The role of phospholipids in nutrition and metabolism of teleost fish

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Abstract

It has been known for almost 25 years now that inclusion of intact phospholipids in the diet could improve culture performance of various freshwater and marine fish species. The primary beneficial effect was improved growth in both larvae and early juveniles, but also increased survival rates and decreased incidence of malformation in larvae, and perhaps increased stress resistance. Determination of absolute dietary requirements has been hampered by the use, in different dietary trials, of a wide range of phospholipid preparations that can vary greatly both in phospholipid content and class composition. Larval studies have been compromised further by the need on many occasions to supply phospholipid through enrichment of live feeds with subsequent re-modelling of the phospholipid and fatty acid compositions. Generally, the levels of phospholipid requirement are around 2 - 4% of diet for juvenile fish and probably higher in larval fish. The effects were restricted to young fish, as a requirement for dietary phospholipids has not been established for adult fish, although this has been virtually unstudied. As the majority of studies have used crude mixed phospholipid preparations, particularly soybean lecithin, but also other plant phospholipids and egg yolk lecithin, that are enriched in several phospholipids, it has been difficult to elucidate which specific phospholipid classes impart beneficial effects. Based on the few studies where single pure phospholipid species have been used, the rank order for efficacy appears to be phosphatidylcholine > phosphatidylinositol > phosphatidylethanolamine > phosphatidylserine. The efficacy of other phospholipid classes or sphingolipids is not known. The mechanism underpinning the role of the phospholipids in larval and early juvenile fish must also explain their lack of effect in adult fish. The role of phospholipids appears to be independent of fatty acid requirements although the presence of an unsaturated fatty acid at the *sn*-2 position may be important. Similarly, the phospholipid requirement is not related to the delivery of other essential dietary components such as the bases choline and inositol. Studies also suggested that the phospholipid effect was not due to generally enhanced emulsification and digestion of lipids. Rather the evidence led to the hypothesis that early developing stages of fish had impaired ability to transport dietary lipids away from the intestine possibly through limitations in lipoprotein synthesis. The current hypothesis is that the enzymic location of the limitation is actually in phospholipid biosynthesis, perhaps the production of the glycerophosphobase backbone and that dietary supplementation with intact phospholipids in larvae and juvenile fish compensated for this. Thus, dietary phospholipids increase the efficiency of transport of dietary fatty acids and lipids from the gut to the rest of the body possibly through enhanced lipoprotein synthesis.

Contents

1. Introduction

Phospholipid is a general term that includes all lipids containing phosphorus. However, it is a term often mistakenly equated with phosphoglycerides, the most common of the phospholipids. Phosphoglycerides are characterised by a common backbone of phosphatidic acid (PA), formed from L-glycerol 3-phosphate with two fatty acids esterified on positions 1 and 2. The major phosphoglycerides of animal including fish tissues phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI), respectively, are formed by the esterification of the "bases" choline, ethanolamine, serine and inositol to the phosphate group of PA. A common generalisation has been that saturated and monounsaturated fatty acids are preferentially esterified on position *sn*-1 of phosphoglycerides with polyunsaturated fatty acids (PUFA) preferentially esterified on position *sn*-2. However, there are many exceptions to this, such as the di-docosahexaenoyl phosphoglycerides that are abundant in the retina of fish, specifically in rod outer membrane segments (Bell and Tocher, 1989; Bell and Dick, 1991). Another phosphorus-containing polar lipid is the sphingolipid, sphingomyelin, a complex lipid based on the long-chain amino alcohol sphingosine. All sphingolipids contain a long chain, generally saturated or monounsaturated fatty acid, linked to the amino group of sphingosine (forming a ceramide), with different polar groups, such as, phosphocholine in the case of sphingomyelin, attached to the primary alcohol group.

For the remainder of this review we will exclusively use the term "phospholipid" when, in actual fact, phosphoglyceride would be the more correct term, as described above. However, the use of phospholipid will be consistent with the vast majority of studies that have been reported in the literature. Similarly, the abbreviations PC, PE, PS, PI and PA, as used above, are not strictly correct but are by far the most commonly used for the major phosphoglyceride classes and so will be used throughout this review.

2. Roles of phospholipids

2.1 Structural

The amphipathic structure of phospholipids, having both hydrophilic (*sn*-3 phosphate/head-group) and hydrophobic (*sn*-1 and -2 fatty acids) regions means they have the same central role in the structure of cell membrane bilayers in fish as they do in mammals. Although not well studied in fish, there is evidence that the asymmetric distribution of phospholipids in cell membranes

established in mammals, with choline-containing phospholipids, PC and sphingomyelin concentrated in the outer leaflet and PE, PS and, to a lesser extent, PI concentrated in the inner leaflet, is also a feature of fish cell membranes (Kagan et al., 1984). The dynamic changes in the composition and metabolism of the phospholipids in biomembranes in response to environmental factors, especially temperature, have been reviewed previously (Hazel and Williams, 1990; Hochachka and Mommsen, 1995). Their amphipathicity is also central to the role phospholipids have in the structure of the lipoproteins that are important in the extracellular transport of lipids in the blood and lymph. The lipoproteins are described in more detail in section 4.3 but, essentially, phospholipids enable hydrophobic lipids such as triacylglycerols and steryl esters to be transported in aqueous environments by forming, along with cholesterol and proteins, the lipid/water interfaces (Tocher, 1985). It can be argued that phospholipids also have an important structural role in the digestion of lipids as they are essential in forming intra-luminal mixed micelles along with bile salts and dietary lipids (see section 4.2) (Olsen and Ringø, 1997). This phospholipid is not entirely of dietary origin as fish bile can contain variable amounts of phospholipid (Moschetta et al., 2005). It is thought that biliary phospholipid has two roles in the bile as the formation of mixed micelles with bile salts not only solubilises biliary cholesterol, but also has a cytoprotective effect, protecting biliary tract epithelium from the cytotoxic effects of bile salts (Moschetta et al., 2005). PC is usually the major phospholipid in fish bile but sphingomyelin can also be a major component, particularly in species with lower biliary phospholipid levels and phospholipid:bile salt ratios (Moschetta et al., 2005).

2.2 Regulation of metabolism and physiology

Phospholipids are important precursors for a range of highly biologically active mediators of metabolism and physiology including eicosanoids, diacylglycerol (DAG), inositol phosphates and platelet activating factors (PAFs). With the possible exception of eicosanoids, these areas of phospholipid metabolism are relatively poorly studied in fish. However, there is evidence that all these pathways occur in fish and that the phospholipid-derived mediators play similar roles in fish as they do in mammals (Tocher, 1995, 2003).

2.2.1 Eicosanoids

Phospholipids are the source of the substrate fatty acids for the formation of eicosanoids, a range of highly bioactive derivatives of, in particular C_{20} highly unsaturated fatty acids (HUFA),

especially arachidonic acid (ARA, 20:4n-6) and eicosapentaenoic acid (EPA, 20:5n-3). Fatty acids released from membrane phospholipids by the action of phospholipase A_2 are converted by either cyclooxygenase enzymes, which produces cyclic oxygenated derivatives, collectively called prostanoids, including prostaglandins, prostacyclins and thromboxanes, or lipoxygenase enzymes which produce linear oxygenated derivatives including hydroperoxy- and hydroxy fatty acids, leukotrienes and lipoxins. Eicosanoids are produced by many tissues in response to various extracellular stimuli and are autocrines, hormone-like compounds of short half-life that act in the immediate vicinity of their production. They are implicated in many physiological processes including blood clotting and cardiovascular tone, immune and inflammatory responses, reproduction, renal and neural functions. The distribution and production of eicosanoids in fish species and tissues and their possible roles have been reviewed previously (Sargent et al., 1989, 2002; Tocher, 1995, 2003).

2.2.2 Phosphoinositides

Phosphorylated derivatives of PI (e.g. PIP_2) are produced by the action of various kinases and phosphatases on the inositol ring. The phosphorylated derivatives of PI may themselves have important roles in metabolism including golgi/lysosome/endosome trafficking, cytoskeleton regulation and cell survival. The most studied pathway though is the cleavage of PIP_2 by phospholipase C-β to produce two intracellular second messengers, DAG, and the inositol phosphate, IP_3, in response to various hormones and effectors (Berridge, 1987). IP_3 induces calcium mobilisation from the endoplasmic reticulum, and increased intracellular Ca^{2+} and DAG are activators of protein kinase C, a threonine/serine kinase and important regulator of metabolism (Kuo et al., 1980). DAG can also be phosphorylated by DAG kinase to form PA and lyso-PA, increasingly being recognised as other important intracellular regulators of a growing number of signalling proteins (Hao et al., 2007). Other phospholipids, including PC and PS, also have important metabolic functions in mammals as specific activators of protein kinase C, along with DAG and Ca^{2+} ions (Kuo et al., 1980). Protein kinase C and PS-activation has been reported in rainbow trout (*Oncorhynchus mykiss*) spleen and dogfish (*Scyliorhinus canicula*) rectal gland (Bell and Sargent, 1987), and a role for protein kinase C has been implicated in the stimulation of steroidogenesis in goldfish (*Carassius auratus*) gonadal tissues (Van Der Kraak, 1990; Wade and Van Der Kraak, 1991). Inositol phospholipid metabolism including the roles of phospholipase C

and Li-sensitive phosphatases have been studied in metabolically active electrocytes from the electric ray (*Discopyge tschudii*) (Arias and Barrantes, 1990).

