# VITELLOGENESIS IN TELEOST FISH

## A study of vitellogenin and egg lipids

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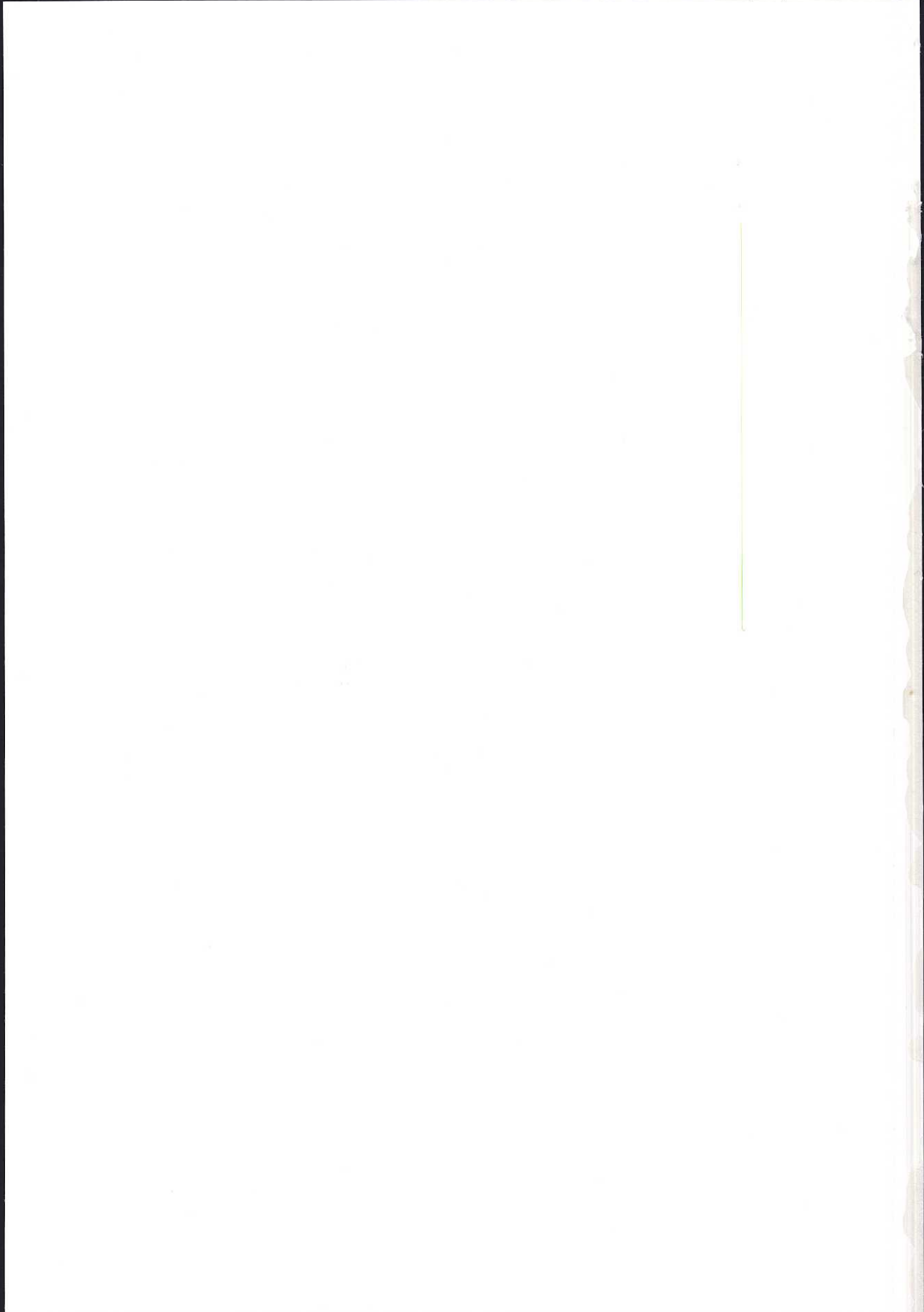
# VITELLOGENESIS IN TELEOST FISH

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*Dissertation abstract*

SILVERSAND, CHRISTER: **Vitellogenesis in teleost fish. A study of vitellogenin and egg lipids**. Department of Zoophysiology, Göteborg University, Medicinaregatan 18, S-413 90 Göteborg, Sweden

The overall objective of the present thesis was to gain information about lipid metabolism in female fish during reproduction. Special attention has been paid to the egg yolk precursor, vitellogenin, and the role this lipoprotein plays in the process of lipid accumulation in growing oocytes. The approach was to comprehensively examine the lipid class and fatty acid composition of vitellogenin and eggs from different teleost species, and to use this information to evaluate the relationship between the lipid moiety of vitellogenin and eggs.

Vitellogenin was purified from plasma of several teleost species by precipitation with EDTA:$Mg^{2+}$, distilled water and high-performance ion-exchange chromatography. Thin-layer chromatography and high performance liquid chromatography combined with light scattering detection was employed for lipid class analysis and gas-liquid chromatography was used for the separation and quantification of fatty acids.

Vitellogenin of cod, rainbow trout, turbot and wolffish contained 16-18 % lipid (dry wt), which was characterised by a high content of polar lipids. The lipids of vitellogenin were rich in polyunsaturated fatty acids (PUFA), predominantly 20:5(n-3) and 22:6(n-3), which comprised about 50 % of the total fatty acids. The lipid content, lipid class composition and distribution of fatty acids of vitellogenin were species specific and highly consistent, indicating a strict control of the lipidation of vitellogenin.

Turbot eggs contained 14 % lipid (dry wt), and were composed of approximately equal amounts of polar and neutral lipids. Most of the neutral lipid classes were confined to the oil globule, which contained triacylglycerol, cholesterol- and wax esters only. Of the total lipids in turbot eggs, more than 50 % was present in the oil globule. Turbot egg lipids were rich in PUFA, with considerably more (n-3) fatty acids than (n-6), and accounted for 45 % of total fatty acids. The abundance of (n-3) PUFA implies that the developing fish embryo has a requirement for high levels of (n-3) PUFA.

Vitellogenin is an important source of egg lipids in teleost fish, and is most likely the major source of polar lipid classes in fish eggs. In cod, a species without egg oil globules, the accumulation of lipids in growing oocytes is suggested to occur principally via the uptake of vitellogenin. In contrast, the differences in the lipid class and fatty acid composition of turbot eggs compared to turbot vitellogenin indicates that the egg lipid deposition can not be considered exclusively as an uptake of vitellogenin. Since turbot vitellogenin mainly transports polar lipids, it can be hypothesised that vitellogenin deposits lipids in the egg yolk, which primarily consists of polar lipids, while lipids of the oil globule originate from other sources.

**Keywords**: vitellogenin, egg, lipid, fatty acid, estradiol-17β, teleost, fish, oocyte development

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## Christer Silversand

# Till Linda, Erik och Sigrid

*En snigel springer i galopp*
*mot salladsbladets högsta topp.*
*Han rusar fram i fjorton dar*
*nu har han bara resten kvar.*

*En snigel vet nog vad han vill*
*han störtar fram en vecka till.*
*Då är han framme vid sitt mål*
*Men trillar rakt ner i ett hål.*

*Han tänker: Så kan det gå*
*för den som bara rusar på!*
*Nu är jag vid min utgångspunkt*
*Jag tror att jag kan ta det lungt.*

(Lennart Hellsing)

Cover photo shows a freshly ovulated turbot egg with the oil globule

discernible in the centre

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This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I.    Silversand, C. and Haux, C. (1989) Isolation of turbot (*Scophthalmus maximus*) vitellogenin by high-performance anion-exchange chromatography. *J. Chromatogr.* 478:387-397.

II.   Silversand, C., Hyllner, S. J. and Haux, C. (1993) Isolation, immunochemical detection, and observations of the instability of vitellogenin from four teleosts. *J. Exp. Zool.* 267:587-597.

III.  Silversand, C. and Haux, C. (1995) Fatty acid composition of vitellogenin from four teleost species. *J. Comp. Physiol.* B 164:593-599.

IV.   Silversand, C., Norberg, B. and Haux, C. (1996) Fatty acid composition of ovulated eggs from wild and cultured turbot (*Scophthalmus maximus*) in relation to yolk and oil globule lipids. *Mar. Biol.* 125:269-278.

V.    Silversand, C. and Haux, C. (1996) Lipid class composition in eggs and vitellogenin from turbot (*Scophthalmus maximus*) as determined by HPLC with light-scattering detection. Manuscript.

# CONTENTS

# INTRODUCTION

## *General introduction*

In oviparous animals, embryonic development occurs outside the maternal body. As a consequence, the embryo is ultimately dependent on compounds stored within the egg for its nutritional requirements. The nutrients stored in the egg are known as yolk and have to contain all necessary supplies for the rapidly growing embryo, until it has developed into a self supporting animal capable of feeding. It is thus obvious that females of oviparous animals must selectively accumulate sufficient amounts of yolk compounds, such as proteins and lipids, in their growing oocytes in order to guarantee the production of a vital offspring.

