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Metabolomics and fish nutrition: a review in the context of sustainable feed development

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Abstract

Aquaculture is facing a strategic challenge to improve feed suitability and support the global increase in fish production. Improvements in diet formulation for sustainable nutritional strategies have focused to date on the partial substitution of marine resources by plant resources but will now include other alternative feed-stuffs. Growth trials and body composition data provide valuable indicators of fish nutritional status, while omics technologies may contribute to a better understanding of fish nutrition and help to demonstrate how feed and nutrients act in fish metabolism. Metabolomic approaches give an insight into fish metabolism through a non-targeted analysis of metabolites in tissues or biofluids that involve multiple factors affecting fish, such as nutrition. In this review, we highlight the outcomes of publications in metabolomics applied to fish nutrition. We explain the concept of metabolomics and discuss specific technical considerations related to sample type, sampling and sample preparation. We show how metabolomic studies help to elucidate the impact of nutrition on fish fillet composition and fish metabolism. Finally, we describe the potential applications of metabolomic approaches for the non-invasive monitoring of fish nutritional status.

Key words: aquaculture, fish nutrition, metabolomics, sampling, sustainable feed.

Introduction

Aquaculture nutrition has continued to evolve over the last two decades with continuous innovation in feed formulation to improve feed efficiency and sustainability. Nutrition of fish with high trophic levels such as salmonids and marine species still relies on fish meal (FM) and fish oil (FO), because these resources adequately cover the nutritional requirements of fish. Diets based on FM and FO have the closest nutritional composition to natural prey consumed by wild fish. Indeed, FM provides high quality and highly digestible proteins with an adequate essential amino acid profile, and the lipid profile of FO meets the essential fatty acid requirements for most fish species, especially marine ones (Guillaume *et al.* 1999; National Research Council (U.S.) 2011; Oliva-Teles *et al.* 2015).

World fishery production levels, including forage fisheries devoted to FM and FO supply, are expected to remain

relatively steady because of fishery quotas, while aquaculture production and other markets for human nutrition, pharmaceuticals and cosmetics using these raw materials are constantly rising, leading to a scarcity of marine resources for aquaculture (Tacon & Metian 2008; Naylor *et al.* 2009; Troell *et al.* 2014; FAO 2016). Thus, the market value of FM and FO in the next few years will no longer be sustainable for the aquaculture industry. Moreover, systematic and non-systematic climatic events, such as El Niño, that affect forage fishery supply will increase the price volatility of FM and FO (Ñiquen & Bouchon 2004; Tacon *et al.* 2011; Troell *et al.* 2014). These price fluctuations will not be absorbed in the same way by all regions in the world, hence curbing the development of aquaculture in emerging countries and compromising small aquaculture businesses.

These constraints call for replacing the FM and FO in fish feed by more sustainable raw materials. Many improvements in terms of formulation of sustainable diets have

been achieved in the last decades through the incorporation of plant ingredients to replace marine ones. Feed formulations from the previous decade incorporated high amounts of FM and FO from pelagic fish strongly impacted by global environmental pollution (Hites *et al.* 2004). With the increase in plant resources in fish feeds, persistent organic pollutants have decreased in both feed and fish. Nevertheless, new pollutants have appeared such as polycyclic aromatic hydrocarbons (Berntssen *et al.* 2015). Moreover, the use of these resources is limited by several drawbacks for fish nutrition. Plant protein feedstuffs are usually characterized by unbalanced essential amino acid profiles and contain numerous anti-nutritional factors (Hardy 2010; Collins *et al.* 2013; Burel & Médale 2014; Hixson 2014; Oliva-Teles *et al.* 2015). The supply of essential fatty acids is the main critical factor of FO substitution. Indeed, because of some limiting enzymatic capacities, most marine species cannot synthesize long-chain poly-unsaturated fatty acids (LC-PUFA) from the dietary poly-unsaturated fatty acids (PUFA) linolenic and linoleic acid (Tocher 2003; Bell & Koppe 2011; National Research Council (U.S.) 2011). Moreover, the substitution by vegetable oils alter the nutritional quality of fish flesh through a decrease in DHA and EPA content although the other factors of fish flesh quality are not depreciated (Oliva-Teles *et al.* 2015). Currently, the remaining proportion of FM and FO to be substituted in fish feed for carnivorous and especially marine species is only about 10–15% for each of the raw materials (Médale *et al.* 2013; Ytrestøyl *et al.* 2015). Thus, the total substitution of fish resources in fish feed is complex for carnivorous species and previous trials have led to reduced growth performances and metabolic alterations that are not compatible with aquaculture production (Geay *et al.* 2011; Collins *et al.* 2013). New feedstuffs should therefore be included in fish feeds to compensate for these drawbacks, but their precise characterization is essential to fully understand their effects on fish metabolism and their suitability for optimal fish growth.

The conventional approach to evaluate new feed formulations usually consists in characterizing the analytical composition and the digestibility of new feeds and then assessing their effects on fish growth performances, feed consumption and other zootechnical parameters. However, these methods might be insufficient to understand the consequence of feeds on fish metabolism. Proximate chemical analysis of fish feeds gives a rough global composition but does not provide information on the profile of small compounds such as non-essential nutrients like taurine and creatine and other nutritional factors as well as numerous anti-nutritional factors such as polyphenols, phytic acid or mycotoxins present in plant feedstuff (Glencross *et al.* 2007). The complete analysis of each of these compounds and factors requires the implementation of numerous and

costly analytical approaches. For example, the analysis of protein content is based on methods for quantifying global nitrogen such as the Kjeldahl method and do not differentiate protein-based nitrogen from non-protein-based nitrogen such as nucleotides. Moreover, the determination of protein content can be fraudulently modified by adding urea or melanin (Jobling 2016). Diet quality also poses another problem since it is assessed in terms of digestibility, either by direct or indirect methods of measurement (Houlihan *et al.* 2001; Glencross *et al.* 2007). In the direct method, measuring the exact amount of feed consumed and collecting all faeces are a huge challenge since leaching can occur quickly in water, leading to large underestimation and hence the misinterpretation of digestibility. The indirect method is based on the quantitative measurement of an inert marker added to the diet. The choice of an appropriate marker is critical as its interactions with nutrients can influence digestibility and even metabolism (Vandenberg & La 2001). Indeed, it has been shown that the common marker chromic oxide may disturb digestive function and has carcinogenic properties (Houlihan *et al.* 2001), which is also probably the case for an alternative such as yttrium oxide. Lastly, growth trials with monitoring of zootechnical parameters and assessment of body composition provide classical indicators of fish condition such as the hepatosomatic index and Fulton's K index, which express fish morphology, feed conversion ratio and carcass composition. They help to demonstrate the main impact of feedstuffs and feeds on fish growth, but do not show how feed and nutrients act on the stimulation or alteration of fish metabolism. Thus, novel methods are needed to further characterize the metabolic modulation that explains feed suitability.

'Omics' technologies enable a new holistic vision of a biological system at the level of genes, transcripts, proteins and metabolites. Nutrigenomics approaches, that is, the relationship between nutrients and targeted gene expression, have become more and more important over the years and have led to new findings including the regulation of genes involved in protein, lipid and carbohydrate metabolism in fish fed plant-based diets (Panserat *et al.* 2009; Geay *et al.* 2011). However, nutrigenomics shares limitations with the transcriptomic approaches. As post-transcriptional modifications or protein activities are not investigated, what truly happens remains partly unknown. On the contrary, metabolomics focuses on the global set of metabolites in a biological system and could provide information about metabolic activities. Combining a feeding trial with metabolomic analyses of tissues and biofluids could thus provide new insights into feed and nutrient effects. Metabolomic studies for nutrition were initially developed for human applications but have recently been extended to livestock animals (Wishart 2008; Brennan 2013; Goldansaz

et al. 2017). In fish, metabolomic research has been reviewed by Samuelsson and Larsson (2008) and Alfaro and Young (2018) who identified different fields of study: environmental metabolomics, metabolomics for health monitoring and metabolomics for fish nutrition.

In this review, we analyse the issues and outcomes of the first set of publications in metabolomics for fish nutrition (Table 1). For a general description of the analytical tools used in metabolomics such as mass spectrometry (MS), nuclear magnetic resonance (NMR), Fourier transform infrared spectroscopy and others, please refer to Young and Alfaro (2018) who have listed the advantages and disadvantages of each technique in the context of aquaculture. First, we explain the concept of metabolomics and describe the technical issues, especially related to samples for fish nutrition. Then, we show how metabolomic studies help to characterize the impact of fish nutrition on fillet composition and to identify the main underlying metabolic mechanisms of how fish adapt to new feeds. Finally, we discuss the potential applications for the non-invasive assessment of fish nutritional status.

Metabolomics: a global view of metabolism subject to specific sample considerations

Metab-'holistic'

Metabolomics is the non-selective chemical analysis of metabolic products (metabolites) and/or contaminants from an external origin (xenobiotics) in a given biological system, that is, cells, biofluid(s), tissue(s). It relies on the high-throughput detection of small compounds (molecular weight < 1500 Da) with or without identification and their absolute or relative quantitation (Wishart 2008; Chin & Slupsky 2013). Metabolites belong to several classes of compounds such as organic acids, carbohydrates, amino acids, fatty acids, nucleosides/nucleotides, steroids and steroid derivatives, terpenoids, carotenoids or flavonoids. In contrast to targeted approaches, the holistic nature of metabolomics helps to investigate the complexity of a biological system responding to direct exposure to an environmental condition such as nutrition (Brennan *et al.* 2015; Sébédio 2017; Alfaro & Young 2018).