2.2.3 Platelet-activating factor (PAF)

PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, is a biologically active phospholipid synthesised by inflammatory cells and a potent mediator of many leukocyte functions, including platelet aggregation, inflammation, and anaphylaxis (Snyder, 1987). PAF is biosynthesized from lyso-PAF and acetyl CoA by the enzyme lyso-PAF acetyltransferase and degraded (thereby terminating activity) by a group of enzymes called PAF acetylhydrolases that are related to phospholipase A_2. In mammals, the reacylation of lyso-PAF is highly specific for ARA, and it is the 1-alky-2-arachidonyl-glycerophosphocholine species that is the substrate for the synthesis of PAF *via* an ARA-specific phospholipase A_2 that also produces ARA for eicosanoid synthesis (Snyder, 1987). Synthesis of PAF has been demonstrated in rainbow trout (Turner and Lumb, 1989) and PAF acetylhydrolase activity has been reported in fish serum (Cabot et al., 1984).

2.3 Energy production

Any lipid class containing fatty acids can act as a source of energy, which is released through β-oxidation of the acyl chains producing acetyl-CoA and NADH that are further metabolised via the tricarboxylic acid cycle and oxidative phosphorylation, respectively. Triacylglycerols are the primary class for lipid storage and energy provision, but phospholipids can serve as a source of energy in fish in certain circumstances, such as embryonic and early larval development.

Upon hatching, the larvae of many fish species have immature mouthparts and intestinal tract and are unable to commence exogenous feeding immediately. Therefore, throughout embryogenesis in the egg and then during early larval development up to first feeding, the larvae gain nutrition from endogenous energy reserves in the yolk. Essentially there are two main types of fish egg, those with oil globule(s) having relatively high levels of neutral lipids (20-50% of egg total lipid), and those without an oil globule, low levels of neutral lipids (< 15% of total lipid) and, consequently, high levels of phospholipids, predominantly PC (Wiegand, 1996; Salze et al., 2006). In eggs of the latter type, including those from Atlantic herring (*Clupea harengus*), cod (*Gadus morhua*), halibut (*Hippoglossus hippoglossus*) and plaice (*Pleuronectes platessa*), phospholipid, predominantly PC, is utilised during both embryogenesis and early larval development (Tocher et al., 1985a; Fraser et al., 1988; Rainuzzo et al., 1992; Finn et al., 1995;

229 Ronnestad et al., 1995 (Finn et al., 1995; Ronnestad et al., 1995). In contrast, neutral lipid-rich
230 eggs such as those from turbot (*Psetta maximus*), utilise primarily triacylglycerols, but also steryl
231 and wax esters where present (Sargent et al., 1989, 2002; Rainuzzo et al., 1992; Weigand, 1996).
232 Another relatively common feature observed during development is the conservation and/or
233 synthesis of PE, as reported in both the phospholipid-rich eggs of cod (Fraser et al., 1988), plaice
234 and halibut (Rainuzzo et al., 1992; Ronnestad et al., 1995), and also the neutral lipid-rich eggs of
235 Atlantic salmon (*Salmo salar*) and turbot (Cowey et al., 1985; Rainuzzo et al., 1992), Senegal
236 sole (*Solea senegalensis*) (Mourente and Vazquez, 1996) and dentex (*Dentex dentex*) (Mourente
237 et al., 1999). This results in a decrease and normalisation of the PC : PE ratio as development
238 proceeds, from the high values seen in most marine fish eggs to values normally observed in fish
239 tissues. This is particularly the case in the phospholipid - rich eggs, dominated by PC, where PC
240 is catabolised during embryogenesis, but has also been observed in Atlantic salmon where PC,
241 originally accounting for over 94% of the phospholipid in the egg, was continuously metabolized
242 so that in the fry a PC : PE ratio approaching that of salmon muscle was obtained (Cowey et al.,
243 1985).
244 These data show a decrease in the absolute amount of phospholipid, usually PC, during
245 embryogenesis and early larval development, particularly in phospholipid-rich marine fish eggs.
246 A possible consequence of complete catabolism of phospholipid for energy would be the loss of
247 important PUFA. However, in an early study in Atlantic herring, much of the PUFA liberated by
248 the catabolism of PC was selectively retained in the neutral lipid pool (Tocher et al., 1985b).
249 About one third of the docosahexaenoic acid (DHA, 22:6n-3) released during PC catabolism in
250 cod eggs was incorporated into neutral lipids (Fraser et al., 1988). Similarly, in halibut, over 60%
251 of the DHA from PC hydrolysis was selectively retained in PE and neutral lipids (Ronnestad et
252 al., 1995). However, DHA was also a quantitatively important fuel in halibut eggs with almost
253 40% of the DHA being catabolised (Ronnestad et al., 1995). Similarly, in cod the fatty acids in
254 PC were catabolised non-selectively with both EPA and DHA being utilised (Finn et al., 1995).
255
256 *3. Phospholipid biosynthesis*
257
258 *3.1 De novo synthesis*
259 In mammals, phospholipids can be synthesised de novo by two main mechanisms (see Lykidis,
260 2007). One utilizes a cytidine diphosphate (CDP)-activated polar head group for attachment to
261 1,2-DAG, and the other utilizes CDP-activated DAG and an inactivated polar head group (Fig.1).

They both initially proceed by esterification of glycerol-3-phosphate with two activated fatty acids (acyl-CoA) to form PA. Action of PA phosphatase on PA results in the production of DAG, which can then react with CDP-choline or CDP-ethanolamine to produce PC and PE, respectively. Alternatively, CTP reacts with PA to form CDP-DAG, which can then combine with serine and inositol to form PS and PI, respectively. Reaction of CDP-DAG with glycerol-3-phosphate leads to phosphatidylglycerol (PG) and combination of PG with a further CDP-DAG leads to the formation of diphosphatidylglycerols that include cardiolipin. PS can also be formed in the membrane bilayer by exchange reactions of serine for ethanolamine or choline in PE and PC, respectively. PS can be converted back to PE by decarboxylation and PC can also be formed by three successive methylations of PE utilizing S-adenosylmethionine.

The pathways of *de novo* phospholipid biosynthesis have not been extensively studied in fish, but all the necessary phospholipid biosynthetic enzymes and protein families can be found in the fugu (*Takifugu rubripes*) and zebrafish (*Danio rerio*) genomes (Lykidis, 2007). The existing biochemical evidence also suggests that pathways are essentially the same as in mammals (Tocher, 1995). Incubation of trout liver microsomes with ^{14}C-glycerol-3-phosphate in the presence of palmitoyl-CoA, resulted in three-quarters of the radioactivity being recovered in total phospholipids, predominantly PA and lyso-PA but also other phospholipid classes, demonstrating glycerol-3-phosphate acyltransferase activity and supporting the conclusion that phospholipid biosynthesis in fish proceeded *via* a PA intermediate (Holub et al., 1975a). Activity of α-glycerophosphate acyltransferase in fish was later confirmed, although it was reported to be extremely low compared to other animals (Iritani et al., 1984). Studies on the incorporation of ^{14}C-palmitate and ^{14}C-glycerol-3-phosphate into lipids in carp (*Cyprinus carpio*) intestinal homogenates in the presence of CTP, CDP-choline and CDP-ethanolamine showed phospholipid biosynthesis from PA and DAG intermediates, indicating synthesis from moieties other than lyso-phospholipids (Iijima et al., 1983). However, little is known about what determines the fate of newly-synthesised DAG (PC or triacylglycerol synthesis) in fish, but it has been suggested that fatty acid composition of the DAG may differentially affect the activities of the biosynthetic enzymes (Oxley et al., 2007). Early studies demonstrated the presence of CDP-choline:DAG choline phosphotransferase activity in trout liver microsomes (Holub et al., 1975b), and brain and liver from goldfish (Leslie and Buckley, 1975), establishing that the choline phosphotransferase (CPT) pathway for the biosynthesis of PC operated in fish. Similarly, synthesis of PE from DAG and the presence of CDP-ethanolamine phosphotransferase (i.e. the EPT pathway) were subsequently demonstrated in trout hepatocytes (Hazel, 1990). The activity of CTP in

295 microsomes from intestinal mucosa in adult Atlantic salmon (~ 1 kg) was comparable to the
296 reported mammalian activity (Oxley et al., 2005). Thus, synthesis via DAG and CPT/EPT was
297 confirmed as the predominant pathway for phospholipid synthesis in fish as in mammals (Hazel,
298 1990). Although not directly studied or demonstrated in fish, the presence in fish of PI, PG and
299 cardiolipin, that cannot be synthesised via the phosphotransferase pathways, implies the presence
300 of the alternative CDP-DAG pathway in fish. Similarly, not all the phospholipid inter-conversion
301 pathways have been studied in fish, but the activities of PE-methyltransferase (PE to PC) and PS-
302 decarboxylase (PS to PE) have been demonstrated in trout hepatocytes (Hazel, 1990).
303
304 *3.2 Phospholipid turnover and remodelling*
305 Remodelling and turnover of phospholipids can involve both head groups and fatty acyl chains.
306 The mechanisms whereby phospholipid classes (PC, PE, PS and PI, etc.) could be inter-converted
307 are described above (see Fig.1), and phospholipid remodelling at this level is certainly involved
308 in homeoviscous adaptation of biological membranes, especially in relation to temperature
309 adaptation. Thus, cold acclimation in fish has been associated with increased proportions of PE
310 and decreased proportions of PC (Hazel and Williams, 1990). Although the activities of CDP-
311 choline and CDP-ethanolamine phosphotransferase were both reduced at lower temperature,
312 synthesis of PC was more dependent upon temperature than that of PE and, in addition, PS
313 decarboxylase activity was increased (Hazel, 1990). As a result, the PC : PE synthesis ratio was
314 positively correlated with temperature and so these mechanisms may underpin the increased PE :
315 PC ratio in cold-acclimated fish (Hazel, 1990).
316 Fatty acid retailoring of phospholipids through acyl exchange reactions has been relatively
317 more studied in fish, again most often in relation to homeoviscous adaptation to environmental
318 changes. The enzymes required for deacylation of phospholipids and reacylation of lyso-
319 phospholipids, and thus for the turnover of phospholipids, have been demonstrated in fish.
320 Intracellular phospholipase A activities have been reported in muscle tissue from a variety of fish
321 species including rainbow trout and cod (see Tocher, 2003), and a review of muscle lipase
322 activities indicated that hydrolysis of phospholipids in fish was primarily under the control of
323 phospholipases A_1 and A_2 (Shewfelt, 1981). The activity of liver microsomal phospholipase A_2
324 has been studied in trout (Neas and Hazel, 1985), whereas cytosolic phospholipase A_2 was
325 demonstrated to be the principal phospholipase activity during zebrafish embryogenesis (Farber
326 et al., 1999). AcylCoA:1-acyl-*sn*-glycero-3-phosphorylcholine acyltransferase activity has also