It is now well established that the yolk of oviparous vertebrates is not synthesised within the egg cell, but rather is derived from exogenously synthesised precursor molecules that are taken up into the growing oocytes (Wallace, 1985). Once inside the oocyte, these precursors are processed and cleaved into yolk. The major yolk protein precursor in oviparous vertebrates, except for birds, is vitellogenin, a complex high-molecular-weight plasma protein containing variable amounts of carbohydrates, lipids, phosphates and metal ions. Vitellogenin is synthesised in the liver in response to estrogens, secreted into the circulation and selectively sequestered by growing oocytes. In birds, an additional yolk precursor is equally important, very low density lipoprotein (VLDL). The process of yolk formation is named vitellogenesis, and the uptake and processing of vitellogenin is a characteristic shared by all oviparous vertebrate groups, including fish, amphibians, reptiles and birds.

In teleost fish, developing oocytes accumulate not only vitellogenin but also large amounts of long-chain polyunsaturated fatty acids (PUFA) of the (n-3) series (Sargent *et al.*, 1989). In fact, few other natural sources are known that are so rich in (n-3) PUFA as the eggs of many fish species. The abundance of PUFA in fish eggs indicates a nutritional requirement for these fatty acids for embryonic and early larval development. Nutritional studies have shown that the dietary lipid composition, particularly the relative amounts of (n-3) PUFA, is crucial to both female fecundity and to embryo and early larval development and survival. Embryonic development is characterised by a considerable membrane synthesis and energy utilisation. It is therefore not surprising that lipids are of particular importance during embryogenesis, since lipids constitute the structural components of biological cell membranes and are also a dominating source of metabolic energy in embryos of many fish species. Although the process of vitellogenesis in fish has been studied for more than 20 years, very little is known about the origin of the egg lipids. Previous research has mainly focused on the yolk proteins and how these are related to the polypeptide of vitellogenin. Much less is known about the interrelationship between egg lipids and vitellogenin. However, the fact that vitellogenin contains lipid and that large amounts of

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vitellogenin are taken up by developing oocytes, indicates that vitellogenin is a key component in the process of lipid accumulation in growing oocytes of fish.

The scope of the present thesis is vitellogenesis in teleost fish, with special emphasis on the lipid moiety of vitellogenin and eggs from marine teleost species. The thesis will start with an introduction that reviews the current knowledge about vitellogenesis and lipid metabolism during reproduction in teleosts. Concerning subjects that are not in direct focus of the present thesis, the reader will often be referred to excellent reviews that discuss the details of the particular themes. Although the results in this thesis are restricted to fish, comparisons will be made to the South African clawed toad (*Xenopus laevis*) and the domestic hen (*Gallus domesticus*). Oocyte development, including vitellogenesis, in these species has been particularly well studied and there are indications of close structural and physiological similarities of oocyte development between all groups of oviparous vertebrates.

## *Vitellogenesis*

### Definitions and general aspects of vitellogenesis in teleost fish

Teleosts constitute almost half of the total number of known vertebrate species with more than 20 000 species (Nelson, 1984). Considering the large number of species and the wide range of habitats to which they have adapted, it is not surprising that several diverse reproductive strategies have evolved and representatives of both oviparous and viviparous fish are found. However, the majority of teleosts are oviparous and only these will be considered in the present thesis. Despite the heterogeneity of reproductive strategies in different species, the vitellogenic processes in oviparous teleosts have many characteristics in common and it is therefore accurate to present a general description of vitellogenesis.

Production of yolky eggs is a feature common to all oviparous vertebrates, and in most species, yolk is the most prominent cytoplasmic component of the egg, discounting the water content (Wallace, 1985). The accumulation of yolk in developing oocytes occurs mainly during later stages of oocyte development, which has been carefully reviewed for fish (Wallace & Selman, 1981; Wallace, 1985; Guraya, 1986; Selman & Wallace, 1989; Wallace & Selman, 1990). During this stage the fish oocyte grows from microscopic to macroscopic dimensions, and becomes fully capable of sustaining the nutritive needs of the growing embryo after fertilization. In many teleosts, the weights of the ovaries increase from less than 1 % of the body weight up to 20 % or more during vitellogenesis, an increase predominantly caused by the accumulation of vitellogenin.

The definition of egg yolk and the modes of its formation is somewhat confusing and several contradictionary definitions are found in the literature. In general terms, yolk is the nutrients present in eggs that nourish the embryo and yolk-sac larva. However, yolk has usually been restricted to the protein components of the yolk platelets or yolk

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spheres, mainly lipovitellin and phosvitin, and has not included the various other components of the oocytes, such as glycogen or lipid inclusions (Wallace & Dumont, 1968; Selman & Wallace, 1989). In the present thesis, the term yolk will be used for protein components, including lipoproteins, confined to the yolk spheres which are used to nourish the embryo and yolk-sac larva. Yolk will not include oil globules or oil droplets that are found in many eggs of teleost species, which will be considered as separate egg components.

Yolk has been reported to be synthesised both within the oocyte (autosynthesis) and outside the oocyte, and then incorporated into the oocyte during vitellogenesis (heterosynthesis). These two synthetic pathways have been referred to as endogenous and exogenous vitellogenesis, respectively (Wallace, 1978). Endogenous vitellogenesis, however, is obscure and stems mainly from early observations of numerous vesicles (cortical alveoli) and lipid inclusions in the cytoplasm of teleost oocytes prior to and concomitant with yolk deposition (reviewed by Wallace & Selman, 1981; Selman & Wallace, 1989). The oocyte development of most teleost species follows a general pattern. Wallace & Selman (1981) divided the teleost oocyte development into four principal stages based on ultrastructural criteria; (1) primary oocyte growth, (2) cortical alveoli stage, (3) vitellogenesis and (4) maturation. These stages do not sequentially replace one another during oocyte development, but rather are sequentially initiated, and once initiated, remain active throughout oocyte growth (Selman & Wallace, 1989). During the cortical alveoli stage, small vesicles appear in the cytoplasm of the oocyte, and by the end of this stage these vesicles fill the oocyte cytoplasm almost entirely (Selman et al., 1988). The cortical alveoli vesicles contain a glycoprotein and are suggested to be synthesised de novo within the oocyte (Korfsmeier, 1966; te Heesen & Engels, 1973; Selman et al., 1986; Inoue et al., 1987). The cortical alveoli stage was therefore referred to in the earlier literature as endogenous vitellogenesis, and the cortical alveoli vesicles were called yolk vesicles, endogenous yolk or intravesicular yolk. However, these terms are inappropriate and misleading since cortical alveoli has nothing to do with the yolk that nourishes the embryo (Wallace & Selman, 1981; Selman et al., 1988). Instead, cortical alveoli migrate to the periphery of the oocyte, fuse with the oolemma and release their glycoprotein content into the perivitelline space during the cortical reaction at fertilization (Inoue & Inoue, 1986; Selman et al. 1988).

The term endogenous vitellogenesis also stems from early ultrastructural observations of lipid bodies or lipid inclusions in the cytoplasm of previtellogenic oocytes from fish (Chopra, 1958; Guraya, 1963; Beams & Kessel, 1973; Shackley & King, 1977). These lipid inclusions were suggested to be endogenously synthesised. However, there is no convincing evidence that the lipid inclusions observed in the early ultrastructural studies are actually synthesised within the oocytes. Moreover, the fact that lipid inclusions also occur in growing oocytes of mammals, and that they disappear in many species prior to, or during subsequent vitellogenesis, indicate that the lipids present in previtellogenic oocytes do not represent true yolk (Guraya, 1986). Instead, the previtellogenic lipid inclusions are

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suggested to play an important role in the developmental process of the oocyte, for example as structural lipids of the oocyte (Wiegand, 1982). However, although the term endogenous vitellogenesis is considered inappropriate, when based on data from the earlier studies (above), endogenous synthesis within the oocyte of various compounds that contribute to nourish the embryo, including lipids, cannot be ignored and must be considered as a potential way for the contribution to yolk formation.

Exogenous vitellogenesis has been defined as the period when the oocytes deposit large amounts of vitellogenin. This process is actually related to the yolk used to nourish the embryo and has therefore also been referred to as true vitellogenesis. Throughout the present thesis, only the term vitellogenesis will be used and refers to the process of yolk deposition by the uptake of exogenously synthesised precursor molecules.