Two common metabolomic approaches can be used to extract pertinent information from biological systems: fingerprinting and profiling usually using NMR and MS (Goodacre *et al.* 2004; Dunn 2005). Fingerprinting is based on the high-throughput analysis of a large set of individuals to build a model based on all analytical variables generated from metabolite detection. It is generally used to assess the impact of xenobiotics or environmental stressors including some farming-related practices, but it is also highly relevant in food or feed authentication. Signal identification is not mandatory in this case as this approach is focused on the

classification of individuals. Profiling is based on the identification and/or quantitation of analytical variables generated from the non-selective analysis of the biological system. Identification of metabolites provides qualitative information on metabolic pathways impacted by internal or external factors. For example, profiling has been used to characterize gut content after a switch from natural diet to manufactured diet in convict grouper (*Epinephelus septemfasciatus*) (Asakura *et al.* 2014). Fingerprinting and profiling are complementary approaches to understand the impact of environmental factors on metabolism. Both approaches have been combined to identify nutritional biomarkers in fasted gilthead sea bream (*Sparus aurata*) and the corresponding metabolic adaptation (Gil-Solsona *et al.* 2017).

NMR and MS are the main analytical platforms used in metabolomics for fish nutritional studies. Although these two analytical strategies are highly complementary in terms of selectivity, sensitivity and type of structural information provided for compound identification, their combination remains rare. Therefore, the choice of an analytical platform should be adapted to the research question. NMR can be relevant in a fish nutritional trial such as a challenge where the comparison of conditions often relies on fingerprinting of several individuals as done by Kullgren *et al.* (2010). Indeed, $^1\text{H-NMR}$ is not selective as the detection of metabolites is based on the presence of non-exchangeable ^1H in their structure. Typically, several dozens of metabolites can be detected by $^1\text{H-NMR}$ up to 200 for human urine (Emwas 2015). Moreover, minimal sample preparation is required; fish muscle can even be analysed intact thanks to magic angle spinning (MAS) that allows revealing the most intense metabolite features, and native fish plasma can be analysed with water suppression sequences (Goodacre *et al.* 2004; Jarak *et al.* 2018). For substitution or supplementation trials, both NMR and MS can be relevant. On the one hand, metabolite identification is easier and profile reproducibility across laboratory is better with NMR than with liquid chromatography (LC)- or gas chromatography (GC)-MS, so results from previous studies can be compared with higher confidence (Ward *et al.* 2010). On the other hand, MS offers much higher sensitivity with the possibility to detect small metabolite amounts (pM to fM compared to μM by $^1\text{H-NMR}$) and reveals consequently a higher number of metabolites present in a given fish extract or biofluid. For LC-MS for instance, the higher sensitivity of MS leads to about 12,500 molecular features (m/z ions), corresponding to much less potential metabolites, here 55 discriminant identified metabolites (Gil-Solsona *et al.* 2019). Thus, the latter analytical strategy can be recommended for biomarker discovery as demonstrated by Gil-Solsona

Table 1 Set of publications related to fish nutrition assessed by metabolomics including (i) classification of trial, (ii) research topic, (iii) experimental diet, (iv) fish, (v) fish natural feeding habit, (vi) sample type, (vii) techniques employed to assess metabolite profile, (viii) type of extracts from sample and (ix) reference work

Trial	Topic	Diet	Fish	Feeding habits	Organ, biofluid	Technique used†	Extract	Reference
Challenge	Microbial and metabolite diversity of different species fed controlled diets or natural feedstuffs	Shellfish/manufactured fish feed	Grouper	Carnivorous	Faeces	NMR	Polar	Asakura et al. (2014)
	Investigate metabolism pattern of starved fish	Fasted	Rainbow trout	Carnivorous	Muscle, liver, serum	MS	Polar & non-polar	Baumgarner and Cooper (2012)
	Identify malnutrition biomarkers	Fasted	Sea bream	Carnivorous	Serum	MS	Polar & non-polar	Gil-Solsona et al. (2017)
	Classification of fish according to muscle profile	Undefined	Atlantic salmon	Carnivorous	Muscle	NMR	Polar & non-polar	Gribbestad et al. (2005)
	Polar compounds profiling of fish fed reduced protein diet fraction	Casein/Gelatin	Grass carp	Herbivorous	Liver, plasma	MS	Polar	Jin et al. (2015)
	Metabolism pattern of starved fish	Fasted	Rainbow trout	Carnivorous	Muscle, liver, plasma	NMR	Polar & non-polar	Kullgren et al. (2010)
	Discrimination of wild and cultured fish	Commercial diets	Sea bass	Mostly carnivorous	Muscle, skin	NMR	Polar & non-polar	Mannina et al. (2008)
	Effects of pellet waste from fish farm on wild fish	Commercial diets	Saithe	Carnivorous	Muscle, liver	NMR	Polar	Maruhenda Egea et al. (2015)
	Fasting/feeding comparison in relationship with circadian cycle	Fasted	Leopard coral grouper	Carnivorous	Muscle	NMR	Polar	Mekuchi et al. (2017)
	Impact of imbalanced diet on fish metabolism	High carbohydrate diets and high fat diets	Wuchang bream	Herbivorous	Liver, plasma	NMR	Polar	Prathomya et al. (2017)
Substitution	Relationship between fish mucus metabolome, fish life history and the environment	Corallivorous vs omnivorous	Butterflyfish	Corallivorous & omnivorous	Mucus	MS	Polar & non-polar	Reverter et al. (2017)
	Classification of fish origin	Undefined	Sea bream	Carnivorous	Muscle	NMR	Non-polar	Rezzi et al. (2007)
	Fish meal replacement by fungal material	Zygomycete	Arctic char	Carnivorous	Liver	NMR	Polar	Abro et al. (2014)
	Fish meal replacement by fungal material	Zygomycete	Atlantic salmon	Carnivorous	Muscle, liver	NMR	Polar & non-polar	Bankfors et al. (2011)
	Effects of substituted diets by cereal	Tritical	Common carp	Omnivorous	Muscle	MS	Polar & non-polar	Cajka et al. (2013)
	Effect of soybean-based protein	Soy-based products	Red drum	Carnivorous	Muscle, liver, plasma	NMR	Polar	Casu et al. (2017)
	Decontaminated fish resources effects on flesh profile and metabolism	Fish meal and fish oil	Arctic char	Carnivorous	Muscle, liver	NMR	Polar	Cheng et al. (2016a)
	Decontaminated fish resources effects on flesh profile and metabolism	Fish meal, fish oil, yeast and mussel	Arctic char	Carnivorous	Muscle, liver, plasma	NMR	Polar	Cheng et al. (2017)

Table 1 (continued)

Trial	Topic	Diet	Fish	Feeding habits	Organ, biofluid	Technique used†	Extract	Reference
	Effects of reduced fish meal and fish oil diets on serum fingerprint	Soy protein, corn gluten, rapeseed and palm oils	Gilthead sea bream	Mostly carnivorous	Serum	MS	Polar & non-polar	Gil-Solsona <i>et al.</i> (2019)
	Assessment of muscle profile in response to dietary carbohydrate on intact and extracted tissue	Gelatinized and raw starch	Sea bass	Mostly carnivorous	Muscle	NMR	Polar & non-polar	Jarak <i>et al.</i> (2018)
	Classification according to different rearing conditions	Blood and plant products	Gilthead sea bream	Mostly carnivorous	Muscle	NMR	Polar & non-polar	Mellis <i>et al.</i> (2014)
	Assess fish health by decreasing fish meal fraction	Fish meal, poultry meal, soy protein	Cobia	Carnivorous	Serum	NMR	Polar	Schock <i>et al.</i> (2012)
	Impact of feather meal as protein source	Feather meal	Rainbow trout	Carnivorous	Liver, plasma	NMR	Polar	Jasour <i>et al.</i> (2017)
	Improve disease resistance by nutrition	High valuable resources (high content of fish derivate products) vs Low	Sea bream	Carnivorous	Liver	IR	Polar & non-polar	Silva <i>et al.</i> (2014)
	Metabolic profiling of fish fed fish meal-based diet, diets including size-fractionated fish protein hydrolysate et plant protein-based diet	Fish protein hydrolysate and plant proteins	Turbot	Carnivorous	Muscle, liver	NMR	Polar	Wei <i>et al.</i> (2017a)
	Impacts of ultrafiltered fish protein hydrolysate in plant-based diets on metabolism	Ultrafiltered fish protein hydrolysate	Turbot	Carnivorous	Muscle, liver	NMR	Polar	Wei <i>et al.</i> (2017b)
Substitution and/or Supplementmentation	Impact of bioactive compound supplementation on fish metabolism	Sesamin	Atlantic salmon	Carnivorous	Muscle, liver	NMR	Polar & non-polar	Wagner <i>et al.</i> (2014)
	Understanding additive effect on metabolic pathways	Butyrate	Sea bream	Carnivorous	Gut content	MS	Polar	Robles <i>et al.</i> (2013)
	Taurine supplementation	Taurine	Nile tilapia	Herbivorous	Muscle	NMR	Polar	Shen <i>et al.</i> , (2018)
Undefined	Flesh profiling according to different rearing conditions	Undefined	Gilthead sea bream	Mostly carnivorous	Muscle	NMR	Polar	Picone <i>et al.</i> (2011)
	Environmental impact assessment including feeding habits	Undefined	Goby, sea bass	Multiple	Muscle, liver, other	NMR, ICP-MS	Polar & non-polar	Yoshida <i>et al.</i> (2014)

†NMR, nuclear magnetic resonance; MS, mass spectrometry, IR, infrared spectroscopy, ICP-MS, inductive coupled plasma mass spectrometry.

et al. (2017) or to focus on a selected fraction of metabolism. MS is often coupled to several selective chromatography separations to cover a broad part of metabolome, from volatile metabolites with GC, non-polar and semi-polar metabolites with reverse-phase LC or after derivatization with GC-MS (Baumgarner & Cooper 2012), to polar metabolites with hydrophilic interaction liquid chromatography (HILIC) columns. Multiple reaction monitoring in tandem mass spectrometry can also be recommended to target specific compounds such as vitamins as done by Gil-Solsona *et al.* (2019). However, feature identification and biological interpretation may prove difficult due to the lack of dedicated metabolite database for fish. For details about molecular feature numbers and identification in a largely studied biofluid, human NIST plasma, using NMR and MS, see Simón-Manso *et al.* (2013).