been demonstrated in trout liver microsomes (Holub et al., 1976). It is presumed that the fatty acyl and head group specificities of the enzymes involved in the deacylation/reacylation turnover processes have important roles in maintaining the characteristic fatty acyl distribution among the phospholipid classes (Tocher, 2003). Thus, PC is characterized by having high 16:0 (higher than any other class) and relatively lower PUFA (i.e. lower than PE and PS); PE generally displays intermediate levels of saturated and monounsaturated fatty acids and high levels of PUFA which are relatively evenly split between C_{20} and C_{22} PUFA; PS is characterized by high 18:0 and high PUFA, predominantly C_{22} PUFA; PI also has high 18:0, but relatively lower PUFA, which is predominantly C_{20}, particularly ARA, and a low n-3 to n-6 ratio (Tocher, 1995).

4. Phospholipid digestion, absorption and transport

4.1 Digestion

Digestion of dietary phospholipids is relatively unstudied in fish, but it is presumed that the mechanisms are generally similar to those in mammals whereby phospholipids are digested by intestinal phospholipase A_2, secreted by the pancreas, resulting in the formation of 1-acyl lyso-phospholipids and free fatty acids that are absorbed by the intestinal mucosal cells (Henderson and Tocher, 1987; Sargent et al., 1989). Lyso-phospholipids have a detergent action and so aid the digestion of other lipids and, indeed, some phospholipid is secreted in the bile as substrate for phospholipase A_2. The lack of a discrete pancreas in most teleost species has hampered studies on intestinal lipolysis in fish in general and, although several studies have demonstrated phospholipase A_2 activities in intestinal tissue of fish, it has been difficult to establish conclusively if they were intracellular activities or secreted (see Tocher, 2003). However, two phospholipase A_2 isoforms were purified from red sea bream hepatopancreas and characterisation confirmed them as low molecular weight, Ca^{2+}-dependent group I (secretory) forms (Ono and Iijima, 1998). Phospholipase A_2 activity was only detected several days after mouth opening in marine fish larvae (Zambonino-Infante and Cahu, 2001). There are few data on rates of phospholipid hydrolysis, but digesta from turbot rectum was shown to hydrolyse PC, although at slower rates than triacylglycerol and sterol esters (Koven et al., 1994).

4.2 Absorption

The mechanisms of absorption of the products of phospholipid digestion have not been extensively studied in fish but are assumed to be generally similar to that in mammals. Thus, the hydrolytic products, 1-acyl lyso-phospholipids and free fatty acids, will be associated with all the other products of fat digestion in mixed micelles with bile salts, that diffuse to the intestinal mucosa where uptake into the enterocytes occurs, probably mainly by passive diffusion.

The concentration of lyso-phospholipids is very low in fish plasma and so it is assumed that in the intestinal mucosa the majority of the 1-acyl lyso-phospholipids are re-esterified with activated free fatty acids in the microsomes before export from the enterocytes into the circulatory system (Sargent et al., 1989). This is supported by a study measuring the absorption of ^{14}C-dioleoyl PC force-fed to carp (Iijima et al., 1990). Radioactivity in plasma lipoproteins 20 - 28 h after force-feeding was associated with various lipid classes including PC, and the radioactivity associated with the *sn*-1 position was more than twice that associated with the *sn*-2 position (Iijima et al., 1990). There appears to be some specificity in the reacylation processes as a study of fatty acid uptake by isolated enterocytes from rainbow trout showed that the recovery of HUFA and 16:0 in phospholipids was higher than that of other fatty acids (Perez et al., 1999).

4.3 Transport

Phospholipids are transported in the blood of most fish as lipoproteins as they are in mammals (see Tocher, 1995). Lipoproteins include chylomicrons, very-low density lipoproteins (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) containing 8, 21, 25 and 29 % of total weight as phospholipids, respectively (Chapman et al., 1978). However, as a percentage of the total lipids, the proportion of phospholipids in trout lipoproteins ranges from around 9% in chylomicrons to 23 % in VLDL, 35 % in LDL and 53 % in HDL (Chapman et al., 1978), although carp lipoproteins contained proportionally more phospholipid than trout, salmon or sardine (*Sardinops*) lipoproteins with the total lipid from VLDL, LDL and HDL containing 30 %, 58 % and 82 % phospholipid, respectively (Iijima et al., 1989). The relative proportions of the plasma lipoproteins in fish are dependent upon age, nutrition and reproductive cycle and also vary with species, but is a constant characteristic of each species depending upon dietary status (Fremont and Leger, 1981).

The re-esterification reactions in the intestine occur primarily in the endoplasmic reticulum leading to the production of chylomicrons and, to a lesser extent, VLDL (see Tocher, 1995). Transport from the intestine to the liver is primarily via the lymphatic system as in trout and

390 tench (*Tinca tinca*) although, in carp, intestinal lipoproteins may be transported directly to the
391 liver via the portal system (Noaillac-Depeyre and Gas, 1974; Sire et al., 1981). Although the
392 effects of phospholipid content and composition on the production of intestinal lipoproteins in
393 fish have not been studied, as chylomicrons and VLDL have very different phospholipid
394 contents, variable proportions of dietary phospholipid could be accommodated by varying the
395 relative proportions of the intestinal lipoproteins produced. In common with most vertebrates, PC
396 appears to be the predominant phospholipid class in fish lipoproteins, although the precise
397 phospholipid class composition in fish plasma lipoproteins has been rarely reported (Nelson and
398 Shore, 1974; Chapman, 1980).
399 The processes of lipoprotein metabolism and remodelling are essentially the same in fish as in
400 mammals and are described in detail previously (Tocher, 1995, 2003) and so are only
401 summarised here. Although some may be produced in the intestine, the majority of VLDL is
402 synthesized in the liver. Triacylglycerols in chylomicrons and VLDL are hydrolyzed by
403 lipoprotein lipase (LPL) and hepatic lipase (HL) at tissue sites with the hydrolysis products being
404 absorbed. Excess surface constituents, including phospholipids, dissociate as nascent HDL
405 particles, leading to the formation of intermediate density lipoprotein (IDL) and LDL. Nascent
406 HDL can take up free cholesterol from peripheral tissues which is then esterified by the action of
407 lecithin:cholesterol acyl transferase (LCAT), a plasma enzyme which catalyzes the esterification
408 of cholesterol using fatty acid from PC, resulting in the production of mature HDL. All the major
409 enzymes of lipoprotein metabolism and remodelling, including LPL, HL and LCAT, have been
410 demonstrated in the plasma of various fish (Tocher, 1995).
411 Although virtually unstudied in fish, by analogy with the system characterized in mammals,
412 phospholipids can probably be taken up into the tissues by two or three main mechanisms. In
413 liver and some other tissues, receptor-mediated endocytosis, *via* receptors for B/E and E
414 apoproteins, are important pathways for LDL (apo B), VLDL- and chylomicron-remnants (apo B
415 and E) and a subfraction of HDL rich in apo E. Non-specific pinocytosis may be an important
416 pathway for all lipoproteins, but particularly LDL and HDL. In liver, around 30 % of LDL uptake
417 is *via* a non-receptor-mediated pathway in mammals. Finally, phospholipids and other surface
418 components of VLDL and chylomicrons may be taken up or exchanged *via* direct interaction
419 with the endothelial cell membranes in the tissues.
420
421 *5. Phospholipid requirements of fish*

All the available evidence suggests that phospholipid metabolic pathways, including those of *de novo* phospholipid biosynthesis, are essentially the same in fish as in mammals. However, there is now substantial evidence that at least some species of fish, both freshwater and marine, may have only a limited capacity to synthesise phospholipids *de novo* both at larval and early juvenile stages (see Coutteau et al., 1997). This may reflect the fact that many fish larvae receive an abundance of phospholipids in their natural diets, whether from yolk sac lipids prior to first feeding or from natural prey at and after first feeding. The same situation may well hold for *de novo* biosyntheses of cholesterol and sphingolipids, which have been scarcely studied in fish.

5.1 Early studies

During studies in the early 1980s investigating microparticulate diets as replacements for live feeds for rearing larval marine fish, Kanazawa and coworkers observed that dietary phospholipids were essential for normal growth and survival of fish larvae such as ayu (*Plecoglossus altivelis*) and red sea bream (*Pagrus major*) (Kanazawa, 1985). Improved growth and survival rates were obtained with 10-100 day-old larval ayu when the diets were supplemented with 3% chicken-egg lecithin, bonito-egg lecithin fraction (PC) or soybean lecithin (Kanazawa et al., 1981, 1983a). Bonito-egg cephalin fraction (PE) was not as effective as the PC fraction (Kanazawa et al., 1981, 1983a). Similar results were found with larval red sea bream (Kanazawa et al., 1983b). These workers also reported that the addition of some phospholipid to the microparticulate diet formulation reduced the incidence of malformations (Kanazawa et al., 1981, 1983a,b). These initial reports stimulated a significant amount of further research into phospholipid requirements of larvae and juveniles of a number of other fish species (see Coutteau et al., 1997). The following sections summarise these studies.