A simplified model that summarises the events that contribute to vitellogenesis in fish is given in Figure 1, and involves; (1) the hepatic synthesis and secretion of vitellogenin in response to circulating estrogens, (2) the transport of vitellogenin via the maternal circulation to the surface of the growing oocyte, (3) the selective uptake of vitellogenin by receptor-mediated endocytosis, and (4) the processing and selective proteolytic cleavage of vitellogenin into the polypeptides comprising the yolk proteins, principally lipovitellin and phosvitin (Wallace & Selman, 1990).
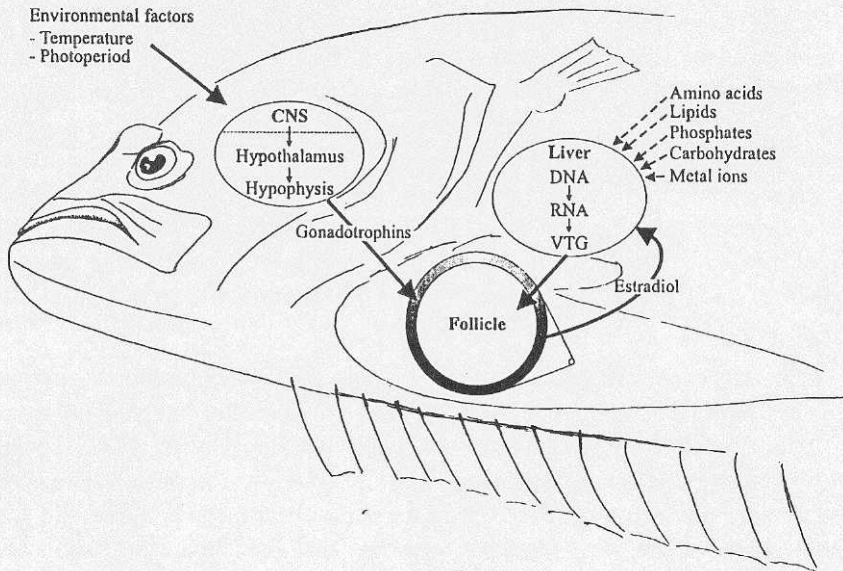


**Fig. 1.** Schematic representation of the events that contribute to vitellogenesis in fish.

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## Endocrine control of vitellogenesis in teleost fish

Vitellogenesis in teleosts, as well as in other oviparous vertebrates, is regulated via the hypothalamo-hypophysis-gonadal axis (reviewed by Nagahama *et al.*, 1993). Briefly, gonadotrophin-releasing hormones are secreted from the hypothalamus in response to environmental factors, such as photoperiod and temperature (Fig. 1.). The gonadotrophin-releasing hormones stimulate the release from the hypophysis into the blood of gonadotrophins, which subsequently stimulate the follicle cells in the ovaries to synthesise and secrete estrogens, mainly estradiol-17β. The estrogens are transported to the liver where they induce the synthesis of vitellogenin.

## Chemical characteristics of teleost vitellogenin

Vitellogenin was first observed in domestic hen by Laskowski (1935), who demonstrated that sera from laying hens contained a phosphoprotein that was absent in non-laying females and males. This phosphoprotein, which was precipitated after dilution of the plasma with distilled water, was given the name serumvitellin since it resembled the egg yolk protein, vitellin, found in eggs of the hen. Similar female specific proteins were later found in the blood of several teleost species during gonadal maturation, as well as in estrogen-treated fish (Laskowski, 1936; Bailey, 1957; Vanstone & Ho, 1961; Plack *et al.*, 1971). Flickinger & Rounds (1956) used $^{32}$P to show that the phosphoprotein originated in the liver of the domestic hen, appeared briefly in the blood, and was finally deposited in the yolk of the eggs. Bergink & Wallace (1974) presented the first evidence that the yolk proteins of oviparous vertebrates, lipovitellin and phosvitin, was actually synthesised as a large common precursor molecule named vitellogenin. The name vitellogenin was coined by Pan *et al.* (1969) for the yolk protein precursor found in insects, and was later adopted by Wallace (1970) for the yolk protein precursor in *Xenopus laevis,* and subsequently as a common name for the specific yolk protein precursor found in vertebrates.

### *Polypeptide backbone of vitellogenin*

Vitellogenin has been isolated and characterised in a large number of non-mammalian vertebrates, including birds (Deeley *et al.*, 1975), reptiles (Gavaud, 1986), amphibians (Wallace, 1970; Redshaw & Follett, 1971; Wiley & Wallace, 1981) and teleosts (reviewed by Wallace, 1985; Mommsen & Walsh, 1988; Specker & Sullivan, 1994). In all oviparous vertebrates investigated so far, vitellogenin is a large glycolipophosphoprotein that binds divalent cations, predominantly calcium. Vitellogenin is assumed to be present in the blood mainly as a dimer, with an estimated native molecular mass of 460-600 kDa for *Xenopus laevis* (Wallace, 1970; Redshaw & Follett, 1971), 450-500 kDa for domestic hen (Deeley *et al.*, 1975) and 300-600 kDa for various teleost species (reviewed by Mommsen & Walsh, 1988; Specker & Sullivan, 1994). The apparent molecular mass of the monomeric

form of teleost vitellogenin, determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), is reported to range between 85 kDa to 200 kDa (Hara *et al.*, 1980; Chen, 1983; Selman & Wallace, 1983; Hamazaki *et al.*, 1987). It is important to recall that the study by Plack *et al.* (1971), referred to in Mommsen & Walsh (1988), is often misquoted in the literature as presenting data on the molecular mass and lipid content of Atlantic cod (*Gadus morhua*) vitellogenin. However, the molecular mass of 440 kDa and the lipid content of 21 % (wt/wt) reported by Plack *et al.* (1971), refer to a yolk protein from the eggs of cod.

Studies on *Xenopus laevis* and *Gallus domesticus* have shown that these species contain multiple vitellogenins, encoded by distinct genes (Byrne *et al.*, 1989). Three different molecular forms of plasma vitellogenin have been isolated and characterised in estrogen-treated roosters (Wang and Williams, 1980; Wang *et al.*, 1983), and at least three forms of vitellogenin have been found to circulate in the blood of estrogen-stimulated *Xenopus* (Wiley & Wallace, 1978). Vitellogenins of these species are synthesised from a family of different but related genes that give rise to several distinct mRNAs (Byrne *et al.*, 1989). The number of vitellogenin genes, transcripts and circulating vitellogenin molecules has not been clearly elucidated for fish. More than one form of vitellogenin has been proposed for several teleost species by electrophoretic and immunochemical detection (Plack *et al.*, 1971; Amirante, 1972; Hara *et al.*, 1983; Ding *et al.*, 1989; Lim *et al.*, 1991; Lee *et al.*, 1992; Kishida & Specker, 1993). However, for some of these species, this can be a consequence of a degradation of the vitellogenin molecule during sample preparation. Teleost vitellogenin is known to be highly sensitive to degradation (Wallace, 1985), and polypeptides described as different forms of vitellogenin may be derivatives from proteolysis of the vitellogenin molecule. A further complication is that vitellogenin may appear in both monomeric and dimeric forms on electrophoresis (deVlaming *et al.*, 1980; Matsubara *et al.*, 1994). Studies on the gene level in tilapia (*Oreochromis aureus*), rainbow trout (*Oncorhynchus mykiss*) and killifish (*Fundulus heteroclitus*) have demonstrated multiple gene transcripts of vitellogenin, indicating that there may be multiple vitellogenin genes (LeGuellec *et al.*, 1988; Ding *et al.*, 1990; Lim *et al.*, 1991; Lee *et al.*, 1992; LaFleur *et al.*, 1996). However, an early study of *in vitro* translation of mRNA of estrogen-stimulated rainbow trout liver suggests that only one form of vitellogenin is synthesised in rainbow trout (Chen, 1983). Obviously, the answer question of multiple forms of teleost vitellogenin has to await further analysis of the expression of vitellogenin gene(s).

*Phosphorus of vitellogenin*

Vitellogenin contains phosphorus, both in the form of phosphate groups covalently bound to serine residues and as components of phospholipids. The amino acid composition of vitellogenin is characterised by a relatively high content of serine residues, of which almost all are phosphorylated (Wallace, 1985; Byrne *et al.*, 1989). In teleost vitellogenin, the content of protein-bound phosphorus is 0.6-0.8 % by weight (Hori *et al.*, 1979; Campbell &

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Idler, 1980; deVlaming *et al.*, 1980; Norberg and Haux, 1985, Norberg, 1995). The serine residues of vitellogenin, and subsequently the protein phosphorus, are enriched in specific regions of the polypeptide. These are cleaved off to form the yolk protein phosvitin once vitellogenin is taken up into the oocyte (Wallace, 1978). In plasma, one calcium appears to be bound to each protein phosphorus group, which is proposed to be a prerequisite to keep vitellogenin in a soluble form in the blood (Wallace, 1970; Wiley *et al.*, 1979). However, the calcium ion is loosely bound to vitellogenin and chromatography causes a loss of the calcium ions (Wallace, 1970). Due to the high degree of phosphorylation, vitellogenin has a high negative surface charge. Compared to vitellogenin from other vertebrates, teleost vitellogenin appears to be less phosphorylated due to a lower content of serine residues. Vitellogenins of *Xenopus laevis* and domestic hen have protein phosphorus content of 1.4 % (Wallace, 1970) and 1.7-1.8 % (Deeley *et al.*, 1975), respectively. Since the level of protein-bound phosphorous is low in plasma from males and non-vitellogenic females, plasma protein-bound phosphorous has been commonly used as a specific indicator of vitellogenin in the blood (Mommsen & Walsh, 1988).