The interpretation of fish metabolomics data still requires a specific database about fish metabolism. Existing metabolomic or metabolism databases such as the Human Metabolome DataBase (HMDB, www.hmdb.ca) or HumanCyc (humancyc.org) provide valuable information about the metabolic pathways and functions of a given metabolite. These information are however based on human data. Specific databases dedicated to animals have started to be developed such as a livestock metabolome database to facilitate inter-species and intra-species comparisons (Goldansaz *et al.* 2017). It would be interesting to develop a fish metabolomic database that contains not only the reference spectra of a given metabolite in a given organ or biofluid but also the existing metadata related to its metabolism and function in the dedicated species. There are, however, comprehensive databases dedicated to the metabolic pathways of metabolites and drugs of animal, plant or bacteria such as the Kyoto Encyclopedia of Genes and Genomes (KEGG, www.genome.jp/kegg). In addition, the scientific community studying fish models such as zebrafish or medaka has already built a database of fish metabolic networks and a fish metabolic model (MetaFishNet) to help the interpretation of omics data, that is, DNA sequencing, transcriptome and proteome (Li *et al.* 2010). Information about metabolic pathway analyses have been even integrated into metabolomic software analysis to directly help interpretation of a given metabolite (Xia *et al.* 2015). Metabolomics as one of the 'omics' approaches should contribute to such fish databases about metabolic networks. This will allow fish nutrition to enlarge the existing view of fish metabolism, mainly based on gene sequences, expression and translation with data of effective metabolism.

The great advantage of metabolomics is its ability to provide a broad outlook on metabolism without preconceptions and renew our vision of metabolism. It has been

implemented in several fields in fish research to understand how fish respond to current challenges.

Metabolomics in fish research

Metabolomics in fish research focuses on the fields of environmental impact on fish, fish health and welfare and fish nutrition (Samuelsson & Larsson 2008; Alfaro & Young 2018).

Environmental metabolomics aims to evaluate how physical, chemical and biological stressors impact on wild and farmed organism metabolisms, including fish (Lankadurai *et al.* 2013). For example, investigations on exposure to pollutants have shown that a metabolomics method was more sensitive than traditional tools to assess the impact of pollutants on Japanese medaka (*Oryzias latipes*) embryos (Viant *et al.* 2005). Other metabolomic studies have investigated the effect of pesticides (Viant *et al.* 2006), endocrine-disrupting chemicals (Samuelsson *et al.* 2006), heavy metals (Santos *et al.* 2010), heavy oils (Kokushi *et al.* 2012) and cyanobacterial blooms (Sotton *et al.* 2017). The impact on fish metabolism of the environmental conditions in which fish are reared has also been assessed, like the exposure to sewage effluents (Samuelsson *et al.* 2011), the effect of temperature (Viant *et al.* 2003; Kullgren *et al.* 2013) and the effect of hypoxia (Lardon *et al.* 2013).

The second main field of metabolomic applications is fish health and welfare monitoring. This is a strategic research field to counteract extensive losses related with farmed fish diseases due to pathogen infection (Noriaki *et al.* 2014; FAO 2016). The main metabolomic applications for fish health concern host–pathogen interactions (Solanky *et al.* 2005; Guo *et al.* 2014; Ma *et al.* 2015; Peng *et al.* 2015), disease characterization (Stentiford *et al.* 2005; Southam *et al.* 2008) and treatment efficiency (Cheng *et al.* 2014; Su *et al.* 2015; Zhao *et al.* 2015). Interestingly, metabolomics approaches help to design feed formulations allowing the efficacy of treatments to be enhanced (Cheng *et al.* 2014; Ma *et al.* 2015; Su *et al.* 2015), or nutraceutical feed that prevents disease to be developed (Robles *et al.* 2013; Silva *et al.* 2014). Fish welfare studies aim to characterize the stress response of fish due to farming practices such as fish handling (Karakach *et al.* 2009) and netting (Mushtaq *et al.* 2014).

Nutrition is a recent field of research in fish metabolomics that has benefited from advances in human research. There are three fields in research on human nutrition: (i) characterization of food chemical composition, (ii) food intake monitoring and (iii) impact of food on metabolic state. Assessment of food composition by metabolomic approaches contributes to evidencing compounds involved in the sensorial properties of

food, for example, sucrose in watermelon (Tarachiwin *et al.* 2008), to identify markers of food impairment, for example, detection of adulterated olive oils, and finally to characterize food processing effects such as the extrusion process (Goodacre *et al.* 2002; Beleggia *et al.* 2011; Mannina *et al.* 2012; Chin & Slupsky 2013; Ye *et al.* 2014). Such studies have, however, not yet been implemented in fish nutrition but could be relevant to assess the characteristics of new feedstuffs. Moreover, fingerprint analyses of food samples are now used to discriminate the origin of foodstuffs and check their integrity. These fields of research have been developed for the characterization of fish products, especially to identify the origin of fish (see chapter feeding habits and fish fillet quality), and less to demonstrate alterations of fish products. Hence, the need for food authenticity and food safety are promoting exciting metabolomics research in food control (Cubero-Leon *et al.* 2014). Food intake monitoring by metabolomics was designed to complement interviews and field surveys in epidemiologic studies of nutrition. It is particularly relevant to demonstrate links between the occurrence of diseases and food consumption habits as they vary greatly in humans and remain difficult to control (Sébédio 2017). In controlled feeding as in animal breeding and aquaculture, this approach is less relevant, although it could be used to assess feeding status in individual fish in view of the wide inter-individual variability in performance generally observed. The metabolomic approach to assess the impact of food on metabolism focuses on the consequences of food habits or food composition on the organism. For example, the effect of dairy intake can be monitored in relationship with human health (Zheng *et al.* 2015). This third use of metabolomics in fish nutrition is relevant to assess the suitability of novel aquaculture feeds. Although there could be confounding factors even in controlled conditions, the quality of a given diet could be clearly established by demonstrating the impairment, maintenance or recovery of metabolic status after consumption of the diet. This will be reviewed in the chapter about dietary modulation of fish metabolism.

Samples and sampling in fish nutrition metabolomics

A classical metabolomic workflow involves five steps: sampling and analytical sample preparation, data acquisition, data processing, statistical analysis and biological interpretation (Brennan 2013; Young & Alfaro 2018). Most of these steps are common to all metabolomic studies, but sampling and analytical sample preparation require particular attention in fish nutritional studies. The choice of sample type, the sampling process and the preparation of the analytical

sample, especially from biofluids, have a strong influence on the validity of metabolomic data (Fig. 1).

A current focus is specific to integrative tissues or biofluids that offer a global insight into metabolism to assess fish performances affected by diets. Fish nutrition assessment by metabolomics has mainly concerned muscle and, to a lesser extent, the liver and plasma (Fig. 2). Muscle is the main tissue of the organism, and muscle and organs such as the liver provide a snapshot picture of metabolism and metabolic pathways impacted by feed. Blood mainly transports circulating compounds that come from digestion of feed and from fish metabolism. Muscle is usually sampled in fish as dorsal muscle without skin. However, skeletal muscle is a complex tissue which comprises a deep fast white muscle and a superficial red slow oxidative muscle with completely different metabolic activities regarding their anaerobic and glycolytic metabolism, resulting in different metabolite profiles (George & Don Stevens 1978; Kiessling *et al.* 2006). Depending on the aim of the research, skeletal muscle, which is the edible part of the fish, is sampled for fish quality studies while deep white muscle is sampled for fish nutrition and metabolism studies. Regarding the liver, no distinction is usually made between the parts of the organ when sampled. Depending on the fish species, the liver is not homogeneous, although a recent study by Cheng *et al.* (2016b) revealed heterogeneity in metabolite profiles from different quarters of Arctic char (*Salvelinus alpinus*) liver. These authors therefore advise sampling the whole liver or the same part of all fish livers to avoid bias, as was the case in the study by Abro *et al.* (2014) who systematically sampled the right side of the ventral lobe. In human and livestock species such as bovine, urine is commonly sampled to conduct metabolomic studies because it comprises end products of metabolism. Therefore, a quantitative assessment of metabolism may be made when a 24-h urine sample is collected. It provides highly significant information on the metabolic processing of food, drinks and contaminants. However, in fish, urine is not the main part of metabolic excretion as excretion of end products occurs mainly through the gills. For this reason, blood is the preferred biofluid to assess metabolism in fish. Moreover, blood sampling as a non-invasive method is a recommended practice that respects animal welfare and allows serial sampling for longitudinal studies. Faeces and mucus can be used as an alternative to monitor metabolism end products in relation to microbiota and immune functions (Asakura *et al.* 2014; Reverter *et al.* 2017).