5.2 Limitations in studies

Studies investigating phospholipid requirements in fish have some limitations based on the requirement for a deficient basal diet. For juvenile fish, deficient diets can be formulated by careful choice of the raw ingredients. However pelleted diets are not suitable for marine fish larvae, and the traditional diets, live feeds, are unsuitable as they contain phospholipids and further enrichment can be difficult (Nordgreen et al., 2007). Therefore, microdiets are required, but they have been difficult to produce and were not available for all the studies (Baskerville-Bridges and Kling, 2000; Yufera et al., 2000; Cahu and Zambonino Infante, 2001; Koven et al.,

455 2001; Robin and Vincent, 2003; Kvale et al., 2006). Irrespective of whether pelleted feeds or
456 microdiets are used, they can both have problems of low palatability as the formulations must be
457 either fish meal-free or use defatted fish meal. As a result the majority of studies have been
458 performed with casein-based diets and the resultant impact on attractant and palatability factors
459 can lead to acceptance problems.
460 Supplementation of phospholipids to basal deficient diets can pose problems as, although
461 commercial phospholipid preparations (lecithins) are available, they are not pure products.
462 Lecithin is, strictly speaking, the trivial name for PC, but has been used commercially to describe
463 a range of phospholipid-enriched products usually obtained either from chicken eggs or as a by-
464 product of vegetable oil processing (see section 7.2.1). The commercially available lecithins can
465 vary greatly in purity, and have highly variable phospholipid contents and class compositions, as
466 well as very different fatty acid compositions. An alternative is to use pure phospholipid species
467 (classes), but these have limited availability and are costly, although it is possible to purify
468 individual phospholipid classes in the laboratory (Geurden et al., 1998a). Irrespective of the
469 specific phospholipid supplemented, replacement of one lipid class with another, i.e.
470 triacylglycerol with phospholipid, will invariably alter the fatty acid composition of the diets and
471 this can be difficult to fully control. This is also the case in comparing different phospholipid
472 preparations. Marine phospholipids were superior to soybean phospholipids in promoting growth
473 in turbot larvae, but fatty acid compositions were not controlled and so the marine phospholipid
474 diets also had far superior fatty acid profiles in terms of n-3 HUFA content (MacQueen Leifson
475 et al., 2003).
476 Finally, another major limitation over all the studies is that older fish have not been
477 investigated. The rationale presumably being that a requirement for intact phospholipids would
478 only manifest itself in fish receiving diets having very low levels of intact phospholipids. Once
479 being fed extruded diets based on fish meal, the diets will contain a luxus of intact phospholipids
480 of all classes and thus a requirement, if any, would be satisfied.
481
482 *5.3 Requirement criteria*
483 The criteria used to measure requirements for phospholipids have generally been gross
484 performance indicators including growth, survival and development. Thus, in most studies, the
485 requirement for intact phospholipid has been increased growth, improved survival and reduced
486 malformations or deformities (Coutteau et al., 1997). Effects of nutritional components, including
487 dietary phospholipids, on skeletal development in fish larvae have been reviewed recently (Cahu

et al., 2003a). A limited number of studies have also looked at stress resistance and showed that this could be increased by phospholipid supplementation (Takeuchi et al., 1992; Kanazawa, 1993; Weirich and Reigh, 2001). Few other physiological, metabolic, biochemical or molecular parameters have been investigated (Fontagne et al., 2000; Koven, 2003; Zambonino-Infante and Cahu, 2007; Hamza et al., 2008).

5.4 Range of species and developmental stages

A relatively defined quantitative requirement for dietary phospholipid has been determined in a number of fish including the marine species red sea bream, Japanese flounder (*Paralichthys olivaceus*), rock bream/knife jaw (*Oplegnathus fasciatus*), European sea bass (*Dicentrarchus labrax*), striped jack (*Pseudocaranx dentex*), gilthead sea bream (*Sparus aurata*) and turbot, and the freshwater species ayu, common carp, rainbow trout and white sturgeon (*Acipenser transmontanus*) (see Table 1 for references). A semi-quantitative requirement has also been determined in Atlantic salmon at the freshwater stage. The requirement for phospholipid has been demonstrated at both larval and juvenile stages for ayu, Japanese flounder, rock bream/knife jaw and European sea bass, in larvae for carp, red and gilthead sea bream, and in juveniles for striped jack, turbot, rainbow trout, Atlantic salmon and white sturgeon. Based on studies comparing a neutral lipid-rich commercial diet and a phospholipid-rich zooplankton diet, Olsen et al. (1991) suggested that polar lipids may promote growth in juvenile Atlantic cod, but further work is required to establish if there is a genuine requirement in this species. As mentioned previously, few studies have been conducted but a requirement for phospholipid has not been conclusively demonstrated in adults of any fish species (Olsen et al., 1999).

5.5 Quantitative requirements

Quantitative requirements have generally been reported in terms of phospholipid levels as a percentage of the diet by weight. However, some data should be regarded as semi-quantitative only, as the actual phospholipid content of the phospholipid preparations used in different studies, which varies as described above, is not always taken into account and so the absolute phospholipid level required may be lower than the value quoted in some cases. This also means that it is not always appropriate to directly compare values in different trials using different phospholipid preparations. Differences in the initial size of larvae or juveniles used, and the durations of the feeding trials also contribute to the semi-quantitative nature of the data. Coutteau et al. (1997) summarised the differences between the trials in all studies published up until the

date of that review. Acknowledging these caveats, values quoted for the quantitative requirements of phospholipids for larval fish range from about 2 - 12 % of diet (Table 1). Requirements were lower in the freshwater fish, carp (2 %) and ayu (3-5 %), and higher in the marine fish red sea bream (5 %), rock bream/knife jaw (5-7 %) and Japanese flounder (7%), with the highest reported value being for European sea bass at 12 % of diet. In juveniles, the values ranged from around 1.5 - 7 % of diet, including 1.5 % for striped jack, 2-3 % for European sea bass and around 4-6 % for Atlantic salmon, and 7 % for Japanese flounder. In Atlantic salmon, studies showed that the phospholipid requirement was 4 - 6 % of diet in fish of initial size 180 mg, 4 % in fish of 1-1.7 g initial weight, whereas in fish of 7.5 g initial weight, no requirement was observed. Similarly, in white sturgeon of 5-10 g, no requirement for dietary phospholipid was detected. Therefore, the data appear to suggest a possible trend with quantitative requirements decreasing with development, at least from larvae to small juveniles. No requirement has been observed in fish (at least salmon and white sturgeon) of greater than 5 g initial weight although this may be due to the short-term nature of the studies. Thus, in Atlantic salmon cultured over a 24-month growth cycle low dietary phospholipid might induce growth depression and other effects over this longer period of culture.

5.6 Qualitative requirements

There are few data available with which to compare the efficacy of different phospholipid classes in satisfying phospholipid requirements. First of all, few phospholipid preparations have been investigated, principally soybean lecithin (SL), egg lecithin (EL), but occasionally also some marine phospholipids (Table 1). As mentioned above, all of these preparations are mixtures of different phospholipid classes, but mainly PC, PE, PI and PS although the proportions vary. For instance, SL can contain 50 - 86 % phospholipid, with typically around 20 – 25 % each of PC, PE and PI. Therefore, it is not possible to draw any conclusions on the efficacy of individual phospholipid classes in trials using commercial crude phospholipid preparations. However, some studies have utilized semi-purified phospholipids, including soybean PC (80 - 98 % PC) or egg PC (95 % PC), soybean PE (99 % PE) and soybean PI (Geurden et al., 1998a). Thus, in the few studies in which comparisons are possible, both PC and PI were effective in meeting phospholipid requirements in larval ayu whereas PE was less effective (Kanazawa, 1983; Kanazawa et al., 1985). In Japanese flounder larvae, growth improved with PC, but not with PI or PE (Kanazawa, 1993). When carp larvae were fed diets containing either purified phospholipid classes (PC, PE, PS and PI), PC gave higher initial growth but induced deformities, whereas PI

554 gave optimal survival and minimal deformities (Geurden et al., 1998a). PE and PS gave
555 intermediate results, and hydrogenated PC or lyso-PC was not as effective as native PC.
556 Therefore, there are no clear patterns other than PC and PI may generally be more effective than
557 PE and PS, with PC perhaps being more important for growth and PI being more important for
558 normal development and prevention of malformations, at least in carp larvae. However, it is
559 possible that different phospholipid classes may play slightly different roles and thus have
560 different effects in different species as PI may have been more effective than PC in improving
561 growth in larval ayu (Kanazawa, 1983).
562
563 *6. Mechanisms of requirement for intact phospholipids in larvae and juveniles*
564
565 *6.1 Improved diet qualities*
566 It has been suggested that phospholipids may help to reduced leaching of water-soluble
567 micronutrients (minerals and vitamins) from semi-purified diets (see Coutteau et al., 1997).
568 Addition of phospholipid influenced the loss of dry matter in casein-based microdiets for post-
569 larval shrimp (Camara, 1994). In a study investigating phospholipid requirements for European
570 sea bass and turbot using extruded diets with or without 2 % EL (69% pure), a diet water stability
571 test showed no effect of phospholipid on dietary fatty acid contents but micronutrients were not
572 investigated (Geurden et al., 1997b). This possible effect of dietary phospholipid requires further
573 study.
574 Although phospholipids contain oxidatively sensitive PUFAs, there is some evidence that they
575 can express an antioxidative effect on various oils and fats (Ishihara, 1997). Satoh and Ishihara
576 (1997) tested various compounds, representative of the major functional groups in phospholipids,
577 for antioxidant activity in a sardine oil system and found choline and ethanolamine strongly
578 inhibited increases in peroxide values in a sardine oil mixture during storage, whereas PA
579 derivatives and glycerol did not. Antioxidant effect was thus attributable not only to the side-
580 chain amino groups but also to the cooperative effect of the hydroxy group in the side chain. The
581 precise mechanism of the antioxidant effect is unknown and various hypotheses include chelation
582 of transition metals, a synergistic effect with vitamin E, and hydroperoxide decomposing activity
583 (Ishihara, 1997).
584 PC enhanced feeding activity and diet ingestion rate of microdiets fed to sea bream larvae up
585 to day 30 post-hatch, which may suggest a role for dietary phospholipids as an age-dependent
586 feed attractant (Koven et al., 1998, 2001). An attractant property is likely to be dependent upon