## *Lipids of vitellogenin*

In contrast to the protein-bound phosphorus content of fish vitellogenin, which appears to be lower than that of vitellogenin from other oviparous vertebrates, the lipid content seems to be higher. The lipid content of teleost vitellogenin has been reported for five teleost species: 21 % (wt/wt) for goldfish (*Carassius auratus*; Hori *et al.*, 1979), 18-21 % (wt/wt) for rainbow trout (Campbell & Idler, 1980; Wiegand & Idler, 1982; Frémont *et al.*, 1984; Norberg & Haux, 1985; Frémont & Riazi, 1988), 19 % (wt/wt) for brown trout (*Salmo trutta*; Norberg & Haux, 1985), 20 % (wt/wt) for halibut (*Hippoglossus hippoglossus*; Norberg, 1995) and 19 % (wt/wt) for barfin flounder (*Verasper moseri*; Matsubara & Sawano, 1995). In all these species, the lipid moiety of vitellogenin consists of about two thirds phospholipids, mainly phosphatidylcholine, and the remaining being triacylglycerol and cholesterol (Hori *et al.*, 1979; Norberg & Haux, 1985; Norberg, 1995; Matsubara & Sawano, 1995). In *Xenopus laevis*, the total lipid content of vitellogenin is 12 % (wt/wt), of which 75 % is phospholipid (Wallace, 1970; Redshaw & Follett, 1971) and in the domestic hen the lipids constitute 15 % (wt/wt) (Banaszak *et al.*, 1991).

The fatty acid composition of teleost vitellogenin has only been documented for rainbow trout (Léger *et al.*, 1981; Frémont *et al.*, 1984; Frémont & Riazi, 1988), but only the partial fatty acid composition was reported. These studies indicate that rainbow trout vitellogenin is rich in long-chain PUFA of the (n-3) series. To date, no comprehensive fatty acid profile of total lipid from teleost vitellogenin, or from any other vertebrate vitellogenin, has been reported.

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## Vitellogenin as a lipoprotein

Lipids are transported in the circulatory system as lipoproteins, *i.e.* macromolecules in which lipids are associated with specific polypeptides, apolipoproteins (for review see Vance & Vance, 1990; Davis, 1991). The basic structure of lipoprotein particles are spherical molecules consisting of a core of non-polar (hydrophobic) lipids surrounded by a shell of polar lipids and apolipoproteins. Lipoproteins are classically divided into chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL), based on their density, *i.e.* the ratio between protein and lipid. The structures, apolipoproteins and lipid class composition of fish plasma lipoproteins are similar to their mammalian counterparts. However, lipoproteins of teleosts contain considerably higher levels of long-chain (n-3) PUFA compared to terrestrial animals in general (Babin, 1987b; Babin & Vernier, 1989).

In relation to other lipoproteins present in the circulation, vitellogenin is classified as a very high density lipoprotein (VHDL) due to the high ratio between protein and lipid (Babin & Vernier, 1989). The lipids of vitellogenin, as the lipids of lipoproteins in general, are non-covalently bound to the polypeptide (Evans *et al.*, 1968; Raag *et al.*, 1988; Davis, 1991). The lipid material of vitellogenin is associated to the lipid-binding domain of the polypeptide, which will form lipovitellin after proteolytic cleavage within the oocyte (Wallace, 1985; Byrne *et al.*, 1989). A three-dimensional model of the lipovitellin-phosvitin complex from lamprey, based on X-ray crystallography, has been presented (Raag *et al.*, 1988; Banaszak *et al.*, 1991; Timmins *et al.*, 1992). These studies indicate that the lipid moiety is bound, presumably by hydrophobic interactions, within a large cavity of the molecule which is surrounded by the polypeptide chain (Fig. 2). The lipovitellin-phosvitin complex contains 21 % (wt/wt) lipid of which approximately 70 % is phospholipids (Raag *et al.*, 1988).
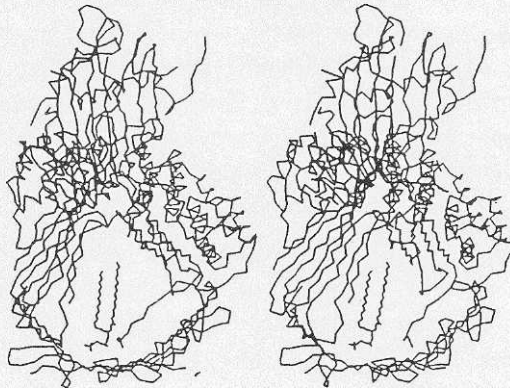


**Fig. 2.** Stereo views of the model of the lipovitellin-phosvitin complex obtained from high resolution X-ray crystallographic analysis (Raag *et al.*, (1988) J. Mol. Biol. 200, 553-569). The protein backbone is shown together with a model phospholipid which has been placed in an arbitrary location within a cavity, believed to contain the lipid.

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The polypeptide of vitellogenin exhibits amino acid sequence similarities to the human apolipoprotein B-100 (apoB-100), the main apolipoprotein of VLDL and LDL (Baker, 1988; Perez *et al.*, 1991). This relatedness of vitellogenin to apoB-100, led Baker (1988) to postulate that vitellogenin is an ancestor to apoB-100. The identity between vitellogenin and apoB-100 may reflect similar overall architecture, possibly illustrating a fundamental mode of protein-lipid interaction (Banaszak *et al.*, 1991). However, vitellogenin and lipoproteins like VLDL differ markedly in one respect. Vitellogenin contain 12-20 % lipid by mass, mostly phospholipids, while VLDL contain > 80 % lipid by mass, principally non-polar lipids such as triacylglycerols and cholesterol esters (Banaszak *et al.*, 1991).

## Isolation of teleost vitellogenin

A prerequisite for studies of vitellogenesis and related processes is the isolation of pure and intact vitellogenin. Vertebrate vitellogenin has been isolated from the plasma of oviparous vertebrates by various methods, including affinity chromatography, gel electrophoresis, gel filtration, ion-exchange chromatography on DEAE- and TEAE-cellulose, density ultracentrifugation and precipitation with dimethylformamide, EDTA:$Mg^{2+}$ and distilled water (Wallace & Jared, 1968; Wallace, 1970; Ansari *et al.*, 1971; Redshaw & Follett, 1971; Deeley *et al.*, 1975; Wiley *et al.*, 1979). However, most of the isolation techniques described have been developed for the purification of vitellogenin from *Xenopus laevis* and domestic hen, and several of these procedures have been reported less suitable for the purification of teleost vitellogenin. This appears to be related to the charge characteristics of teleost vitellogenin, *i.e.* that vitellogenin of fish is less negatively charged compared to vitellogenin from amphibians and birds (deVlaming *et al.*, 1980; Norberg & Haux, 1985; Wallace, 1985). Hence, vitellogenin of some teleost species may not have a sufficiently negative surface charge to allow methods like precipitation with EDTA:$Mg^{2+}$ and ion-exchange chromatography to purify vitellogenin properly (deVlaming *et al.*, 1980; Selman & Wallace, 1983; Kanungo *et al.*, 1990; Tyler & Sumpter, 1990a). Thus, precipitation with EDTA:$Mg^{2+}$ may fail to precipitate vitellogenin from plasma while ion exchange chromatography may not provide entirely pure vitellogenin because vitellogenin appears to be less easily resolved from other plasma proteins.

Isolation of teleost vitellogenin is further complicated by the fact that it can be highly susceptible to proteolytic degradation. There are a number of reports in the literature emphasising the instability of teleost vitellogenin and the difficulties in obtaining undegraded vitellogenin (Hickey & Wallace, 1974; deVlaming *et al.*, 1980; Selman & Wallace, 1983; Norberg & Haux, 1985; Tyler & Sumpter, 1990a; Goodwin *et al.*, 1992). It has been demonstrated that even when great precautions to prevent proteolysis of vitellogenin were taken, degradation of fish vitellogenin could not be completely eliminated (deVlaming *et al.*, 1980; Selman & Wallace, 1983; Norberg & Haux, 1985).

Another feature that has to be considered during the isolation of vitellogenin is the fact that the polypeptide chains and the lipids of lipoproteins are combined by relatively weak, non-covalent bindings. This indicates that the lipid composition of vitellogenin can be affected by the method used for the isolation (Evans *et al.*, 1968). In an early study on *Xenopus laevis,* the chemical composition, including the lipid content, of vitellogenin was reported to be altered by the different isolation methods used (Redshaw & Follett, 1971). Finally, care must also be taken during preparative and analytical work of teleost vitellogenin to prevent oxidation of PUFA. It is well known that PUFA are highly susceptible to oxidative degradation (Ackman, 1980), and since PUFA are particularly abundant in lipids of marine teleost species, this could be accentuated in studies on marine lipids.

## Hepatic synthesis and secretion of teleost vitellogenin

*Induction and plasma levels of vitellogenin*

In teleosts, as in all other oviparous vertebrates, vitellogenin is synthesised in the liver in response to estrogens (Plack & Frazer, 1971; Wallace, 1985; Mommsen & Walsh, 1988). Normally, vitellogenin is synthesised in female fish during sexual maturation when circulating levels of estradiol-17β are elevated. In naturally vitellogenic fish, plasma vitellogenin reaches levels of approximately 1 to 50 mg/ml, depending on species (Copeland *et al.*, 1986; Copeland and Thomas, 1988; Methven *et al.*, 1992; Mañanós *et al.*, 1994; Matsubara *et al.*, 1994). Although vitellogenin normally is synthesised in females, the hepatic synthesis of vitellogenin can be induced in males, as well as in sexually immature and juvenile females, by administration of estradiol-17β (Wallace, 1985; Mommsen & Walsh, 1988). Since males and sexually immature animals contain no ovaries that sequester vitellogenin from the circulation, large amounts of vitellogenin are accumulated in the blood during estradiol-17β-treatment. This has been utilised in a large number of studies where a high titre of vitellogenin in the blood is required or is advantageous, for example during the isolation of vitellogenin.