Specific care is required when sampling in metabolomics, as sampling methods can dramatically impact the metabolic profiles of a sample and provide spurious findings. It is preferable to sample as many individuals as possible because the more individuals there are, the more powerful the statistics will be. Among the fish nutrition studies using

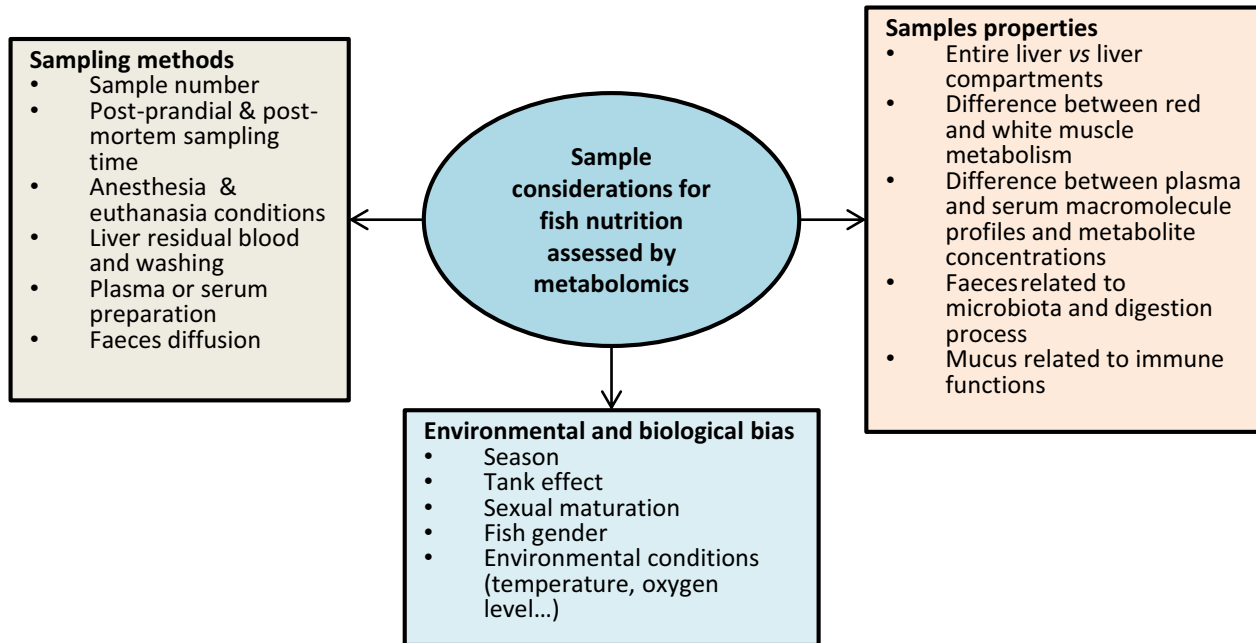


Figure 1 Considerations about samples for fish nutrition studies using a metabolomic strategy.

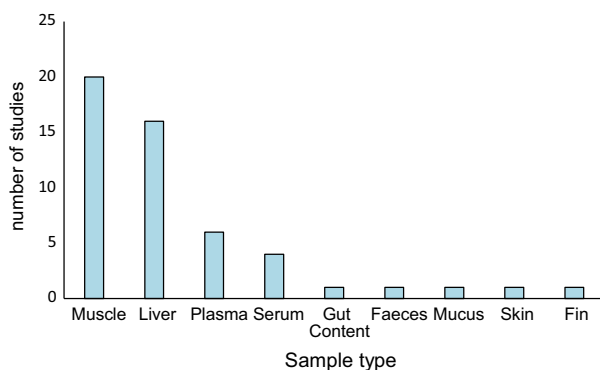


Figure 2 Frequency of tissues and biofluids sampled in metabolomic studies on fish nutrition listed in Table 1.

metabolomics and listed in Table 1, the median of fish analysed per condition tested is 12 (Fig. 3). This is relatively low in comparison to metabolomic studies in human that frequently exceed 100 individuals. However, for livestock animals reared in controlled conditions, most studies use < 30 individuals both to respect the standards of animal welfare by minimizing the number of experimental animals and also to reduce the cost of animal experiments (Goldansaz *et al.* 2017). This is almost three times more than the standard practice in fish, even though a wide individual variability in metabolism is generally observed. Interestingly, some authors compensate the low number of fish per treatment by collecting several samples of the same

tissue (Mannina *et al.* 2008). Metabolomics for fish nutrition is probably in its early stages by aiming to demonstrate the feasibility of metabolomics and to study large feeding effects. Moreover, the sample sizes used in fish studies may simply have been transposed from previous studies on the control of the origin and quality of fish based on the composition of lipids, which is relatively stable and reproducible. Moreover, metabolic processes occurring both in live fish, for example, due to circadian rhythm, and in dead fish due to numerous post-mortem alterations are dynamics. Thus, it is recommended to have precise control over external factors such as temperature and oxygen that affect the dynamics of metabolites during rearing and fishing. Metabolomics is a time-sensitive technique and we suggest carefully considering sampling time relative to the last feeding, which can radically affect the sample characteristics during the post-prandial period. Short post-prandial sampling times are suitable for studying the feed metabolome (compounds originating from feed digestion), while long times are more suitable for studying fish metabolism. The post-prandial sampling time should be adapted to several factors affecting the gut transit time, for example, species, gut capacity, temperature and season. Post-mortem sampling time also contributes to metabolite variability. Anaerobic reactions occur in excised tissue and modify metabolite concentrations, especially phosphorylated compounds, lactate and biogenic amines (Picone *et al.* 2011). Thus, samples should be snap-frozen in liquid nitrogen immediately after slaughtering to avoid tissue degradation.

Furthermore, euthanasia may have a strong effect on tissue metabolite profiles, especially with time-consuming methods such as hypothermia. Such methods implemented in farm conditions stress fish and lead to prolonged swimming activity, inducing a rise in ATP which is further degraded in IMP (Özogul & Özogul 2004; Overmyer *et al.* 2015). Generally, experimental fish are rapidly slaughtered for animal welfare considerations and most tissue sampling in fish nutrition is conducted just after euthanasia. This is not the case, however, for wild fish caught in the natural environment. In the case of anaesthetized fish, anaesthetic compounds can also affect the concentration of several metabolites such as glucose in fish anaesthetized by benzocaine (Sneddon 2012). When liver is sampled without preparation, it is complicated to discern liver metabolites from blood residual metabolites in the subsequent metabolic analysis. This is especially important if sampling is done early after the last meal because blood is still carrying feed nutrients. In mammalian species such as mice, transfusion with saline solution can be used to remove residual blood, but this practice takes time and seems complex to set up for a metabolomic study (Ly-Verdú *et al.* 2014). In fish, rinsing liver with saline solution generally helps to reduce contaminations from blood and eventually the gall bladder (which could provide bile salts) and ensure optimal sample quality.

The volume of blood samples is another issue if a non-invasive sampling method is set up. Approximately 0.5% of total body weight (i.e., 0.5 ml for a 100 g fish) can be collected without or with low mortality (Iwama *et al.* 2011; Zang *et al.* 2015). In general, metabolomic approaches use millilitre samples of blood although microlitre samples may suffice depending on the techniques employed or the instrumental setup. This is particularly important in small organism studies (Dunn 2005). Blood sampling methods produce plasma or serum, but these resulting biofluids do not exhibit the same metabolite profile in human and this also needs to be considered in fish (Yin *et al.* 2015). Moreover, the presence of macromolecules in native plasma (mainly proteins and lipoproteins) varies depending on the post-prandial sampling time. In NMR, plasma lipoproteins interfere with the detection of small metabolites and impair direct injection of plasma in LC-MS. Consequently, specific sample preparation for LC-MS or specific acquisition sequences in $^1\text{H-NMR}$ should be used. Sample preparation for MS involves deproteinization, ultrafiltration and solvent-based extraction. Results are convincing despite important drawbacks. For example, with ultrafiltration, lipoproteins can clog the membrane and prevent the passage of small compounds (Tiziani *et al.* 2008). In NMR, specific sequences such as Carr–Purcell–Meiboom–Gill are required to reduce macromolecule signals, but these sequences also impact

small molecule signals heterogeneously and alter absolute metabolite quantification. Finally, fish serum preparation is complex regarding the control of clotting because of the presence of nucleated erythrocytes in fish blood as opposed to unnucleated erythrocytes in mammals. The clotting process, which is species specific, should be taken into account when preparing serum in fish. This is especially the case in cold water species which are reared at low temperatures and have a lower blood cell count (Witeska 2013).

Other experimental, environmental and biological factors that may affect fish metabolism should be considered and controlled more carefully. The tank effect has been shown to affect growth indices in fish, especially tanks positioned next to the main walkways (Speare *et al.* 1995). Environmental factors such as oxygen and temperature have been demonstrated to affect the fish metabolome (Kullgren *et al.* 2013; Lardon *et al.* 2013). Finally, biological factors such as sexual maturation induce changes related to decreasing muscle fat content (Melis & Anedda 2014; Zhou *et al.* 2017), and amino acid and unsaturated fatty acid profiles differ in zebrafish (*Danio rerio*) liver according to their gender (Ong *et al.* 2009).