587 the phospholipid head group as lecithin and certain L-amino acids had previously been tested for
588 attractant activity for aquatic organisms including weatherfish (*Misgurunus anguillicaudatus*)
589 and juvenile yellowtail (*Seriola quinqueradiata*) (Harada, 1987). The attraction activity of amino
590 acids and their derivatives was ascertained to largely depend on both alpha -carboxyl and alpha -
591 amino groups, but especially the former.
592
593 *6.2 Improved digestion*
594 Phospholipids are surface-active agents, or surfactants, and the main industrial use of lecithins is
595 as lipid emulsifiers particularly in processed foods. Fish diets, of course, do not require the
596 presence of phospholipids as emulsifiers per se, but their presence in the formulation may
597 improve lipid emulsification and aid digestion in the intestine of the fish after consumption.
598 Improved lipid digestibility in salmon fed diets containing soybean lecithin has been attributed to
599 emulsification properties of the phospholipid (Hung et al., 1997), and other studies have shown
600 that dietary phospholipids increased digestibility in juvenile fish (Craig and Gatlin, 1997; Kasper
601 and Brown, 2003). However, supplementation with bile acids did not mimic the effect of dietary
602 phospholipid in promoting growth in larval ayu (Kanazawa, 1983). In a study in gilthead sea
603 bream larvae, a lecithin–supplemented diet substantially increased the uptake of labelled lipids
604 from the diet and this was initially suggested to be via improved emulsification (Koven et al.,
605 1993). However, this result was not substantiated in subsequent studies on gilthead sea bream
606 fed with phospholipid-supplemented diets (Hadas et al., 2003). Phospholipid supplements also
607 aided the absorption of dietary neutral lipids in European sea bass post-larvae, but this was not
608 via a mechanism involving increased emulsification as diets containing other emulsifiers or lyso-
609 phospholipid, which has greater emulsification properties than phospholipid, did not replicate the
610 effect (Geurden et al., 1997c, 1998a). Therefore, there is little evidence to support a role for
611 dietary phospholipids as aids to digestion.
612
613 *6.3 Provision of essential components*
614
615 *6.3.1 Fatty acids (energy and essential fatty acids)*
616 Phospholipids can be an important source of energy (fatty acids) in fish, particularly during
617 embryonic and early larval development in species that produce phospholipid-rich eggs (Tocher,
618 1995). Larval fish at first feeding may be predisposed to digestion and metabolism of
619 phospholipids and the use of fatty acids from phospholipids for energy (Sargent et al., 1997).

620 Dietary lipid is also important as a source of essential fatty acids (EFA), and phospholipids tend
621 to be a richer source of EFA than neutral lipids such as triacylglycerols (Tocher, 1995). In
622 addition, phospholipids may be superior to neutral lipids as a source of EFA in larval fish due to
623 improved digestibility (Sargent et al., 1997, 1999b). Recently it was shown that phospholipids
624 were the more efficient mode of supply for dietary EPA and DHA to sea bass larvae (Gisbert et
625 al., 2005). However, in many experiments on phospholipid requirement, diets have been carefully
626 formulated to discriminate a phospholipid effect from a fatty acid effect with, for instance, diets
627 formulated to be EFA sufficient from neutral lipid alone (see Coutteau et al., 1997). In any case,
628 most of the studies on phospholipid requirement have been performed with soybean lecithin and
629 egg lecithin that are relatively rich in 18:2n-6 (and may have some 18:3n-3), but are deficient in
630 the n-3 HUFA, EPA and DHA, required in abundance by larval fish (Coutteau et al., 1997;
631 Sargent et al., 2002). In addition, further evidence that the beneficial effects of dietary
632 phospholipids are not due to the provision of EFA is that soybean lecithin, lacking n-3 HUFA is
633 as effective or more effective as lecithins from marine sources including fish eggs which are rich
634 in n-3HUFA (Kanazawa et al., 1981, 1983a; Geurden et al., 1995b). Therefore, there is good
635 evidence that the effects of dietary phospholipids on growth-promotion, survival and the
636 prevention of malformations are not due to the provision of EFA. Indeed, diets containing very
637 high levels of marine phospholipids and n-3HUFA actually induced skeletal malformations in sea
638 bass larvae (Villeneuve et al., 2005, 2006).
639
640 *6.3.2 Phosphorous*
641 Phosphorous is a nutritionally important mineral due to its requirement for growth, bone
642 mineralization, reproduction, nucleic acid synthesis, and energy metabolism (Lall, 2002).
643 Deficiency signs include reduced growth and skeletal deformities and quantitative requirements
644 have been determined for several fish species (Lall, 2002). As phosphate is low in most aquatic
645 environments (non-polluted), feed is the main source of phosphorous, mainly from meals or
646 premixes, with meat/bone meal > fish meal > plant meals. Bioavailability varies, but generally
647 the inorganic (calcium and potassium salts) and organic (phospholipids) forms found in fish
648 meals are more readily available to fish than the phytates (phytic acid salts) found in plant meals
649 (Lall, 2002). Many studies on phospholipid requirements have used casein- or soy protein-based
650 diets, but it is unlikely that this would be a problem with mineral premixes (Coutteau et al.,
651 1997). In the only study to date, the effects of dietary phosphorus and phospholipid level on
652 growth and phosphorus deficiency signs were investigated in juvenile (1 g) Japanese flounder

(Uyan et al., 2007). The results showed there was no interaction between dietary phosphorous and phospholipid suggesting that supply of phosphorous was not a mechanism for the growth promoting effects of dietary phospholipids.

6.3.3 Choline or inositol

It is known that choline cannot be synthesised in animals (Lykidis, 2007), but there may be evidence that some inositol can be synthesised in carp intestine and channel catfish (see Halver, 2002) and may not be required by juvenile sunshine bass (Deng et al., 2002). However, both choline and inositol are regarded as vitamins for fish, as there are known deficiency signs for both, and quantitative requirements for growth have been defined, at least for choline (Halver, 2002). Both choline and inositol are quite ubiquitous in feed ingredients including wheat germ, fish and plant (bean) meals, but they are often also supplemented in vitamin premixes. In studies with carp larvae, the growth-promoting and prevention of malformation effects of PC and PI were not mimicked by choline and inositol (Geurden et al., 1995b).

6.4 Provision of intact phospholipid

Over twenty years ago, based on his own series of studies with larval ayu, red sea bream and Japanese flounder, Kanazawa speculated that phospholipid biosynthesis itself may be limiting in larval fish. He reasoned that, as larval fish require an abundance of phospholipid for new tissue growth, "biosynthesis of phospholipids may not take place at a sufficiently fast rate to meet phospholipid requirement" and "requirement for dietary phospholipids may diminish with increasing age" (Kanazawa, 1985). However, it was another 10-15 years before there was a sufficient body of evidence to support the hypothesis of limited phospholipid biosynthetic ability in early life stages of fish.

Several studies showed that larval diets deficient in phospholipid could lead to the accumulation of lipid vacuoles (droplets) in the intestinal enterocytes (Fontagné et al., 1998; Olsen et al., 1999; Salhi et al., 1999; Liu et al., 2002). For example, intestinal steatosis (fat drop accumulation) was induced in carp larvae fed phospholipid-deficient diets and PC and, to a lesser extent, PI, prevented this (Fontagné et al., 1998). These studies led to the suggestion that intact dietary phospholipids may be required for the efficient exportation of dietary lipids from the enterocytes (Fontagné et al., 1998; Geurden et al., 1998b; Olsen et al., 1999; Salhi et al., 1999). Supporting this, in sea bream larvae fed phospholipid-deficient diets containing radiolabelled 18:1n-9, increased transport of ^{14}C-18:1n-9 free fatty acid from enterocytes to tissues was