*Metabolic changes during vitellogenin synthesis*

The onset of vitellogenin synthesis is reflected by profound alterations in the liver metabolism in fish. In response to estradiol-17β, liver cells undergo major ultrastructural changes, such as increased cell size, massive proliferation of rough endoplasmic reticulum, enlarged Golgi apparatus and mitochondria, and increased amounts of secretory vesicles, all to enhance the synthesis and secretion of vitellogenin and other proteins induced by estradiol-17β (Van Bohemen, 1981; Selman & Wallace, 1983; Peute *et al.*, 1985; Pacoli *et al.*, 1991). Vitellogenin synthesis is also accompanied by changes in the biochemical composition of the liver, including increased RNA and lipid content and decreased

glycogen content (Korsgaard & Petersen, 1979; Haux & Norberg, 1985; Mommsen & Walsh, 1988). Altogether, the increased metabolic activity in the liver during vitellogenin synthesis is reflected in a pronounced enlargement of the liver.

## Biosynthetic pathways of vitellogenin

It is generally believed that the final form of the circulating vitellogenin in fish, as well as in other oviparous vertebrates, is completely synthesised in the liver, *i.e.* carbohydrates, lipids, phosphorus and different ions are added to the polypeptide before secretion to the blood (Mommsen & Walsh, 1988). Vitellogenin appears to follow the same biosynthetic and transport pathway as essentially all secretory proteins undergo, briefly summarised as: (1) synthesis of the protein backbone by translation of mRNA on membrane ribosomes, (2) translocation of the synthesised polypeptide across the endoplasmic reticulum into the lumen, (3) co- and posttranslational modifications, *i.e.* glycosylation, lipidation and phosphorylation, (4) concentration into transporting vesicles and (5) secretion out of the cell (Wallace, 1985; Davis, 1991). Although teleost vitellogenin is known to be a glycolipophosphoprotein, the intracellular location for co- and posttranslational modifications of vitellogenin, its packing and the mechanism of secretion have not yet been established. In *Xenopus laevis*, phosphorylation of the protein backbone of vitellogenin begins in the rough endoplasmic reticulum, while glycolysation occurs just prior to secretion (Gottlieb & Wallace, 1981, 1982). In the domestic hen, on the other hand, glycosylation appears to take place before phosphorylation, which occurs immediately prior to secretion (Wang & Williams, 1982).

While some information exists concerning the mechanisms of phosphorylation and glycolysation of vertebrate vitellogenin, no information is available on the process of lipidation of vitellogenin. This is partly related to the fact that lipidation is difficult to study because of the weak bindings between the lipid moiety and the polypeptide of vitellogenin (Wallace, 1985). A molecular mechanism for the lipidation of another hepatically synthesised lipoprotein, the human apoB-100 containing VLDL, has been suggested (Olofsson *et al.*, 1987; Borén *et al.*, 1991). According to this model, the binding of lipid to the apolipoprotein (apoB-100) occurs cotranslationally rather than posttranslationally. Thus, apoB-100 is integrated into the membrane of the endoplasmic reticulum during translation. In the membrane, the polypeptide interacts with certain membrane regions where sequestration of lipids occurs and the lipoprotein is assembled. After assembly, the mature lipoprotein is ejected into the lumen of the endoplasmic reticulum and subsequently enters the secretory pathway. Although vitellogenin and apo-B-100 containing lipoproteins differ in their biochemical composition, similar overall principles for lipoprotein assembly may exist and vitellogenin may possibly be lipidated by a similar mechanism to that suggested for VLDL. Considering the large size of the polypeptide of vitellogenin and the hydrophobic character of lipids, cotranslational lipidation of vitellogenin seems reasonable.

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# Oocyte uptake of vitellogenin and conversion to yolk in teleosts

## Oocyte uptake

Vitellogenin is selectively taken up into growing oocytes by receptor-mediated endocytosis. Much of our knowledge of the mechanisms of vitellogenin uptake into oocytes of oviparous vertebrates comes from studies on *Xenopus laevis* (Wallace *et al.*, 1972; Opresko & Wiley, 1987) and domestic hen (Stifani *et al.*, 1988, 1990a). A series of recent studies have shown that vitellogenin uptake in teleost oocytes follows the same pattern. Experiments, on teleosts both *in vivo* and *in vitro*, using radiolabelled vitellogenin have demonstrated that the incorporation of vitellogenin into growing oocytes is a selective process in killifish (Selman & Wallace, 1982, 1983; Kanungo *et al.*, 1990) and in rainbow trout (Tyler *et al.*, 1988a, b; 1990b; Shibata *et al.*, 1993). Based on earlier ultrastructural studies, showing abundant endocytotic activity at the surface of vitellogenic oocytes of teleosts, *i.e.* extensive numbers of coated pits and vesicles, the selective vitellogenin sequestration in teleosts was proposed to occur by means of receptor-mediated endocytosis (Upadhyay *et al.*, 1978; Selman & Wallace, 1982, 1983). More recently, an oocyte-surface receptor for vitellogenin has been reported and partly characterised in coho salmon (*Oncorhynchus kisutch*; Stifani *et al.*, 1990b), tilapia (Chan *et al.*, 1991) and rainbow trout (Tyler & Lancaster, 1993).

The oocyte uptake of vitellogenin in teleosts is developmentally regulated. Once a teleost oocyte reaches a certain size, or developmental stage, it begins to incorporate vitellogenin (Selman & Wallace, 1983; Kanungo *et al.*, 1990; Tyler *et al.*, 1991; Hyllner *et al.*, 1994b). In rainbow trout, for which oocyte uptake of vitellogenin has been particularly well studied, the endocytotic uptake starts when the oocytes reach a diameter of approximately 600 µm, which occurs about 9 months prior to ovulation (Fig. 3) (Tyler *et al.*, 1991; Hyllner *et al.*, 1994b). Furthermore, the uptake of radiolabelled vitellogenin into rainbow trout and killifish oocytes is correlated with the oocyte size, being highest in the largest oocytes, even when the rate was expressed relative to surface area (Kanungo *et al.*, 1990; Tyler *et al.*, 1990a, 1990b).



**Fig. 3.** Immunofluorescence micrographs showing a sectioned oocyte from a female rainbow trout (Hyllner, Silversand & Haux (1994) Molec. Reprod. Dev. 39, 166-175). The oocyte diameter is 600 µm and was sampled from a female with a gonadosomatic index of 0.25. Homologous antiserum directed against vitellogenin (diluted 1:800) was used for immunohistochemical staining. Vesicles inside the oocyte showed a positive crossreaction to the antiserum (arrows).
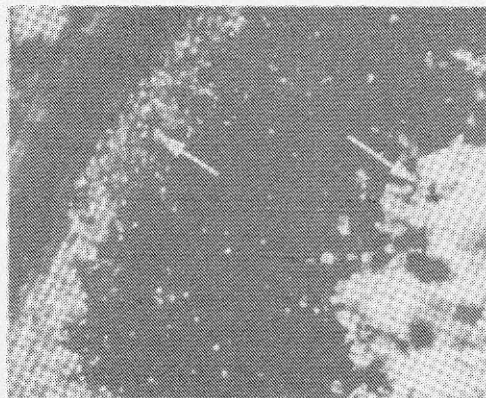
*Oocyte translocation and proteolytic processing of vitellogenin*

After internalisation by the oocyte, vitellogenin accumulates in small vesicles in the oocyte cortex. These vesicles subsequently migrate centripetally where they fuse to form larger yolk spheres, which in turn, eventually coalesce into a continuous, central mass of fluid yolk in most teleosts (Selman & Wallace, 1989; Hyllner *et al.*, 1994b) (Fig. 3). Crystalline yolk has been described in the oocyte of a few teleost species (Lange, 1985), but in most fish, yolk proteins are accumulated in fluid-filled yolk spheres (Selman & Wallace, 1989). The cytoplasmic translocation in the oocyte is accompanied by a proteolytic processing of the vitellogenin molecule (Sire *et al.*, 1994). The endocytotic vesicles containing vitellogenin appear to fuse with modified lysosomes, that contain a specific enzyme which cleaves vitellogenin into the yolk proteins. Cathepsin D has been demonstrated to be the catalyst for intraoocytic processing of vitellogenin in domestic hen, *Xenopus laevis* and rainbow trout (Retzek *et al.*, 1992; Sire *et al.*, 1994; Yoshizaki & Yonezawa, 1994). In most oviparous vertebrates, the formed yolk proteins remain apparently unchanged until fertilization, when the yolk is utilised for embryonic development (Wallace, 1985). However, in oocytes of several teleost species, particularly marine species with pelagic eggs, the yolk proteins undergo additional proteolysis at the time of final oocyte maturation (Wallace & Selman, 1985; Greeley *et al.*, 1986; Norberg, 1987; Matsubara and Sawano, 1995). In marine species with pelagic eggs, the final oocyte maturation is accompanied by a massive uptake of water which makes the egg translucent and enhances buoyancy in sea water (Wallace & Selman, 1981). The water uptake has been suggested to be achieved by the yolk protein cleavage. This proteolytic cleavage generates a large increase in the intracellular concentration of free amino acids, which increases the osmotic pressure and thereby facilitates water uptake.