Consequently, the control of experimental and analytical parameters is of great importance to ensure the reliability of metabolomics data. Such reliability can also be reconsidered in terms of analytical accuracy, precision, repeatability and reproducibility; however, this is beyond the scope of this review. One can argue that metabolomics only provides superficial information on an entire organism, which is not enough to fully depict the modifications induced by diets. As other omics, metabolomics represents a part of the organism interactions, but it is more relatable to functional activities (Goodacre *et al.* 2004), including for fish. Therefore, metabolomics can be the entry point to understand metabolic functions, and its combination with other omic approaches should foster confidence in the results. Mekuichi *et al.* (2017) have, for example, used transcriptomics and metabolomics to assess the influence of circadian rhythm on nutritional state of leopard coral grouper (*Plectropomus leopardus*).

Feeding habits and fish fillet quality

Comparison between wild and farmed fish

Metabolomics fingerprinting and profiling strategies have been used separately or in association with discriminate fish on their feeding habits. This is especially to distinguish farmed from wild fish and to analyse the effect of lipid composition of feed.

An $^1\text{H-NMR}$ -based fingerprinting strategy on lipid muscle extracts successfully separated wild sea bream from farmed sea bream (Rezzi *et al.* 2007; Melis & Anedda

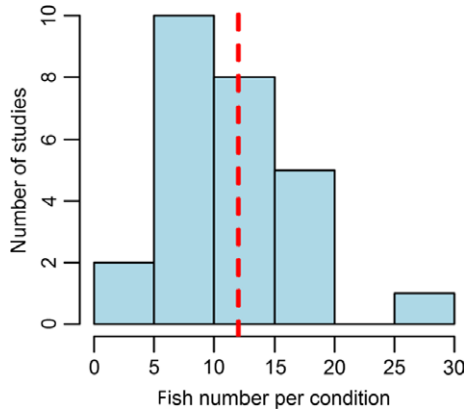


Figure 3 Frequency of number of fish per tested conditions in metabolomic studies on fish nutrition listed in Table 1. The dashed red line represents median fish number.

2014). Moreover, multivariate analysis on NMR muscle profiles partially discriminated fish geographic origins. The authors concluded that feeds were the main discriminative factors. This conclusion was supported by the different feeds distributed among the test farms. Similarly, an ^1H and ^{13}C NMR-based study on organic and aqueous extracts of muscle and skin was carried out in sea bass (*Dicentrarchus labrax*) to discriminate wild fish from farmed fish (Mannina *et al.* 2008). The multivariate analysis effectively differentiated the fish on both tissues although not all the discriminative metabolites found in muscle were found in skin, especially in aqueous extract. However, feeding habits were found to be the main discriminative factor. The manufactured feeds destined for farmed salmon but which are lost around farms also have a strong influence on the muscle and liver lipid profiles of the wild saithe (*Pollachius virens*) populations that eat the non-consumed pellets (Maruhenda Egea *et al.* 2015). This influence of a manufactured diet on wild fish was monitored even on faeces metabolite profiles of convict grouper (*Epinephelus septemfasciatus*) during the transition from natural to manufactured diet (Asakura *et al.* 2014).

It clearly appears through the discrimination of wild and farmed fish that feeding habits deeply impact fish metabolism. We investigate in the next subsection which functions are altered by manufactured diets, especially those related to lipid profile.

Fish as sources of PUFA

Fish is a healthy product for human consumption not only because of its high protein content and well-balanced amino acid protein profile but also because it constitutes a high valuable source of essential fatty acids (EFA): docosahexaenoic acid (DHA) and

eicosapentaenoic acid (EPA) (Swanson *et al.* 2012; Tacon & Metian 2013). However, owing to the inclusion of an increasing proportion of plant-based resources in fish feed, the lipid profile of farmed fish has been modified. ^1H -NMR and ^{13}C -NMR analyses were able to characterize lipid profiles of fish fillets and to identify and/or quantify valuable fatty acids well before the advent of metabolomics for nutritional studies (Sacchi *et al.* 1993; Gribbestad *et al.* 2005). Consequently, metabolomic profiling strategies have gained interest for investigating the impact of manufactured feeds on the lipid profile of fish muscle. ^1H -NMR profiles of apolar extracts of fillet of farmed sea bass fed commercial feed showed a large reduction in EFA and an increase in mono- and di-unsaturated fatty acids (MUFA and DUFA) compared to wild fish (Mannina *et al.* 2008). In this study, the contents of DHA and EPA were nearly twofold higher in wild fish muscles. A further accumulation of MUFA and DUFA was also observed in ^1H -NMR profiles in muscle of gilthead sea bream fed highly substituted FM by plant meals and vegetable oils (Melis & Anedda 2014). However, in this experiment, there was a potential interaction with other confounding factors (season combined with sexual maturation, water temperature, feed change), so the effect of diet should be interpreted with caution. Indeed, sexual maturation can be responsible for DHA depletion in muscle by its transfer into the gonads. The authors also observed a weak capacity of marine fish to synthesize LC-PUFA from dietary linolenic and linoleic acid precursors, in agreement with previous results (Tocher 2003; Bell & Koppe 2011). The substitution of marine resources concerns not only carnivorous species but also herbivorous and omnivorous ones. Muscle profiles of common carp (*Cyprinus carpio* L.) fed manufactured or natural feed also showed a reduction in PUFA coupled to an increase in saturated fatty acids (SFA) and MUFA in fish fed the manufactured diet (Cajka *et al.* 2013). These metabolomic assessments are in line with traditional approaches based on fish muscle composition after dietary challenges with plant-based diets and are related to the deficiency of EFA in these manufactured feeds (Guillou *et al.* 1995; Turchini *et al.* 2011; Anedda *et al.* 2013; Dernekbaşı *et al.* 2017).

The polar fraction can also provide information on changes in other characteristics of fish fillet quality related to the presence of specific metabolites. Polar compounds found such as trimethylamine, hypoxanthine and inosine-monophosphate can be indicators of impairment in fillet quality related to freshness of the fish (Gribbestad *et al.* 2005; Picone *et al.* 2011).

Fish fed manufactured diets with an increasing content of plant-based products have shown metabolic disturbances, as illustrated by a low PUFA content in their

muscles related to the composition of vegetable oils. Furthermore, metabolomics can also highlight other metabolic disturbances occurring in fish fed with plant-based diets and new diets based on alternative feedstuffs.

Dietary modulations of fish metabolism

What is tested?

Metabolic profiles of fish fillet are clearly impacted by feeds, suggesting that metabolic changes occur depending on diets. To evaluate these responses, the following section reports the types of studies carried out by metabolomics on experimental diets, then focuses on which key metabolic functions are modulated.

We have identified three different classes of trials for metabolomic assessment in fish nutrition studies:

- (i) challenge, when two different feeds or fish conditions (including different feeding habits) are compared to evaluate the whole dietary impact on fish metabolism. It commonly involves the assessment of manufactured diets of farmed fish compared to natural food of wild fish.
- (ii) substitution, when the experimental nutrition trial is designed to evaluate the substitution of one or more feedstuffs, most often FM and FO. The reference diet is generally a diet based on incorporation of FM or an FO.
- (iii) supplementation, when one compound/ingredient or feedstuff is added to complement or improve the feed formulation. The reference diet is generally a current commercial diet still containing a part of FM and FO and a blend of plant protein and oil sources, although some experiments use a full plant-based diet as a reference.

For the challenge experiments, diets tested in the metabolomics literature contain several formulations that obviously match species requirements and feeding habits (omnivorous, herbivorous or carnivorous) (National Research Council (U.S.) 2011). For the substitution experiments, FM and/or FO are replaced by plant-based proteins and vegetable oils, animal protein, fish-derived resources such as decontaminated FM or fish protein hydrolysates but also microorganism feedstuffs such as zygomycete biomass or baker's yeast (*Saccharomyces cerevisiae*).

Most of the experiments are based on relatively short-term feeding tests (12–20 weeks). They start to be long-term experiments where diets are tested throughout the fish life cycle, but none of them use metabolomics to assess the adaptation of fish metabolism to these diets. Moreover, metabolomics assessment is almost exclusively performed at the end of the feeding test, although very few studies to date have analysed the time course of changes during the feeding test, demonstrating trajectories of adaptation (Schock *et al.* 2012; Asakura *et al.* 2014; Casu *et al.* 2017).

Disturbances in muscle energy metabolism

The main biological function of fish muscles is mobility both for rest and burst swimming. The red slow and white fast muscle fibres are specialized to ensure these two swimming modes respectively. White muscle fibres which represent almost 80% of muscle mass are mainly recruited when fish need to burst for a few seconds only (Beamish 1978). Thus, the supply of adequate energetic substrates to muscles to ensure swimming activity is essential. Moreover, the skeletal muscle of fish which are ectothermic is characterized by a low metabolic activity compared to that of endothermic animals, especially the white muscle. Thus, the relatively low turnover of metabolites in fish muscle is helpful to discern the long-term effects of dietary variations (Yoshida *et al.* 2014).

Challenge studies, where natural food sources are compared to manufactured feeds with plant-based products, show consistent results concerning the disturbances of energy metabolism in muscle both in energy store of muscle and in the metabolites involved in the production of energy. Indeed, fumaric and malic acids, which are major intermediates of the tricarboxylic acid (TCA) cycle, were found to be upregulated in sea bass muscle extracts of fish fed a commercial diet (Mannina *et al.* 2008). Moreover, wild saithe populations surrounding salmon farms which had consumed lost pellets presented higher lactate contents in muscle extracts (Maruhenda Egea *et al.* 2015). Lactate is the main end-product of anaerobic energy metabolism and this suggests a disturbance in the energy status of muscle due to diet, although an interaction with the effect of fishing and storage of wild fish in different conditions could be suspected. Lactate production can be due to several factors. Lactate is a good marker of fish activity because burst swimming increases lactate content in muscle (Goolish 1989; Pearson *et al.* 1990). This is demonstrated by the effect of fish handling which stimulates muscle activity and thus lactate production. Chronic stress is also associated with higher lactate production (Karakach *et al.* 2009). Furthermore, fish muscle retains lactate, especially in rainbow trout (Gleeson 1996; Weber *et al.* 2016). The duration of post-mortem sampling time also affects lactate content through rapid depletion of glycogen stores. Owing to anaerobic reactions occurring in the muscle following slaughtering, glycogen stores are preferentially used to supply glucose for energy supply, so lactate, one of the end-products of glycolysis, accumulates in the muscle (Bankefors *et al.* 2011). Higher lactate levels can also result from rich carbohydrate diets that contribute to increase glycogen stores and therefore to preferential glycogen degradation for energy requirements (Maruhenda Egea *et al.* 2015).