686 observed upon supplementation with PC (Hadas et al., 2003). As described in section 4.3, dietary
687 lipids are transported from the intestine in the form of the large, neutral lipid-rich lipoproteins,
688 chylomicrons and, to a lesser extent, VLDL and so it appeared that, in fish fed phospholipid-
689 deficient diets, there may be insufficient lipoprotein synthesis. Lipoprotein production in the
690 enterocyte involves a series of biosynthetic processes whereby neutral lipids, triacylglycerol and
691 cholesteryl esters, form a large hydrophobic core surrounded by a thin coat of protein
692 (apoproteins), phospholipid (particularly PC) and cholesterol (Vernier and Sire, 1986). Clearly,
693 intact phospholipid is required for lipoprotein assembly, and so it was proposed that the
694 stimulating effects of phospholipids in larval fish growth were due to the fish larvae having a
695 limited ability to biosynthesise phospholipids de *novo* (Geurden et al., 1995b, 1999; Coutteau et
696 al., 1997; Fontagné et al., 1998). However, the Golgi are intimately involved in the secretion of
697 lipoproteins from the enterocytes, which may implicate another role for phospholipids in this
698 mechanism. De novo PC synthesis has been shown to be required to replenish the Golgi-
699 associated PC pool and to support Golgi-mediated secretion of tumour necrosis factor α in mouse
700 macrophages (Tian and Jackowski, 2007).
701 Although the limiting biosynthesis hypothesis is quite widely accepted now, there is actually
702 very little biochemical or enzymological data to support it. The major question is, what
703 enzymatic step or steps are responsible for the limitation in phospholipid biosynthesis in the
704 enterocyte? There is plenty of evidence that fish larvae readily reacylate dietary glycerides
705 including lyso-phospholipids and so there is no limitation in acylation reactions (see Sargent et
706 al., 2002). Thus, Sargent et al. (2002) speculated that the limitation in phospholipid biosynthesis
707 in larvae is in forming the glycerophosphobase backbone (e.g. glycerophosphocholine or
708 glycerophosphoethanolamine) of the phospholipid molecule. Unfortunately, due to the
709 complexity of the phospholipid synthesis pathways (Lykidis, 2007), this does not narrow the
710 search down significantly as there are many possible enzymatic steps that could be involved.
711 Possibly the most obvious candidates are the CDP-choline and CDP-ethanolamine
712 phosphotransferases (CPT and EPT) involved in the conversion of DAG to PC and PE,
713 respectively. Further enzymes are required to produce the CDP-choline and CDP-ethanolamine
714 required by the phosphotransferases, but synthesis of CDP-choline is not limiting as it was
715 shown that CDP-choline could not mimic the effect of PC on the improvement of survival rates
716 in larval ayu (Kanazawa, 1983). However, limitations in CPT and EPT cannot account for the
717 observed effects of PI, as it is not formed via the CPT/EPT pathways. This suggests that the

718 limiting step would have to be further back in the phospholipid synthesis pathway, but there is no
719 single enzyme that could be responsible for limiting both PC and PI synthesis. PA is the common
720 intermediate in the production of PC and PI but different enzymes, phosphatidate phosphatase
721 and CDP-DAG synthase, convert PA to DAG and CDP-DAG, the precursors of PC and PI,
722 respectively (see section 3.1). There is no direct evidence and no studies investigating the
723 pathways of phospholipid biosynthesis in larval fish and so the enzymic location(s) of any
724 limitation in these pathways in larval and early juvenile fish is still unknown.
725 Sargent et al. (2002) also speculated that such apparent limitations in the ability of fish larvae
726 to synthesise phospholipids *de novo* may not be surprising since, in their natural environment,
727 larvae ingest live feed whose lipid is predominantly phospholipid. Thus, the larvae will seldom if
728 ever be required to biosynthesise phospholipids extensively *de novo*. It is possible that early
729 developing fish larvae have limited lipid biosynthetic capabilities in general, whether for fatty
730 acids or lipid classes, including cholesterol.
731
732 *7. Dietary phospholipids and sustainability*
733
734 *7.1 Sustainability*
735 Exploitation and, indeed, over-exploitation of wild fisheries has meant that an increasing
736 proportion of fish for human consumption is provided by aquaculture, which has been expanding
737 at around 10% per year over the last decade (Tidwell and Allan, 2002). Paradoxically, diets for
738 aquaculture have been based traditionally on fishmeals and oils, themselves derived from
739 industrial feed-grade or reduction fisheries, as the predominant protein and lipid sources (Sargent
740 and Tacon, 1999; Pike, 2005). This is certainly the case in Europe where intensive farming is
741 based largely on carnivorous species that feed high up in the food chain, particularly salmonid
742 and marine species. The demand for these marine products is rapidly increasing and current
743 estimates suggest requirements for aquaculture feeds could exceed global supplies of fish oil and
744 fish meal within the next two to eight years, respectively (FAO, 2006). Therefore, if aquaculture
745 is to continue to expand and supply more of the global demand for fish, alternatives to fish oil
746 and meal must be found (Barlow, 2000; Tacon, 2004). Under the present regulations, other
747 animal-based meals such as feather, meat, bone and blood meals are not permitted for use in fish
748 diet formulations in the European Union. The only obvious, and sustainable, alternative to marine
749 meals are plant meals, but these contain very little, if any, phospholipid in comparison with fish
750 meals (Sargent et al., 2002). Therefore, replacing the fish meal component of diets with plant

meals will result in greatly reduced phospholipid concentrations in diets. This means that possible phospholipid requirements, if any, of adult and ongrowing fish, may become an important and significant issue in the future as plant meals comprise an increasing proportion of diet formulations.

As mentioned previously, there have been no studies investigating phospholipids requirements in adult fish, or individuals of much more than 5 g. However, interestingly, accumulation of lipid droplets was reported in the hindgut of juvenile gilthead sea bream fed diets based on plant proteins and fish oil (Sitjà-Bobadilla et al., 2005). Similar histological alterations in gilthead sea bream intestine had been reported earlier in studies investigating replacement of fish oil with vegetable oils (Caballero et al., 2003). Similarly, substantial accumulation of lipid droplets was observed in the gastrointestinal tract of salmonids fed with vegetable oils, and this condition was reversed by phospholipid supplementation (Olsen et al., 1999, 2003). Liver steatosis was also observed in gilthead sea bream fed plant meals (Sitjà-Bobadilla et al., 2005) or vegetable oils (Caballero et al., 2004; Wassef et al., 2007). Perhaps related, there is some evidence that soybean PC may alleviate similar signs of liver disease in human and animal studies (Canty and Zeisel, 1994; Ipatova et al., 2004). Therefore, there is circumstantial evidence that perhaps may indicate that dietary phospholipid supplementation may be beneficial in larger (on-growing juvenile or adult) fish particularly when fed diets high in plant products.

7.2 Dietary sources of phospholipids

7.2.1 Oils

The phospholipid contents of refined oils, both fish oils and vegetable oils, are very low as they are removed during the normal refining processes. Degumming separates the phospholipids as a gum. Crude soybean oil contains around 1.5 - 3.1 % total phospholipid that is removed to become the by-product, soybean lecithin (Daniel, 2004). As mentioned above, soybean lecithin can have very variable phospholipid contents and class compositions but most commonly contains around 50 – 60 % total phospholipid, and around 13 - 18% PC, 10 - 15% PE, 10 – 15 % PI and 5 – 12 % PA (Daniel, 2004). Crude sunflower oil can have a phospholipid content of 0.5 – 1 % and is also used to produce lecithin having similar phospholipid class profile to soybean lecithin. Most other crude vegetable oils have less phospholipid, up to about 0.5 % total phospholipid before degumming, but due to the large production, rapeseed/canola lecithin is also commercially available (Daniel, 2004).

784

785 *7.2.2 Meals*

786 Unless rather crude oils are used, the vast majority of phospholipids in fish diets will be provided
787 by meals and other essentially "protein" components. Residual lipid contents in fish meals can be
788 reasonably high, varying from 5 – 13 % of weight, with a triacylglycerol/phospholipid ratio of
789 around 2:1 (de Koning, 2005). Phospholipid content of meals has been suggested as a quality
790 index as fresh fish generally contain a rather constant amount of phospholipids that decrease
791 rapidly upon storage (de Koning, 2005). In fish feeds, phospholipid can account from 5 – 25 % of
792 the total lipid depending on lipid content and formulation of the feed, and analytical method used
793 (Johnson and Barnett, 2003). Individual phospholipid class compositions for fish meal are
794 difficult to find, but should generally reflect the phospholipid composition of fish (PC, PE, PS,
795 PI, PA and lyso-phospholipids) (see Tocher, 1995). Generally, most plant meals have much
796 lower levels of residual phospholipids, mainly due to the original products (seeds/beans etc.)
797 having much lower phospholipid contents. However, soybean has the highest levels with full-fat
798 soybean meal (FFSM or soy flour) having about 20 – 25 % lipid with only around 0.3 - 0.6 %
799 phospholipid, primarily PC, PE, PI and PA. Defatted soybean meal, prepared by solvent
800 extraction, has < 1 % lipid with soybean cake (produced by pressing) possibly having slightly
801 higher lipid content. Very little information is available about the lipid and phospholipid contents
802 and compositions of other plant meals.

803

804 *7.3 Analysis of phospholipid content and composition in fish diets and ingredients*

805 Although not an exhaustive list, Table 2 indicates that the phospholipid content of commercially
806 available fish meals can vary considerably. In this sample of four meals, the phospholipid content
807 varied from around 17 to 27 % of total lipids. The phospholipid content appears to be largely
808 independent of lipid content of the meals, which varied from around 6 to 10 % of the wet weight
809 giving phospholipids contents of between 1.2% and almost 2.4% of wet weight of the fish meals.
810 The predominant lipid classes were PC and PE in a roughly 2:1 ratio, followed by around 1 - 3 %
811 of the other phospholipids classes PI, PS and sphingomyelin. Standard, commercial feeds
812 formulated predominantly with fish meal will therefore show a range of phospholipid contents
813 from around 1 % to around 4 % depending upon lipid content of the diet (Table 2). Thus, diets
814 with lower lipid contents (lower levels of added oil) have a higher phospholipid content that is
815 predominantly PC, with PE also detected in the diets with higher phospholipid content.