*Yolk proteins*

The structural alteration of vitellogenin after internalisation by the oocyte is well documented in *Xenopus laevis* and domestic hen (Wallace, 1985). Detailed biochemical studies of yolk proteins in teleosts are few and available data are mainly limited to salmonid species. However, a feature common to all oviparous vertebrates, including teleosts, is that vitellogenin is cleaved into two main classes of yolk proteins: lipovitellins and phosvitins (Wallace, 1985; Mommsen & Walsh, 1988). In addition to lipovitellin and phosvitin, another class of yolk protein, the β′-component, has been reported in some teleost species, foremost in salmonids (Markert & Vanstone, 1971; Campbell & Idler, 1980; Hara *et al.*, 1993; Matsubara & Sawano, 1995). In teleosts and amphibians, lipovitellins constitute the bulk of the yolk proteins (Wallace, 1985). Amino acid analyses indicate that lipovitellin accounts for approximately 90 % of the vitellogenin molecule of *Xenopus laevis* (Redshaw & Follett, 1971). Similar proportions have been reported for vitellogenin of rainbow trout (Campbell & Idler, 1980). Teleost lipovitellin is a high molecular weight lipoprotein that contains 20 to 25 % lipid (wt/wt) and is present as a dimer in its native state (Plack *et al.*, 1971; Campbell & Idler, 1980; Matsubara & Sawano, 1995). The lipovitellin monomer

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consists of two subunits: $LV_1 \sim 100$ kDa and $LV_2 \sim 20$ kDa (Chen, 1983; Babin, 1987a; Matsubara & Sawano, 1995). These are usually referred to as heavy and light chain lipovitellin. Phosvitins are smaller and more heterogenous peptides (about 10 to 40 kDa), that contain no lipids but are highly phosphorylated (deVlaming *et al.*, 1980; Chen, 1983; Wallace & Begovac, 1985; Murakami *et al.*, 1990; Matsubara & Sawano, 1995). The β'-component is a peptide (20-30 kDa) that contains neither lipids nor phosphorus, and is suggested to be derived from vitellogenin (Campbell & Idler, 1980; Hara *et al.*, 1993). The fact that the yolk proteins in several marine teleost species undergo proteolytic cleavage during final oocyte maturation is a complicating factor in biochemical studies of teleost yolk proteins (Greeley *et al.*, 1986). Another complicating factor is the existence of multiple forms of vitellogenin in some species, such as *Xenopus laevis*, which give rise to multiple yolk proteins in the oocyte (Wiley & Wallace, 1981). Interpretation of the relationship between vitellogenin and the mixture of cleavage fragments in species with several different vitellogenin molecules, demands that one is able to distinguish between overlapping fragments and fragments derived from different parts of the vitellogenin molecule (Wiley & Wallace, 1981).

## Interrelationship between vitellogenin and yolk proteins

The interrelationship between vitellogenin and yolk proteins in teleosts has been confirmed chemically, immunochemically and electrophoretically (Hara & Hirai, 1978; Campbell & Idler, 1980; deVlaming *et al.*, 1980; Chen, 1983; Kishida *et al.*, 1992; Hara *et al.*, 1993; Hyllner *et al.*, 1994b; Kishida & Specker, 1993; Matsubara & Sawano, 1995). Direct evidence for the processing of vitellogenin into lipovitellins and phosvitins has also been shown by the use of radiolabelled vitellogenin (Tyler *et al.*, 1988a; Tyler, 1993).

Quantitative analyses performed with eggs of *Xenopus laevis* have demonstrated that more than 99 % of the yolk proteins are derived from vitellogenin (Wallace *et al.*, 1972). The yolk proteins constitute 80-90 % of the total protein in *Xenopus* eggs (Callen *et al.*, 1980). In contrast, yolk deposition in bird eggs results essentially from the oocyte uptake of two classes of lipoproteins, VLDL and vitellogenin (Wallace, 1985; Kuksis, 1992). In fact, in terms of weight, the major yolk protein precursor is not vitellogenin, but rather VLDL in domestic hen. The relative contribution from vitellogenin versus other sources to the yolk formation in teleosts is less well defined. Unlike the case in amphibians and birds, a more precise quantification of the interrelationship between vitellogenin and yolk has not yet been performed. However, since large amounts of vitellogenin are incorporated into growing oocytes of fish, it has been suggested in the literature that vitellogenin sequestration, as in *Xenopus*, represents the major mechanism for yolk deposition in teleosts. This is confirmed by several studies, showing that vitellogenin derived proteins constitute by far the greatest proportion of the yolk proteins in eggs of many teleost species (deVlaming *et al.*, 1980; Chen, 1983; Babin, 1987a; Tyler, 1993; Matsubara & Sawano, 1995). Although vitellogenin seems to be the principal yolk protein precursor, other sources must be considered,

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including oocyte uptake of other bloodborne proteins and endogenous synthesis. Evidence for the presence of yolk proteins derived from precursors distinct from vitellogenin has only been reported for one teleost species, the winter flounder (*Pleuronectes americanus*) (Nagler & Idler, 1990). This species will be further discussed below when accumulation of egg lipids is considered.

# Nature and origin of egg lipids in teleost fish

## General aspects of lipids: definition, classification biochemistry

The term lipid has traditionally been applied to a multitude of substances of differing chemical structure and biological function, whose only common feature is that they are readily soluble in organic solvents. Lipids derive this distinctive property from long hydrocarbon chains that form a major proportion of their structure. Solubility alone, however, is a very broad and vague criterion to define lipids. A more specific definition is preferable and the term lipid is nowadays usually restricted to: "Fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds" (Christie, 1987). This definition treats cholesterol as a lipid but excludes other steroids, lipophilic vitamins, carotenoids and terpenes.

Fatty acids are hydrocarbon chains with a terminal acidic carboxyl group that are synthesised in nature via condensation of malonyl coenzyme A units by a fatty acid synthetase complex (Christie, 1987). Most fatty acids in animals are unbranched, even-numbered with 14-22 carbon atoms. Fatty acids can be fully saturated, which means that they have no double bonds, or they can contain one up to six double bonds. Fatty acids with two or more double bonds are referred to as polyunsaturated fatty acids (PUFA). When double bonds are present, they are nearly always in the *cis* configuration, and if there is more than one, they are almost exclusively spaced at three-carbon intervals. To simplify the discussion of fatty acids, a shorthand designation has been developed to describe a fatty acid in terms of number of carbon atoms and number and position of double bonds (Fig. 4). Because the double bonds in the majority of unsaturated animal fatty acids are almost always separated by a $CH_2$-group, notion of the double bond closest to the terminal methyl group of the fatty acid is sufficient to describe the molecule.

The nature of lipids containing fatty acids is to a large extent determined by the length of the fatty acid chains and the number and position of the double bonds within these chains (Bell *et al.*, 1986; Cullis & Hope, 1991). Alterations in the chain length and degree of unsaturation of fatty acids are used in biological systems to control physical and chemical properties of lipid systems and thereby the functional capacities of biological membranes. Biophysical and biochemical properties of fatty acids will be further discussed below under the section "Functional role of polyunsaturated fatty acids in teleost fish".
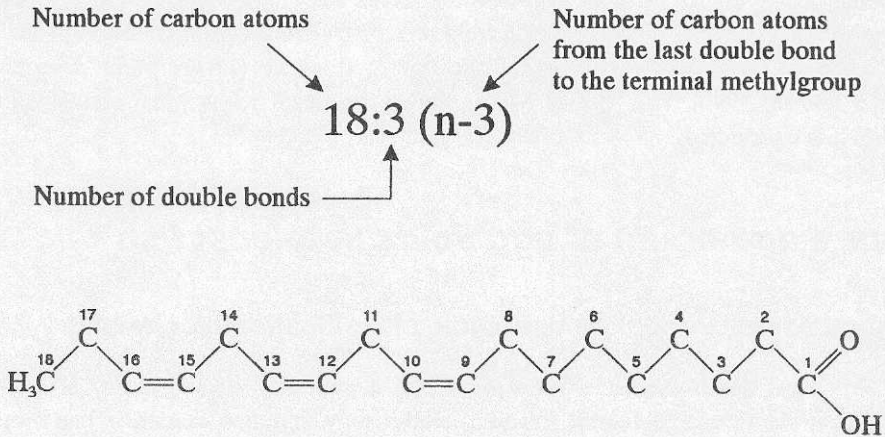
Fig. 4. A common polyunsaturated fatty acid (PUFA), linolenic acid, and its shorthand nomenclature.