The omnivorous species common carp fed with triticale as main source of energy displayed energy disturbances, as

illustrated by a reduced creatine content in the muscle extract compared to natural food (Cajka *et al.* 2013). Creatine is a precursor of phosphocreatine, a rapidly mobilizable reserve of energy in muscle used during intense muscle activity. Due to fast degradation of phosphorylated compounds in muscle during fishing, slaughtering of fish and sampling of muscle, the creatine/phosphocreatine system has to be considered as a potential energy store (van den Thillart *et al.* 1989). Creatine can be synthesized by fish, but the main source comes from the diet either in the form of FM and other animal meal or by supplementation. Thus, changes observed in this metabolite could be explained by a disturbed energy metabolism but also by the reduced supply of creatine due to plant-based diets or other alternative ingredients. Interestingly, levels of glutamine, which is also a precursor of TCA cycle intermediates, were found to be modulated in muscle extracts in the three studies mentioned above.

The impact of the substitution of marine resources for fish nutrition has also been investigated. We distinguish plant-based feedstuff from alternative feedstuffs such as fungal materials, decontaminated fish resources and fish protein hydrolysate. The formulation of experimental diets tested in controlled conditions generally overcomes some of the biases of previous studies, that is, non-isoenergetic, non-isoproteic or non-isolipidic diets, control of fish sexual maturation, constant water temperature. However, their results tend to confirm that such diets disturb energy metabolism and suggest a state of energy deficiency due to upregulation of catabolic pathways.

Red drum (*Sciaenops ocellatus*) fed for 12-week isoproteic and isolipidic diets with soy protein concentrate as main protein source was found to have a higher content of the energetic metabolites creatine and phosphocreatine, malate and even ATP in muscle (Casu *et al.* 2017). Furthermore, several free amino acids including alanine, glycine and serine had a lower content in fish muscles. Glycine, serine, alanine and even glutamine play several important roles in metabolism, but their modulation in fish fed with plant-based diet can result from adaptation of energy metabolism (Li *et al.* 2009; Casu *et al.* 2017). These three amino acids are known to be primary substrates of the TCA cycle, which may suggest their greater use to counterbalance the energy-deficient state suggested by the authors (Wei *et al.* 2017a) and their higher supply by protein catabolism supported by reduced growth (Li *et al.* 2009).

In juvenile turbot (*Scophthalmus maximus* L.) fed for 68 days with a high content of plant-based proteins (57% of dry matter), muscle had higher contents of malate, fumarate and glycine which, as previously mentioned, are related to energy metabolism (Wei *et al.* 2017a). Indeed, fumarate and malate are important TCA cycle

intermediates and their upregulation either suggests a TCA cycle inhibition or a stimulation of energy metabolism.

The lower body weight of fish fed a plant-based protein diet is also in accordance with a disturbed energy metabolism (Wei *et al.* 2014). To offset the inclusion of vegetable oils in salmonid diets, especially the enhanced conversion of linolenic and linoleic acids into PUFA, Wagner *et al.* (2014) supplemented vegetable oil-based diets with sesamin. Sesamin is a lignan found in sesame seed which has been previously shown to increase the proportion of DHA compared to linolenic acid. However, sesamin probably has other functions as high levels of sesamin alter fish energy metabolism. Indeed, the authors found higher levels of lactate and creatine/phosphocreatine in muscle extracts.

It is interesting to compare the metabolic consequences of these plant-based diets with those in fasted fish which have a clear energy-deficient status. In fasted rainbow trout, the first metabolic consequences were the preferential use of stored lipids to maintain energy supply (Kullgren *et al.* 2010; Baumgarner & Cooper 2012). Secondly and notably, as revealed by NMR, the fasted fish presented energetic metabolite variations in muscle other than those of fatty acids such as an increase in phosphocreatine and alanine and a decrease in lactate and betaine (Kullgren *et al.* 2010). As revealed by GC-MS, depleted levels of oxalate and malonate, which are inhibitors of specific TCA cycle enzymes, also suggest an alteration in energy status in fasted fish (Baumgarner & Cooper 2012). The effect of food deprivation combined with the influence of circadian rhythm led to an increase in leucine and isoleucine amino acids as a potential signal at cell level to regulate energy metabolism (Mekuchi *et al.* 2017). Such energetic metabolites variations are also observed in fish fed with diets in which marine resources are replaced by plant products, suggesting that energy status become deficient in these fish. However, the mechanisms could be different in fasted fish in which energy substrates are supplied only by catabolism compared to fed fish in which energy substrates are supplied mainly by feed. Together, these metabolomics results demonstrate an energy-deficient status in fish fed plant-based products with an adaptation of muscle metabolism to maintain their primary functions such as swimming activity, as observed in fasted fish (Kullgren *et al.* 2010).

Other feedstuff alternatives have been assessed by metabolomics to explore the metabolic consequences on muscle tissue. A partial substitution of dietary fish meal by fungal-based ingredients modulated lactate and creatine metabolites in Atlantic salmon muscle (*Salmo salar* L.), although no difference in growth was observed (Bankefors *et al.* 2011). The partial substitution of crude FM from the Baltic sea by baker's yeast (*Saccharomyces cerevisiae*) and deshelled blue mussel (*Mytilus edulis*) in Arctic char led to higher

contents of amino acids such as glycine and alanine in muscle (Cheng *et al.* 2017). The partial substitution by fish protein hydrolysate in juvenile turbot tended to increase creatinine and glucose and reduced glutamine (Wei *et al.* 2017a). Ultrafiltered fish protein hydrolysate to replace crude FM especially tended to decrease lactate, fumarate and alanine (Wei *et al.* 2017b). The total substitution of crude Baltic FM and FO, which accumulate persistent organic pollutants, by decontaminated FM and semi-purified FO highlighted the role of energy-related metabolites such as pyruvate. It suggested an interaction with energy supply for metabolic pathways involved in decontamination (Cheng *et al.* 2016a). Finally, $^1\text{H-NMR}$ analysis of taurine supplementation in Nile tilapia (*Oreochromis niloticus*) showed modulation in carbohydrate, amino acids, lipids and nucleotide metabolic pathways (Shen *et al.* 2018).

Thus, metabolomics analysis of fish fed with alternative ingredients to a plant-based diet demonstrates changes in energy status of muscle together with modifications of the different metabolic pathways involved (Fig. 4). However, these changes are less extensive but are more complex to interpret in supplementation experiments.

Impairment of liver metabolism

Liver is the central organ in fish nutrition as its main function is to collect the large dietary nutrient supply through the portal vein which is directly connected to the digestive

tract and to deliver these nutrients to the other tissues (Rust 2002). The delivery function comprises the production of lipoproteins for the redistribution of lipids to the peripheral tissues. Liver functions also include lipid and carbohydrate storage, production of bile for lipid digestion and detoxification processes (Brusle 1996). Thus, liver plays a key role in metabolism regulation that has been extensively studied in omics studies, and metabolomics analysis of the liver could provide relevant information on metabolic modulations by dietary variations.

Challenge and substitution studies have demonstrated similar findings with regard to the adaptation of glucose metabolism. In fish, glucose metabolism is quite similar to that in mammals although many pending questions remain, especially in carnivorous fish in which glucose seems to be poorly metabolized. This is especially the case of carnivorous fish fed over 20% of carbohydrate in their diet, which exhibit a post-prandial hyperglycaemia sometimes associated with 'fatty liver' disease (Polakof *et al.* 2012). In carnivorous species, plant-based diets seem to modulate glycolysis and gluconeogenesis in liver compared to fish fed marine resource-based diets. Indeed, red drum fed soybean products, juvenile turbot fed plant-protein based diets and Atlantic salmon fed sesamin-supplemented diets all showed modulation of hepatic glucose content (Wagner *et al.* 2014; Casu *et al.* 2017; Wei *et al.* 2017a). Depending on the diet, glucose content either increased in juvenile turbot and Atlantic salmon or decreased in red