The phospholipid contents and compositions of a variety of feed ingredients including alternative protein sources are presented in Table 3. The two fish oils analysed (northern and southern hemisphere oils) did not contain any detectable phospholipids. However, the rapeseed oil contained around 1.7% phospholipids, specifically PE and PC. The cereal products, which had reasonably high lipid contents (~ 6 to 10%), showed markedly different phospholipid contents with corn gluten having very low levels (0.5%) whereas the phospholipid content of wheat gluten was almost 7% of total lipids, although it also contained high levels of galactolipids and other glycolipids (20%). Sunflower cake, which also had a relatively high lipid content at 8 %, contained phospholipids at 9 % of total lipid and a lower amount of galactolipid. The high protein soy products analysed (HP soya and SPC 60) contained only 1 – 2 % lipid, but it was relatively rich in phospholipids amounting to 28 – 40 % of total lipid. Peas were not lipid-rich (1.8%) but almost 30% was present as phospholipids. The animal-derived protein alternatives showed markedly different phospholipids contents. The haemoglobin and blood meals, although relatively low in lipids contained phospholipids at between 22 and 31% of total lipid, whereas the feather meals contained 6-7% lipid, but had very low or no phospholipids. Binders (field beans and kidney beans) were also low fat products with 12-28% of the total lipid being phospholipids. The rank order for the phospholipid classes in the soy and legume products was generally PC > PE > PS > PI. The legumes and soy products also contained galacto- and other glycolipids at around about 10% of total lipid. The lecithin analysed (BergaPur) was 75 % lipid of which two-thirds was phospholipids (PC, PE, PS and PI in roughly similar proportions), along with around 15 % glycolipids and 17% other neutral lipids, mainly triacylglycerol.

7.4 Dietary cholesterol

Although not the subject of the present review it is pertinent to mention that, in addition to reducing the level of dietary phospholipids, the increasing proportion of plant meals in dietary formulations will also reduce the level of dietary cholesterol. This may not be obvious from the lipid class compositions presented in Tables 2 and 3, as these data were generated by thin-layer chromatography (TLC) followed by staining and densitometric analysis (Henderson and Tocher, 1992), and this method cannot distinguish between cholesterol and other sterols. However, cholesterol is the predominant sterol in animals, whereas other sterols are present in plant tissues (Padley et al., 1986). The TLC/densitometric method also has other confounding factors such as co-eluting lipids (e.g. diacylglycerols) and the fact that sterols are generally overestimated as a result of their different staining compared to fatty acid-containing lipids (Henderson and Tocher,

849 1992). Indeed, quantitative cholesterol determination remains a difficult analysis (Christie, 1989).
850 The cholesterol (as opposed to total sterol) content of traditional salmonid feeds and a variety of
851 alternative feed ingredients as determined by gas chromatography (see Christie, 1989) is listed in
852 Table 4. This clearly shows that plant products, both oils and meals, contain virtually no
853 cholesterol. Feeds traditionally formulated with fish meal and fish oil will provide at least 1 g of
854 cholesterol per kg feed. Substitution of fish meal and/or fish oil will greatly reduce the level of
855 cholesterol supplied by the feed. The only alternatives to fish meal and oils that could supply
856 dietary cholesterol are the other animal-derived products, the blood and feather meals (Table 4).
857 Although there have been a few early studies investigating dietary supplementation of
858 cholesterol in salmonids, primarily in relation to the development of coronary arteriosclerotic
859 lesions (Farrell and Munt, 1981; Farrell et al., 1986), there are few reports on the effects of
860 dietary cholesterol on growth or metabolism in fish. However, the effects of dietary cholesterol
861 supplementation on growth, organ indices, digestibility of cholesterol and macronutrients, and
862 tissue fatty acid compositions have been investigated in Atlantic salmon (Bjerkeng et al., 1999).
863 This study showed that dietary cholesterol level had no significant effect on specific growth rate
864 (SGR), mortality, apparent digestibility coefficients of macronutrients, and total lipid content.
865 However, both hepatic cholesterol concentration and hepatosomatic index were increased by the
866 dietary cholesterol supplement. In a very recent study, it was shown that genes of the cholesterol
867 biosynthesis pathway in liver were up-regulated in salmon fed a vegetable oil blend compared to
868 fish fed a diet containing fish oil (Taggart et al., 2008). The effects on cholesterol metabolism
869 suggest this may also be an area requiring consideration in the future as dietary cholesterol
870 concentrations decline as the proportion of plant meals and oils in dietary formulations increases.
871
872 **8. Conclusions**
873
874 Intact phospholipids are required for optimal growth, survival, prevention of skeletal deformities
875 and, possibly, stress resistance in larval and early juvenile fish, of both marine and freshwater
876 species. The quantitative requirement appears to diminish with age, and no requirement has been
877 demonstrated in fish greater than 5 g, although there are very few studies and most are of short
878 duration. Qualitative requirements suggest that PC may be more important for growth and PI for
879 development. The mechanism of apparent essentiality of phospholipids in larval and early
880 juvenile fish is not clear. Most potential mechanisms can be discounted, leaving “limiting
881 biosynthesis” as the leading hypothesis. It is suggested that phospholipid biosynthesis pathways

882 are inefficient or not fully developed in larvae of a number of (possibly all) fish species. In larvae
883 fed diets rich in triacylglycerol, lack of sufficient dietary phospholipid limits lipoprotein
884 synthesis in enterocytes, leading to impaired transport of lipid (energy supply) nutrients to
885 tissues. Dietary phospholipid may provide intact glycerophosphobase backbones, which bypasses
886 this limitation. Thus, growth stimulation is due to improved transport, assimilation and utilization
887 of dietary lipid.
888
889 **References**
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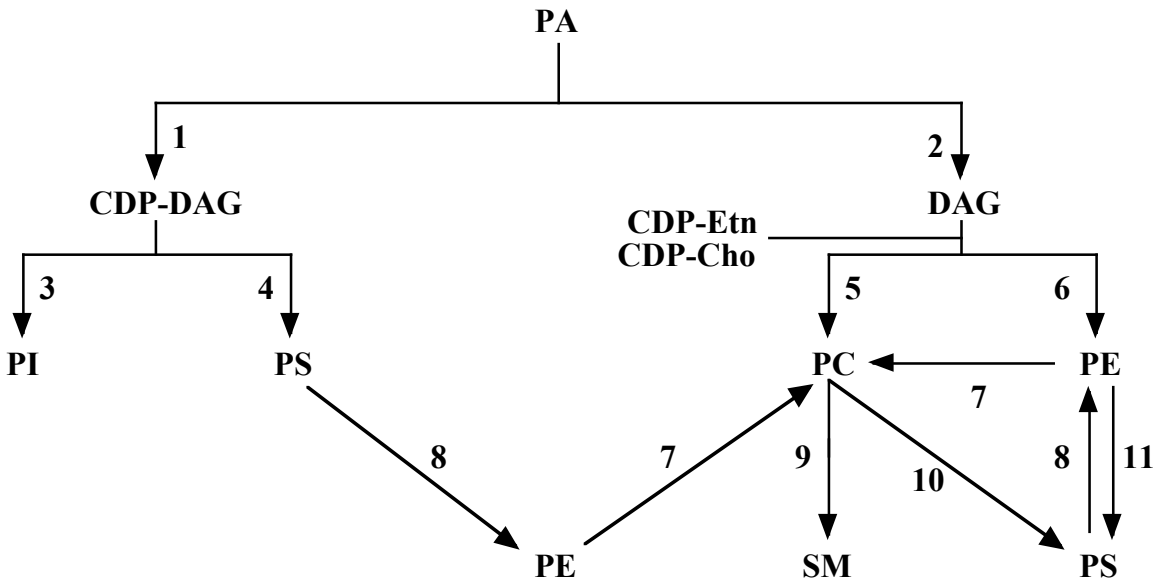

Fig.1. Biosynthetic pathways for the major phospholipid classes. CDP-Cho, CDP-choline; CDP-DAG, CDP-diacylglycerol; CDP-Etn, CDP-ethanolamine; DAG, diacylglycerol; PA, phosphatidic acid: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin. The enzymes are;- 1, CDP-DAG synthases; 2, PA-phosphatases; 3, PI synthase; 4, PS synthase; 5, CDP-choline:DAG phosphotransferase; 6, CDP-ethanolamine:DAG phosphotransferase; 7, PE methyltransferase; 8, PS decarboxylase; 9, SM synthase; 10 and 11, PS synthases via base exchange.