Lipids can be subdivided into two main groups, neutral and polar lipid classes (Table 1). Neutral and polar lipids are also frequently referred to as simple and complex lipids, respectively (Christie, 1987). Simple lipids yield one or two hydrolysis products per molecule, whereas complex lipids yield three or more products upon hydrolysis. The classification in neutral and polar lipids is based on chemical properties and depends on the presence of polar groups on the molecules. Polar lipid classes are amphiphatic molecules, which means that they have both a polar or hydrophilic head group region and a non-polar or hydrophobic region . Neutral lipid classes, on the other hand, are relatively hydrophobic, or non-polar, although some neutral lipid classes exhibit slightly amphiphatic characteristics. The biological function of lipids is largely related to their chemical and physical properties, and hence the subdivision into neutral and polar lipids is also of physiological significance. In general, neutral lipids mainly serve as sources of metabolic energy in vertebrates, whereas polar lipids predominantly have structural functions, being the principal constituents of biological cell membranes. The structural function of polar lipids are related to its amphiphatic characteristics (Cullis & Hope, 1991). Cholesterol is an exception, since it is classified as a neutral lipid and yet has a structural function in biomembranes. In addition to the functions mentioned above, several other vital physiological functions are ascribed to lipids, including buoyancy, thermal isolation, waterproofing and as precursors to biologically active signal molecules such as eicosanoids (Hadley, 1985; Henderson et al., 1985; Sargent, 1995).

**Table 1**. The principal lipid classes of animal tissue

| Neutral lipid classes | Polar lipid classes |
|---|---|
| Cholesterol | **Glycerophospholipids** |
| Cholesterol ester |   Phosphatidic acid |
| Free fatty acid |   Diphosphatidylglycerol |
| Triacylglycerol |   Phosphatidylcholine |
| Diacylglycerol |   Phosphatidylethanolamine |
| Monoacylglycerol |   Phosphatidylinositol |
| Wax ester |   Phosphatidylserine |
| |   Lyso-phosphatidylcholine |
| | **Glyceroglycolipids** |
| |   Monogalactosylglycerol |
| |   Digalactosylglycerol |
| | **Sphingolipids** |
| |   Sphingosine |
| |   Ceramides |
| |   Sphingomyelin |
| |   Glycosylceramides |
| |   Gangliosides |

## Lipid composition of teleost eggs

Following protein, lipids are the second most abundant dry constituent of most teleost eggs (Henderson & Tocher, 1987; Sargent *et al.*, 1989). The importance of lipids during early ontogeny in fish has been pointed out in numerous studies, and particular emphasis has been laid upon the function of PUFA (Sargent *et al.*, 1989). Despite this, there is a lack of knowledge concerning the detailed lipid requirements during embryonic and early larval development in fish. Nutritional studies have mainly been conducted on larval, juvenile and adult fish, with much less attention given to developing fish embryos and yolk-sac larvae. Considering that the initial nourishment to the developing embryo and yolk-sac larva is drawn from the egg, the nutritional requirements during these developmental stages are assumed to be indicated by the biochemical composition of the nutritive reserves stored in eggs just prior to fertilization (Henderson & Tocher, 1987; Sargent *et al.*, 1989; Sargent, 1995). Therefore, one approach to get an insight into the lipid requirements during embryonic development in fish, is to analyse the lipid composition of ovulated eggs.

## Lipid content and class composition

In accordance with the foregoing, the total lipid content and composition of eggs and ovaries have been reported for numerous teleost species. A summary of the lipid data found in the literature on eggs and ovaries, is presented in Table 2. This table clearly illustrates that the relative content of total lipid and the lipid composition of eggs and ovaries vary considerably with species. The total egg lipid content ranges between 6 % and 56 %, as a percentage of the dry weight. The lipids in the eggs are stored in two major fractions: (1) lipids associated to proteins within the yolk sphere (lipovitellin), and (2) free lipids in discrete oil globules. The oil globules vary in number and size among species, from several small scattered droplets to one large oil globule in the millimetre range (Russel, 1976; Ahlstrom & Moser, 1980). However, oil globules are not present in eggs of all teleosts, and the species listed in Table 2 are divided into those producing eggs with and without distinct oil globules. From the data in the table, it is apparent that eggs possessing oil globules contain higher relative lipid contents than eggs without oil globules.

Polar lipids dominate in eggs without oil globules (61 % to 86 % of total lipid, by wt), whereas eggs with oil globules contain more neutral lipids (49 % to 98 % of total lipid, by wt) (Table 2). In eggs without oil globules, the lipids are principally represented by the lipids associated to the yolk proteins. Hence, yolk lipids in these species are mainly comprised of polar lipids. Polar lipids are also predominant in the yolk fraction of eggs harbouring oil globules (Nakagawa & Tsuchiya, 1976; Léger et al., 1981; Eldridge et al., 1983; Moodie et al. 1989; Ando & Hatano, 1991). Phosphatidylcholine is the principal polar lipid class in fish eggs, followed by phosphatidylethanolamine (Tocher & Sargent, 1984; Cowey et al., 1985; Falk-Petersen et al., 1986; White & Fletcher, 1987; Mourente & Odriozola, 1990; Chu & Ozkizilcik, 1995). Phosphatidylcholine accounts for 40 % to 62 % of the total lipids in eggs without oil globules (Table 2). Concerning species possessing oil globules in their eggs, less information on the content of phosphatidylcholine is available, although the data indicate a lower level. In addition to phosphatidylcholine and phosphatidylethanolamine, teleost eggs contain minor amounts of lysophosphatidylcholine, phosphatidylinositol, phosphatidylserine and sphingomyelin (Tocher & Sargent, 1984; Falk-Petersen et al., 1986; White & Fletcher, 1987).

The major neutral lipid classes in fish eggs are generally triacylglycerol and cholesterol (Kaitaranta & Ackman, 1981; Tocher & Sargent, 1984; Cowey et al., 1985; Falk-Petersen et al., 1986; White & Fletcher, 1987). The eggs of some species with oil globules, contain appreciable relative amounts of cholesterol ester and/or wax ester (Iyengar & Schlenk, 1967; Kaitaranta & Ackman, 1981; Eldridge et al., 1983; Vetter et al., 1983; Moodie et al., 1989; Anderson et al., 1990; Mourente & Odriozola, 1990; Planas et al., 1993b).

The variation in lipid content and composition of eggs between different fish species has been considered an adaptation to the time interval of the endogenous nutrition period (Kaitaranta & Ackman, 1981; Tocher & Sargent, 1984). This opinion arises from the finding that the time interval between fertilization and hatching, or depletion of the yolk-sac, is

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correlated with both total and neutral lipid content of eggs of some teleost species. That means that eggs with longer development times contain larger lipid reserves, mainly stored as neutral lipids that are utilised for embryonic and early larval development. The time from fertilization to hatching can vary from less than 24 h, to several months in fish. Thus, fish like the salmonids with long incubation periods (about 20 weeks), have relatively large eggs, with large lipid reserves rich in neutral lipid classes (Kaitaranta & Ackman, 1981; Cowey *et al.*, 1985; Ashton *et al.*, 1993). In contrast, many marine species producing relatively small eggs with shorter incubation periods has a lower lipid content which is dominated by polar lipids, *e.g.* cod, haddock (*Melanogrammus aeglefinus*), plaice (*Pleuronectes platessa*), saithe (*Pollachius virens*) and whiting (*Merlangius merlangus*) (Russel, 1976; Tocher & Sargent, 1984; White & Fletcher, 1987). Although it is suggested that high percentage of neutral lipids in fish eggs is related to longer incubation periods, like in the salmonids, several species diverse from this. Eggs of red drum (*Sciaenops ocellata*), striped bass (*Morone saxatilis*) and turbot (*Scophthalmus maximus*), for example, are all rich in neutral lipids but have noticeably short development time from fertilization to hatch (< 24 h - 5 days) (Eldridge *et al.*, 1983; Vetter *et al.*, 1983; McEvoy *et al.*, 1993).

The generally higher relative lipid content and the greater proportion of neutral lipids in eggs with oil globules appears to be related to the composition of the oil globules. In the few teleost species, where oil globules and yolk lipids have been analysed separately; rainbow trout (Nakagawa & Tsuchiya, 1976; Léger *et al.*, 1981), the cyprinodontid fish (*Nothobranchius guentheri*; Brind *et al.*, 1982), striped bass (Eldridge *et al.*, 1983), walleye (*Stizostedion vitreum*; Moodie *et al.*, 1989) and three *Oncorhynchus* species, (*O. keta, O. nerka* and *O. masou*; Ando & Hatano, 1991), the oil globules are comprised exclusively of neutral lipids, although Ando & Hatano (1991) observed very minor amounts of phosphatidylcholine in the oil globules. In the cyprinodontid fish and the *Onchorhynchus* species, 73 % to 95 % of the oil globules were triacylglycerol (Nakagawa & Tsuchiya, 1976; Brind *et al.*, 1982; Ando & Hatano, 1991). In walleye, the oil globule contains 45 % cholesterol ester and/or wax ester and the rest is triacylglycerols (Moodie *et al.*, 1989), and in striped bass, 90 % of the oil globule is cholesterol ester and/or wax ester (Eldridge *et al.*, 1983). The oil globules make up a considerable amount of the total egg lipids, 89 %, 97 % and 67 % in the cyprinodontid fish, striped bass and walleye, respectively (Brind *et al.*, 1982; Eldridge *et al.*, 1983; Moodie *et al.*, 1989), and 27 % to 47 % in the salmonid species (Léger *et al.*, 1981; Ando & Hatano, 1991).