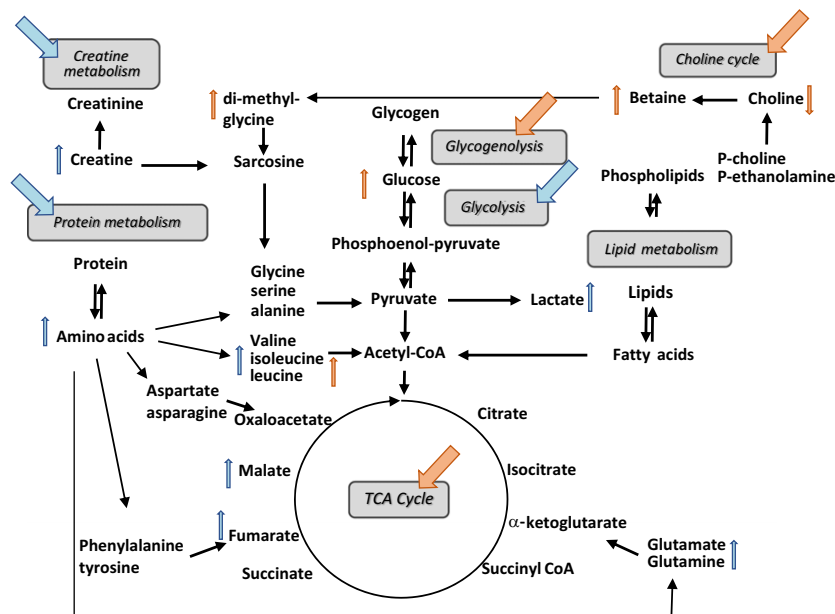


Figure 4 Affected metabolic pathways in response to dietary incorporation of plant-based feedstuff in muscle and liver. Muscle-affected metabolic pathways (blue) are related to glycolysis, creatine and protein metabolism. Liver affected metabolic pathways (orange) are related to choline cycle, glycogenolysis and TCA cycle. Wide arrows correspond to metabolic functions disturbed, thin arrows correspond to metabolite modulation.

drum. However, the relatively short post-prandial sampling time in juvenile turbot suggests that the higher glucose content in liver was more related to feed metabolome than fish metabolism. Glucose can also be associated with stress by the control of catecholamine on glycogenolysis. This mechanism was suggested by Cheng *et al.* (2016a) in fish fed contaminated FM and FO to produce energy for liver detoxification. Furthermore, the lower glucose content in red drum liver was associated with higher glycogen content. This pattern has previously been seen in two extreme conditions: those of fasted rainbow trout and herbivorous species fed high-carbohydrate diets, which showed a higher hepatosomatic index, suggesting enhanced glycogen accumulation (Kullgren *et al.* 2010; Prathomya *et al.* 2017). These findings of the use of carbohydrate resources for glycogenesis suggest that other sources of energy are preferentially used in an energy-deficient state induced by plant-based diet. Finally, the lactate modulations in liver found in fasted rainbow trout, in red drum fed soybean protein concentrate and in wild saithe partially fed manufactured pellets lost from salmon farms provide evidence of the altered glucose metabolism. The modulation of glucose metabolism in liver is consistent with the altered energy metabolism in muscle.

A second set of important metabolic functions affected in liver concern lipid metabolism and transport. Carnivorous fish fed plant-based diets have shown metabolite variations such as an increase in glycerol-3-phosphate, suggesting enhanced lipid catabolism (Casu *et al.* 2017). On the other hand, fish fed a diet supplemented with sesamin showed a decrease in hepatic glycerol that could result from lipid retention in liver and thus from an inhibition of lipolysis (Wagner *et al.* 2014). As a substrate of mitochondrial glycerol-3-phosphate dehydrogenase, glycerol-3-phosphate is to be found at the interface between lipid catabolism and glycolysis in mammals (Mráček *et al.* 2013). Indeed, the higher content of this metabolite suggests an increase in lipid catabolism to provide a substrate for gluconeogenesis (Casu *et al.* 2017).

The choline content and its metabolites N,N dimethylglycine, dimethylamine and phosphocholine were shown to be altered in fish fed manufactured diet and plant-based diets (Maruhenda Egea *et al.* 2015; Casu *et al.* 2017; Wei *et al.* 2017a). Although choline metabolism is essential in muscle, it is thought that choline metabolism in mammals is enhanced to limit the metabolic pressure on liver induced by lipid accumulation linked to the stimulation of cholesterol metabolism and lipoprotein synthesis (Cole *et al.* 2012). Furthermore, choline and its metabolite inosine are constitutive of the main phospholipids, namely phosphatidyl-choline and phosphatidyl-inositol. Alterations in liver choline in fish could thus also account for altered hepatic lipid metabolism and lipid redistribution

to peripheral tissues due to large differences in lipid composition between marine ingredients and terrestrial ingredients. Finally, choline is also a donor of methyl radicals and could compensate an unbalanced supply of other methyl donors such as methionine in alternative ingredients (Maruhenda Egea *et al.* 2015). In line with these results observed in liver, choline and its metabolites were also modulated in muscle of fish fed plant-based or commercial diets (Mannina *et al.* 2008; Melis & Anedda 2014; Maruhenda Egea *et al.* 2015).

Liver is involved in amino acid deamination and transamination pathways, thus supplying carbon backbones to gluconeogenesis and *de novo* fatty acid synthesis. Moreover, carbon backbones are also used by other metabolic pathways such as the TCA cycle. The TCA cycle is a central mechanism in energy homeostasis which is connected to the main metabolic pathways through the supply of glucose, certain amino acids and fatty acids to provide ATP for further energy requirements. Carnivorous fish species are known to use amino acids primarily, then fatty acids rather than glucose to supply the TCA cycle (National Research Council (U.S.) 2011).

In metabolomic studies on liver, the amino acid modulations are quite complex to disentangle because amino acids are involved in several metabolic functions. Thus, an increase in the level of amino acids could suggest an unbalanced amino acid profile of the alternative fish feed, inducing an accumulation of these amino acids that might not then enter the TCA cycle. Another hypothesis posits that accumulation is the result of an increasing amino acid supply either from the diet or from protein catabolism to support energy demand.

This contradiction can be illustrated by different examples. The first hypothesis was supported by two experiments on Atlantic salmon fed with a high level of sesamin (Wagner *et al.* 2014) and the other on rainbow trout fed a diet supplemented with feather meal (Jasour *et al.* 2017). The content in liver of the branched chain amino acids leucine and valine measured by ¹H-NMR was higher in two case studies suggesting TCA inhibition, because these compounds can normally be transaminated to produce glutamate, pyruvate and alanine, the entry points into the TCA cycle through the production of acetyl-CoA and succinyl-CoA (Wagner *et al.* 2014). The second hypothesis is supported by the higher level of branched chain amino acids found in the liver of juvenile turbot fed a plant-based diet supplemented with ultrafiltrated fish protein hydrolysate (Wei *et al.* 2017b). The authors suggested that a large supply of these amino acids by hydrolysates stimulates their use for energy supply associated with a reduced gluconeogenesis. Furthermore, such an accumulation of branched chain amino acids is noteworthy as they are involved in several important functions in mammals such as the

activation of the mTOR cellular signalling pathway involved in protein synthesis, immune functions and glucose consumption (Zhang *et al.* 2017).

Liver metabolite variations thus provide valuable information regarding metabolism and it appears that plant-based diets modulate energy metabolism in the liver (Fig. 4). Moreover, lipid metabolism and transport seem to provide a key response to dietary modulations. The amino acid metabolites are more puzzling to interpret in liver compared to muscle due to their involvement in several metabolic pathways.

Non-invasive monitoring of fish nutritional state by metabolomics

Tissue analysis provides valuable information on the nutritional impact on fish, but sampling requires fish slaughtering and the post-mortem metabolic processes can create confounding factors for metabolomic interpretation depending on the condition of sampling. Biofluids such as blood, mucus and faeces can be collected without slaughtering the animal. Moreover, they allow the evolution of metabolic status over time to be monitored in the same animal. Metabolomic studies require specific considerations as described previously. Since the prerequisite conditions are fulfilled in most studies, we now assess the consistency of metabolomic results on non-invasive samples to date with those from tissues and discuss the viability of monitoring the nutritional state by these approaches.

Agreement between biofluids and tissues

There are few recent metabolomic studies where both blood and tissues, namely liver and muscle, are considered simultaneously (NMR: Kullgren *et al.* 2010; Casu *et al.* 2017; Cheng *et al.* 2017; Jasour *et al.* 2017; Prathomya *et al.* 2017; GC-MS: Jin *et al.* 2015). Generally, the results obtained in blood, plasma or serum are consistent with those obtained in tissues not only for lipid metabolites during fasting experiments (Kullgren *et al.* 2010) but also for other metabolites such as amino acids, betaine and creatine, which demonstrated similar changes induced by challenge and substitution treatments (Jin *et al.* 2015; Cheng *et al.* 2017; Jasour *et al.* 2017). Monitoring fish at several time points after feeding offers the opportunity to compare relative changes in blood and in tissues, but in some cases, changes in plasma profiles are too erratic to be compared to those in tissues (Casu *et al.* 2017). None of the studies until now considered the predictability of blood metabolomics because the use of the metabolomics approach is too recent in fish and because a specific experimental design is required for such purposes. Thus, we could only analyse the literature in which the results observed in blood are

consistent with those observed in tissues. They showed that commercial and plant-based diets impact several metabolic pathways: energy, lipids and amino acids.

Fasted rainbow trout exhibited energy metabolism disturbances both at tissue and plasma level with a decrease in glucose and lactate content (Kullgren *et al.* 2010). Disturbances in glucose metabolism in fasted fish were also shown by an increase in catecholamine content involved in gluconeogenesis in gilthead sea bream (Gil-Solsona *et al.* 2017).

In the serum of Cobia (*Rachycentron canadum*) fed diet in which FM was mainly substituted by soybean meal and poultry meal, metabolites related to energy metabolism were impacted (Schock *et al.* 2012). Indeed, glucose decreased when betaine and tyrosine increased. Moreover, lactate was present in low concentrations. Similarly, the energy metabolism was affected in Wuchang bream (*Megalobrama amblycephala*) fed high carbohydrate diets resulting in a glucose and creatine increase in plasma (Prathomya *et al.* 2017). These results are consistent with the impaired energy status of fish fed plant-based diets, as shown by the modulation of energy-related metabolites in muscle and liver: glucose, lactate, creatine, glycine, alanine, serine and the TCA cycle intermediates fumarate and malate.