Table 1. Quantitative and qualitative phospholipid requirements of teleost fish

Species	Developmental stage	Phospholipid supplement[a] and levels studied[b]	Optimal requirement and criteria used[c]	Feeding period	Reference
Ayu	Larvae	0 and 3 % SL or EL	3 % (G,S,M)	20 days	Kanazawa et al. 1981
(*Plecoglossus altivelus*)	Larvae	0, 1, 3 and 5 % SL	3 % (M), 5 % (G,S)	50 days	Kanazawa et al. 1983a
	Larvae	0 and 3 % EL or BPL	3 % (G,S,M)	50 days	Kanazawa et al. 1983a
	Juvenile	0 and 3 % SL or BPL	3 % (G)	33 days	Kanazawa et al. 1981
	Juvenile	0, 1, 3 and 5 % EL	3 % (G)	33 days	Kanazawa et al. 1981
Japanese flounder	Larvae	0, 3, 5 and 7 % SL	7 % (G,S)	30 days	Kanazawa 1993
(*Paralichthys olivaceus*)	Juvenile	0, 3, 5 and 7 % SL	7 % (G)	30 days	Kanazawa 1993
Knife jaw	Larvae	0, 2.5, 5 and 7.4 % SL	7.4 % (G,S)	22 days	Kanazawa et al. 1983b
(*Oplegnathus fasciatus*)	Larvae	0, 3, 5 and 7 % SL	5 % (G,S,R)	28 days	Kanazawa 1993
	Juvenile	0, 3, 5 and 7 % SL	3 % (G)	60 days	Kanazawa 1993
European sea bass	Larvae	3, 6, 9 and 12 % SL	12 % (G,S,M)	40 days	Cahu et al. 2003b
(*Dicentrarchus labrax*)	Juvenile	0 and 3 % SL	3 % (G)	40 days	Geurden et al. 1995a
	Juvenile	0 and 2 % EPC or SPC	2 % (G)	40 days	Geurden et al. 1995a
Red sea bream	Larvae	0 and 5 % SL	5 % (G,S)	20 days	Kanazawa et al., 1983b
(*Pagrus major*)					
Common carp	Larvae	0 and 2 % EL	2 % (G,S)	25 days	Geurden et al. 1995b
(*Cyprinus carpio*)	Larvae	0 and 2 % PL	2 % (G,S)	21 days	Geurden et al. 1995b
	Larvae	0 and 2 % SPC, SPI or EL	2 % (G,S,M except EL)	25 days	Geurden et al. 1997a
	Larvae				Geurden et al. 1998
Pikeperch	Larvae	1, 5 and 9 % SL	9 % (G)	24 days	Hamza et al. 2008
(*Sander lucioperca*)					
Gilthead sea bream	Larvae	9, 11 and 15 % SL	> 9 % (G,S)	23 days	Seiliez et al. 2006
(*Sparus aurata*)					
Striped jack	Juvenile	0, 0.5, 1, 1.5 and 2 % SPC	1.5 % (G,S,R)	6 weeks	Takeuchi et al. 1992
(*Pseudocaranx dentex*)	Juvenile	0 and 1.5 % SPE	1.5 % (G)	6 weeks	Takeuchi et al. 1992
Rainbow trout	Juvenile	0, 2, 4 and 8 % SL	4 % (G)	20 weeks	Poston 1990a
(*Oncorhynchus mykiss*)	Juvenile	0 and 14 %	14% (G)	8 weeks	Rinchard et al. 2007
Turbot	Juvenile	0 and 2 % EL	2% (G)		Geurden et al. 1997b
(*Psetta maximus*)					
Atlantic salmon	Juvenile (180 mg)	0, 2, 4, 6 and 8 % SL/CPL	6 % (G)	14 weeks	Poston 1991
(*Salmo salar*)	Juvenile (180 mg)	0 and 4 % SL	4 % (G)	16 weeks	Poston 1990b
	Juvenile (1.0 g)	0 and 4 % SL	4 % (G)	12 weeks	Poston 1990b
	Juvenile (1.7 g)	0 and 4 % SL	4 % (G)	12 weeks	Poston 1990b
	Juvenile (7.5 g)	0 and 4 % SL	0 % (no requirement)	12 weeks	Poston 1990b
White sturgeon	Juvenile (5-10 g)	0 and 8% SL	0% (no requirement)	6 weeks	Hung and Lutes 1988
(*Acipenser transmontanus*)					

[a] BPL, bonito egg polar lipid; CPL, corn polar lipid; EL, chicken egg lecithin; EPC, purified egg phosphatidylcholine ; PL, various phospholipid sources supplemented to supply 2 % dietary phospholipids including EL, SL, sunflower, rapeseed and marine phospholipids: SL, soybean lecithin; SPC, purified soybean phosphatidylcholine; SPE, purified soybean phosphatidylethanolamine; SPI, purified soybean phosphatidylinositol; [b] Percentage of diet weight; [c] G, growth; S, survival; M, malformations; R, stress resistance.

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Table 2. Lipid content (percentage of wet weight) and lipid class composition (% total lipid) of commercially available fishmeals and traditional feeds for salmonids based on high levels of fishmeal

	Fishmeals				Feeds	
Lipid class	1	2	3	4	A	B
Lipid content	10.4	10.0	8.5	5.9	16.5	34.4
Phosphatidylcholine (PC)	7.5	12.1	11.8	9.3	2.5	1.1
Phosphatidylethanolamine	4.1	5.8	6.7	3.9	1.4	0.0
Phosphatidylserine	0.9	1.4	1.6	0.7	0.0	0.0
Phosphatidylinositol	1.2	1.8	2.1	1.9	0.0	0.0
CL/PG	0.6	1.6	1.5	2.7	0.0	0.0
Sphingomyelin	1.6	1.3	1.5	1.5	0.0	0.0
Lyso-PC	1.0	1.3	1.5	0.5	0.0	0.0
Total polar lipids	16.9	23.4	26.7	20.4	3.9	1.1
Total neutral lipids	83.2	76.6	73.3	79.6	96.1	98.9
Triacylglycerol	55.0	44.3	47.7	66.5	69.2	78.6
Free fatty acids	15.3	14.2	15.1	5.6	11.9	9.3
Sterols	9.3	8.9	9.9	7.5	10.6	8.8
Steryl esters	3.6	9.1	0.7	0.0	4.5	2.2

Values are means of duplicate analyses. CL, cardiolipin; PG, phosphatidylglycerol.

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Table 3. Lipid content (percentage of wet weight) and lipid class composition (% total lipid) of feed ingredients

	Oils				Meals											Binders			Lecithin
Lipid class\diet	RO	SO	NFO	SFO	Corn gluten	Wheat gluten	SF cake	Soy SPC60	HP soya	Peas	Rice protein	Blood meal	Hb meal	German FeaM	French FeaM	Field beans	Raw KB	Cooked KB	BergaPur
Lipid content	~100	~100	~100	~100	5.6	9.9	8.0	0.8	1.9	1.8	9.6	1.4	1.0	6.8	6.6	1.5	1.7	1.4	74.5
Phosphatidylcholine (PC)	0.5	0.0	0.0	0.0	0.5	2.2	4.7	9.6	14.6	13.6	0.5	7.8	9.8	0.0	0.6	12.4	3.8	7.9	19.8
Phosphatidylethanolamine	1.2	0.0	0.0	0.0	0.0	2.8	1.4	6.7	7.8	7.6	0.3	3.7	5.5	0.0	0.0	7.2	1.5	5.9	17.6
Phosphatidylserine	0.0	0.0	0.0	0.0	0.0	0.0	2.0	6.3	9.4	4.7	1.0	0.6	1.3	0.0	0.0	5.2	2.9	6.1	15.0
Phosphatidylinositol	0.0	0.0	0.0	0.0	0.0	1.8	0.6	4.8	5.5	1.5	0.7	1.7	2.6	0.0	0.0	1.6	2.2	0.8	12.6
PG/PA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.3	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0
Sphingomyelin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.9	11.8	0.0	0.3	0.0	0.0	0.0	0.0
Lyso-PC	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.8	1.6	0.5	4.9	1.1	0.0	0.0	0.2	0.7	1.1	1.0	1.0
Total phospholipid	1.7	0.0	0.0	0.0	0.5	6.8	9.0	28.2	38.9	28.9	7.7	21.8	31.0	0.0	1.1	27.8	11.5	21.7	66.1
MGDG	0.0	0.0	0.0	0.0	0.0	2.4	3.3	4.1	4.2	3.1	1.7	0.0	0.0	0.0	0.0	2.7	4.0	3.9	9.8
DGDG	0.0	0.0	0.0	0.0	0.0	9.7	0.0	0.9	2.0	2.0	0.4	0.9	0.0	0.0	0.0	2.1	1.8	0.8	2.7
Other glycolipids	0.0	0.0	0.0	0.0	0.0	7.8	0.0	2.2	4.3	3.6	0.9	0.2	1.2	0.4	0.0	3.6	6.9	4.9	4.3
Total polar lipids	1.7	0.0	0.0	0.0	0.5	26.7	12.4	35.4	49.4	37.6	10.7	22.8	32.1	0.4	1.1	36.1	24.2	31.3	82.9
Total neutral lipids	98.3	100.0	100.0	100.0	99.5	73.3	87.6	64.6	50.6	62.4	89.3	77.2	67.9	99.6	98.9	63.9	75.8	68.7	17.1
Triacylglycerol	74.0	83.3	83.9	73.3	52.1	46.3	64.8	47.6	33.0	38.6	39.0	15.5	6.6	31.7	34.0	37.2	30.0	40.5	12.7
Free fatty acid	9.6	8.0	9.3	12.9	17.0	12.4	7.1	6.2	4.2	6.2	36.1	13.5	10.6	39.1	31.8	9.8	32.9	14.1	1.6
Total sterols	8.2	7.9	6.8	10.0	19.3	7.4	5.7	4.0	8.1	10.2	12.0	29.4	35.4	17.6	17.9	8.2	11.8	10.9	2.8
Steryl ester	6.5	0.8	0.0	3.9	11.1	2.1	3.4	3.9	0.5	3.0	0.0	18.9	15.2	11.1	15.2	3.0	1.0	0.5	0.0
Unknown neutral lipid	0.0	0.0	0.0	0.0	0.0	5.2	6.6	2.9	4.8	4.4	0.0	0.0	0.0	0.0	0.0	5.7	0.0	2.7	0.0

Values are means of duplicate analyses. BergaPur is a deoiled lecithin (98%) (Berg+Schmidt, Hamburg, Germany); DGDG, digalactosyldiacylglycerols; FeaM, feather meal; Hb, Heamoglobin; KB, kidney beans; MGDG, monogalactosyldiacylglycerols; NFO, North Atlantic fish oil; PA, phosphatidic acid; PG, phosphatidylglycerol; RO, rapeseed oil; SF, sunflower; SFO, South American fish oil; SO, soybean oil.

Table 4. Cholesterol content (mg/100g product) of traditional feeds and alternative feed ingredients

	Product	Cholesterol content
Feeds	Feed A	100.2
	Feed B	112.5
Oils	Rapeseed oil	5.9
	Soybean oil	4.8
	North Atlantic fish oil	359.9
	South American fish oil	324.3
Meals	Corn gluten	1.0
	Wheat gluten	8.1
	Sunflower cake	1.0
	SPC 60	0.2
	High protein soya	0.1
	Peas	0.4
	Rice protein	6.8
	Blood meal	218.7
	Haemoglobin meal	251.8
	German feather meal	134.3
	French feather meal	142.9
Binders	Field beans	4.3
	Raw kidney beans	1.1
	Cooked kidney beans	0.4
Lecithin	Bergapur	7.4

Values are means of duplicate analyses.