous fish to the gut of a carnivorous fish and investigate the overall effect in carbohydrate utilisation, though there are constraints such as short intestinal tract and lack of place for fermentation.

In a more personal perspective, commercial culture of carnivorous fish in India is only in formative years with pilot scale production of Asian sea bass (in brackish water ponds) and cobia (in marine cages). Minor quantities of indigenous carnivorous fish such as snakeheads/murrels (family: Channidae) or stinging catfish (*Heteropneustes fossilis*) are produced by extensive farming without using formulated feeds. Nutritional aspects for the indigenous fish are yet to be standardised in clear terms. However, based on their documented feeding habits and nutrient requirements (Rao et al., 1998; Arockiaraj et al., 1999), they appear to be distinct **new carnivorous fish models suited to study carbohydrate metabolism and use.** Given the extreme physiological diversity in teleosts, comparative metabolic studies using the indigenous warm water fish of India belonging to different trophic levels (e.g. carp *Labeo rohita vs.* murrel *Channa striatus*) may possibly provide hitherto unknown insights on carbohydrate metabolism in fish.

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Résumé de la thèse:

L'objectif de la thèse était d'identifier s'il existait des différences d'utilisation digestive et métabolique des glucides alimentaires entre deux lignes de truite sélectionnées pour leur teneur divergente en lipides intramusculaires: la lignée maigre (L) à faible taux de lipides dans le muscle et la lignée grasse (F) à teneur élevée de lipides musculaires. Dans ce but les deux lignées ont été nourries avec deux aliments, un contenant des glucides alimentaires (20% d'amidon), l'autre ne contenant pas. Chez les deux lignées, la présence d'amidon dans les régimes n'a pas eu d'effets sur la croissance, a été associée à une épargne protéique, et a provoqué une hyperglycémie postprandiale modérée, en induisant les acteurs impliqués dans la signalisation cellulaire (TOR/AMPK). Aucune différence entre les deux lignées n'a été observée pour la digestion de l'amidon, le niveau d'expression des gènes codant les transporteurs de glucose dans l'intestin, la glycémie postprandiale et l'utilisation périphérique du glucose (muscle et tissu adipeux). Par contre, la lignée F se caractérise par une croissance et une efficacité alimentaire plus faibles, des capacités plus élevées de stockage du glucose sous forme de glycogène hépatiques et de lipogenèse (sans impact sur l'utilisation des glucides) et un métabolisme lipidique spécifique (plus faible catabolisme des acides gras, plus fortes capacités à synthétiser des chylomicrons et à bio-convertir des acides gras). Dans une seconde étude, le remplacement de l'huile de poisson de l'aliment par un mélange d'huiles végétales a supprimé la plupart des différences moléculaires observées précédemment entre les deux génotypes. Toutefois, l'apport de glucides alimentaires a alors entrainé des modifications métaboliques au niveau intestinal et hépatique avec, par exemple, l'induction des capacités moléculaires de bioconversions des acides gras. Globalement, nos données ont donc démontré qu'il existait des différences métaboliques importantes entre les deux lignées mais que cela n'aboutissait pas à une meilleure utilisation des glucides alimentaires. En outre, nos travaux soulignent l'importance de la composition des aliments dans les réponses' des différents génotypes de poissons.

Summary of the thesis:

The aim of the thesis was to characterise the differences in digestive and metabolic utilisation of dietary carbohydrates between two lines of rainbow trout divergently selected for muscle fat content (Fat-F and Lean-L), when fed diets with (20%) or without gelatinised starch. In both lines, starch intake did not adversely affect growth, resulted in a moderate postprandial hyperglycemia, enhanced protein/lipid retention and a distinct intracellular signalling pattern in the liver involving the energy sensor AMPK and nutrient sensor TOR-S6. No difference between the two lines was observed for the apparent digestibility of starch, the mRNA levels of genes encoding glucose transporters in the intestine, regulation of postprandial glycemia and utilisation of glucose in the peripheral tissues (muscle and adipose tissue). However when compared to the L line, the F line was characterised by lower growth and feed efficiency; better capacity to store excess glucose as indicated by higher glycogen levels and mRNA levels of lipogenic markers in the liver (with no impact on glucose homeostasis); and a specific lipid metabolism (a greater potential to synthesise chylomicrons and to bioconvert fatty acids, coupled with a lower potential to oxidise fatty acids). In a separate study, replacement of fish oil in the diet with a blend of vegetable oils was found to suppress most of the molecular differences previously observed between the two genotypes. Starch intake under the vegetable oil diet regime led to different metabolic changes in the intestine and liver, for example, elicited a higher transcriptional response of key desaturase and elongase enzymes in both lines. Overall, our data demonstrated the existence of significant metabolic differences between the two trout lines, but it did not lead to better utilisation of dietary carbohydrates. In addition, our work highlights the importance of diet composition in the growth and metabolic response of different genotypes of fish.