In the study by Baumgarner & Cooper 2012 on fasted rainbow trout, the level of SFA was lower compared to fish fed recurrently. This result is consistent with the study of Kullgren *et al.* (2010) where fasted rainbow trout preferentially used MUFA as energy supply. Similar results were found in fasted gilthead sea bream (Gil-Solsona *et al.* 2017). Indeed, the use of lipid as energy supply was supported by carnitine upregulation, which is involved in the transport of lipids into the mitochondrial membrane for their oxidation. Moreover, phosphocholine and phosphatidylcholine metabolites have been shown to be modulated in fasted fish. These metabolites may be considered to express lipid transport as they are involved in lipoprotein biosynthesis or as a marker of phospholipid degradation for energy supply. Lipid transport is also enhanced in fasted rainbow trout plasma by the higher content of lipoproteins and choline (Kullgren *et al.* 2010).

Juvenile grass carp (*Ctenopharyngodon idella*) fed an unbalanced diet with a low-protein content presented a higher concentration in SFA and MUFA and arachidonic acid (Jin *et al.* 2015). These results are consistent with those observed in muscle whose metabolic profiles demonstrated the difference in lipid composition, especially the higher presence of SFA, MUFA and DUFA. Furthermore, in liver, the modulation of choline and its derivative end products glycerol and glycerol-3-phosphate demonstrated lipid transport and lipid catabolism.

The agreement between amino acid modulation in tissues and biofluid can be highlighted by the juvenile grass carp

fed an unbalanced diet with low-protein content, which had a lower content in leucine and valine (Jin *et al.* 2015). In fasted fish, however, the modulation of glycine, serine and alanine in the serum rainbow trout was not found in the plasma of gilthead sea bream and rainbow trout (Baumgartner & Cooper 2012; Gil-Solsona *et al.* 2017), although alanine was similarly modulated in the liver and muscle of fasted rainbow trout (Kullgren *et al.* 2010).

An interesting result observed only once in the LC-MS analysis of serum is the modulation of several Meister's cycle metabolites (glutathione, pyroglutamic acid) involved in the improvement of oxidative capacity (Gil-Solsona *et al.* 2017).

Alternative samples could also be considered for non-invasive investigation of metabolism by metabolomics. Metabolomic analysis of faeces has been used as a marker of microbiote activity in different fish species fed with different diets (Asakura *et al.* 2014). Indeed, some correlations between plasma metabolites detected by $^1\text{H-NMR}$ and host microbiota species have been observed in rainbow trout fed commercial and plant-based diets by Gatesoupe *et al.* (2018). NMR-based analyses of faeces of convict grouper (*Epinephelus septemfasciatus*) fed with a natural diet then with a manufactured diet showed an increase in carbohydrates, phospholipids and fatty acids and a decrease in branched-chain amino acids (Asakura *et al.* 2014). Mucus MS-based metabolic profiles were analysed to characterize fish life-history traits (Reverter *et al.* 2017). Results indeed highlighted diet as a major factor for fish discrimination based on mucus non-polar fraction profiles. Thus, mucus should be used to assess the impact of a controlled diet. Finally, fish fins could be used to some extent for non-invasive sampling, although this has been done only once to study mineral profiles (Yoshida *et al.* 2014).

The results of metabolomic analyses on non-invasive biofluids in fish nutrition showed a good match with those of tissues. Indeed, several common metabolites such as glucose, creatine and choline derivatives are modulated in both plasma and serum and in the liver and muscle of fish fed plant-based diets. However, it is complex to establish a clear link with diet because these metabolites create several responses. Faeces and mucus have not been extensively studied and do not show the same metabolite profiles, so they are probably more suitable for other types of studies.

Non-invasive samples account for other fish responses

Plasma and serum reflect fish nutritional status by carrying dietary nutrients to the different organs after processing in the liver. However, their composition can also be affected by several other biological factors, thereby creating a bias in interpretation. Indeed, lactate, glucose, creatine or phosphocholine have been identified as discriminant

metabolites in fish stressed by handling and in fish fed a diet contaminated with heavy oils (Karakach *et al.* 2009; Kokushi *et al.* 2012). Other metabolites such as tyrosine, betaine and glycine have been found to be affected in pathological states of cobia fed with a plant-based diet (Schock *et al.* 2012). Therefore, it is not possible to differentiate the effects of stress and health from those of diet.

Faeces are the undigested part of the feed and metabolic waste. Their analysis reveals nutrient absorption and microbiota functions that contribute both to dietary modulation and immune functions (Asakura *et al.* 2014). However, faeces still contain significant bacterial mass with its own metabolites and also compounds from bacterial degradation (Clements *et al.* 2014). Thus, the metabolites in faeces result from digestion and account for the activity of residual microbiota.

Mucus reflects the feeding habits of corallivorous and omnivorous fish, so it is possible to have an overview of the dietary impact by analysing this type of sample (Zamzow 2004; Reverter *et al.* 2017). However, mucus in fish is rather devoted to other functions such as osmoregulation, immune defence mechanisms and locomotion (Shephard 1994). Moreover, high contents of macromolecules such as glycoproteins and muco-polysaccharides require specific sample preparations and are out of scope of metabolome (non-polymeric metabolite and molecular weight < 1500 Da).

Finally, plasma and serum are good indicators of fish nutritional status because of the metabolite content arising from internal metabolism and feed. However, metabolites could result from factors other than dietary intervention such as various stressors and disease. Among the alternative samples, faeces seem better than mucus for studying metabolism because faeces are the result of digestion by the host and microbial population, whose role in nutrition seems to be increasingly important (Clements *et al.* 2014; Vernocchi *et al.* 2016).

Contribution of metabolomics to fish nutrition

Metabolomics, as a holistic approach, contributes to fish nutrition studies by assessing the direct or indirect impact of diet on metabolism. This approach may serve to classify fish according to diets, identify biomarker of disturbed state, or shed light on the metabolic pathways affected, as exemplified below. The global variations of fish metabolites affected by diets can be used to build classification models of groups of individuals. Several studies in line with this strategy have demonstrated that metabolomics can contribute to assess fish origin based on muscle lipid profiling (Rezzi *et al.* 2007; Mannina *et al.* 2008; Melis *et al.* 2014). Metabolites important for fish classification may also be relevant to identify biomarkers of metabolic modifications.

The serum NMR study by Schock *et al.* (2012) is a promising example revealing an early shift of metabolites that differentiate fish fed mostly plant-based diets from fish fed marine resources on a 98-day feeding trial. The serum LC-MS study of Gil-Solsona *et al.* (2019) revealed unsuspected alterations of vitamin metabolites of fish fed low FM and FO and so demonstrated the advantage of a metabolomic approach to identify nutritional deficiency markers. In some cases, the identification of metabolites sheds light on specific metabolic pathways impacted by a diet of interest. Casu *et al.* (2017) have established by NMR metabolomics that higher concentrations of glycine, serine and threonine metabolites in liver and muscle in fish fed soy-based diets are related to protein catabolism pathway. Finally, metabolomics may contribute to understand the mechanisms involved in the utilization of novel feedstuffs in a controlled environment, and help to decipher a central question in fish energy metabolism, that is, which precursors supply intermediary metabolism. Metabolomic studies have already highlighted the contribution of amino acids, fatty acids and even alternative substrates as compared to that of glucose to energy metabolism in fish fed plant-based diet and alternative feed stuffs (e.g., Casu *et al.* 2017; Jarak *et al.* 2018). Some metabolic network maps with up- or down-regulated metabolites were also reported (Casu *et al.* 2017; Gil-Solsona *et al.* 2017; Prathomya *et al.* 2017), which is promising.

Conclusions

Metabolomics provides a global insight into metabolism by the identification of multiple metabolites involved in biological responses of individuals exposed to different factors such as nutrition. In fish nutrition, metabolomics studies demonstrate the interest of a non-targeted global analysis and could be used for fish authenticity. They not only confirm the impact of plant feedstuffs on muscle fatty acid composition by the overexpression of SFA, MUFA and DUFA but also reveal other mechanisms related to lipid metabolism and transport through choline or glycerol. Moreover, metabolomic studies highlight metabolites such as glucose, lactate and creatine that have been related to impaired energy metabolism similar to the state of energy deficiency in fasted fish. These studies also demonstrate intermediate metabolic disturbances both by TCA cycle intermediates and metabolites such as amino acids that are the entry point to the TCA cycle. However, interpretation of metabolites requires caution because of the numerous metabolic functions in which one metabolite may be involved. The establishment of a specific database dedicated to fish metabolomics would offset this problem.

Specific care should be taken with sample type and sampling methods since they may introduce a bias in the

interpretation of the data, especially time-dependent factors such as post-prandial and post-mortem sampling times. Plasma and serum offer the great advantage of being non-invasive, but the complexity of obtaining fish serum should be taken into account.

In the future, it could be interesting to use fluxomic studies to confirm hypotheses generated by metabolomic approaches. Moreover, metabolomics for fish nutrition will probably focus on interactions with host microbiota. Thus, the combination of metagenomics and metabolomics to characterize bacterial populations, metabolites and host metabolites should gain in importance. Attention to feed characterization is another key point to establish a clear link between novel feedstuff and modulated metabolic fish functions. Although several challenges remain, such as the transposition of results from one species to another, metabolomics has begun a promising integration into the research landscape of fish nutrition.

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