The functional role of oil globules in teleost eggs is uncertain. Since triacylglycerols and wax ester have a low specific gravity, the oil globules have been suggested to play a role in egg buoyancy, *i.e.* to facilitate flotation of pelagic eggs and early larvae until formation of functional swimbladders. However, since the presence of oil globules is poorly correlated with the pelagic or demersal nature of fish eggs (Table 2), oil globules appear not to function primarily in buoyancy. Instead, it is believed that the oil globules serve as a special source of nourishment to the developing embryo and the emerging larva, as further discussed below, although they must clearly add to buoyancy (Blaxter, 1988).

**Table 2**. Lipid content and composition of teleost follicles and eggs. Data assembled and calculated from the references given in the table

| Species | TL | PL | NL | PC | PUFA | DHA | Pelagic or * demersal eggs | Reference |
|---|---|---|---|---|---|---|---|---|
| **Eggs without oil globules** | | | | | | | | |
| • Atlantic cod (*Gadus morhua*) | 13 | 72 | 28 | 46 | 45 | 25 | Pelagic | Tocher & Sargent (1984) |
| • Atlantic herring (*Clupea harengus*) | 15 | 69 | 31 | 58 | 44 | 27 | Demersal | Tocher & Sargent (1984) |
| • Baltic herring (*Clupea harengus*) | — | 86[a] | 14[a] | — | 50[b] | 29[b] | Demersal | [a] Kaitaranta & Ackman (1981) [b] Kaitaranta & Linko (1984) |
| • Haddock (*Melanogrammus aeglefinus*) | 11 | 71 | 29 | 46 | 46 | 24 | Pelagic | Tocher & Sargent (1984) |
| • Halibut (*Hippoglossus hippoglossus*) | 12 | 71 | 29 | 62 | 40 | 25 | Pelagic | Falk-Petersen et al. (1986) |
| • Plaice (*Pleuronectes platessa*) | 6-7 | 63-73 | 27-37 | 58-62 | — | — | Pelagic | White & Fletcher (1987) |
| • Roach (*Rutilus rutilus*) | — | 78[a] | 22[a] | — | 42[b] | 27[b] | Demersal | [a] Kaitaranta & Ackman (1981) [b] Kaitaranta & Linko (1984) |
| • Saithe (*Pollachius virens*) | 15 | 67 | 34 | 50 | 41 | 23 | Pelagic | Tocher & Sargent (1984) |
| • Whiting (*Merlangius merlangus*) | 11 | 61 | 39 | 40 | 47 | 27 | Pelagic | Tocher & Sargent (1984) |
| **Eggs with oil globules** | | | | | | | | |
| • Atlantic salmon (*Salmo salar*) | 36[a] | 44[b] | 56[b] | 41[b] | 37[b] | 17[b] | Demersal | [a] Hamor & Garside (1977) [b] Cowey et al. (1985) |
| • Burbot (*Lota lota*) | — | 13[a] | 87[a] | — | 51[b] | 21[b] | (Pelagic) | [a] Kaitaranta & Ackman (1981) [b] Kaitaranta & Linko (1984) |
| • Capelin (*Mallotus villosus*) | 26 | 51 | 49 | 38 | 42 | 19 | Demersal | Tocher & Sargent (1984) |

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| Species | | | | | | | Habitat | Reference |
|---|---|---|---|---|---|---|---|---|
| • Chinook salmon (*Oncorhynchus tshawytscha*) | 30-32 | 41-44 | 56-59 | — | 36-44 | 16-18 | Demersal | Ashton et al. (1993) |
| • Gilthead sea bream (*Sparus aurata*) | 15-20 | 33 | 67 | 18 | 33-41 | 13-26 | Pelagic | Mourente & Odriozola (1990a, b) |
| • Perch (*Perca fluviatilis*) | — | 14[a] | 86[a] | — | 52[b] | 31[b] | Demersal | [a] Kaitaranta & Ackman (1981) [b] Kaitaranta & Linko (1984) |
| • Rainbow trout (*Oncorhyncus mykiss*) | 22[a] | 50[b] | 50[b] | — | 43[c] | 21[c] | Demersal | [a] Léger et al. (1981) [b] Kaitaranta & Ackman (1981) [c] Kaitaranta & Linko (1984) |
| • Red drum (*Sciaenops ocellata*) | 34 | 33 | 67 | — | — | — | Pelagic | Vetter et al. (1983) |
| • Red sea bream (*Pagrus major*) | 30 | 26-38 | 62-74 | — | 30-37 | 7-16 | Pelagic | Watanabe et al. (1984b) |
| • Sand eel (*Amodytes lancea*) | 20 | 23 | 77 | 17 | 43 | 20 | Demersal | Tocher & Sargent (1984) |
| • Sea bass (*Dicentrarchus labrax*) | 26-33 | 24-27 | 73-76 | 15-22 | — | — | Pelagic | Devauchelle et al. (1982) |
| • Sole (*Solea solea*) | 13-19 | 38-47 | 53-62 | 20-29 | — | — | Pelagic | Devauchelle et al. (1982) |
| • Striped bass (*Morone saxatilis*) | 56 | 2 | 98 | — | 25 | 10 | Pelagic | Eldridge et al. (1982, 1983) |
| | 51 | 9 | 91 | 7 | 24 | 6 | | Chu & Ozkizilcik (1995) |
| • Turbot (*Scophthalmus maximus*) | 14-17 | 35-50 | 50-65 | — | 36-37 | 16-20 | Pelagic | McEvoy et al. (1993) |
| | 15 | 50 | 50 | — | 35 | 13 | | Planas et al. (1993a, b) |
| • Whitefish (*Coregonus albula*) | 26[a] | 32[b] | 68[b] | — | 53[b] | 15[b] | Demersal | [a] Dadrowski & Luczynski (1984) [b] Kaitaranta (1980) |

TL, total lipid content (expressed as a percentage of egg dry weight); PL, polar lipid content (expressed as a percentage of total lipid); NL, neutral lipid content (expressed as a percentage of total lipid); PUFA, polyunsaturated fatty acids (expressed as a percentage of total fatty acids); DHA, docosahexaenoic acid [22:6(n-3)] (expressed as a percentage of total fatty acids); * Data from Russel (1976), Ahlstrom & Moser (1980) and the references given in the table

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*Fatty acid composition*

As for the total lipid content and lipid class composition of fish eggs, there is a considerable interspecific variation in the relative fatty acid composition of teleost eggs. However, although the fatty acid profiles of egg lipids differ between species, general patterns common to all species can be discerned. Lipids of teleost eggs are characterised by a high content of long-chain PUFA, with considerably more (n-3) fatty acids than (n-6) (Henderson & Tocher, 1987; Sargent *et al.*, 1989). Although fish lipids in general are known to be highly unsaturated compared to most other species in the animal kingdom, lipids of fish eggs are in most cases richer in PUFA than the corresponding maternal body lipids (Sargent *et al.*, 1989). Total PUFA in eggs of the species listed in Table 2, range from 24 % to 53 % of the total fatty acids. Particularly high levels of PUFA have been reported for eggs of wild marine teleost species from high latitudes, including Atlantic herring (*Clupea harengus*), capelin (*Mallotus villosus*), Atlantic halibut, sand eel (*Amodytes lancea*) and four Gadoids; cod, haddock, saithe and whiting (Tocher & Sargent, 1984; Falk-Petersen *et al.*, 1986). Equally high levels of PUFA, or even higher, have also been documented for eggs from wild stock of chinook salmon (*Oncorhynchus tshawytscha*) (Ashton *et al.*, 1993), and for eggs from fish caught in the brackish water of the Baltic sea, including herring burbot (*Lota lota*), roach (*Rutilus rutilus*), perch (*Perca fluviatilis*) and whitefish (*Coregonus albula*) (Kaitaranta, 1980; Kaitaranta & Linko, 1984). Total lipid of eggs from cultured rainbow trout was also noticeably rich in PUFA, although (n-6) fatty acids constituted a greater proportion of the total PUFA, compared to the others (Kaitaranta & Linko, 1984). In all the species mentioned above, PUFA range from 40 % to 53 % of the total fatty acids of total egg lipids, of which 88 % to 99 % belongs to the (n-3) series, except for the rainbow trout in which (n-3) fatty acids accounts for 70 % of total PUFA. Eggs of teleosts inhabiting freshwater is reported to contain generally higher relative levels of (n-6) fatty acids than marine species (Ackman, 1967; Henderson & Tocher, 1987). Among the (n-6) PUFA in fish eggs, both marine and freshwater species, 18:2(n-6) and 20:4(n-6) (arachidonic acid) are the major fatty acids in most cases.

The major PUFA in teleost eggs is 22:6(n-3), followed by 20:5(n-3) (Kaitaranta, 1980; Eldridge *et al.*, 1983; Kaitaranta & Linko, 1984; Tocher & Sargent, 1984; Watanabe *et al.*, 1984b; Cowey *et al.*, 1985; Falk-Petersen *et al.*, 1986; Mourente & Odriozola, 1990; Ashton *et al.*, 1993; McEvoy *et al.*, 1993). In eggs without oil globules, 22:6(n-3) accounts for 23 % to 29 % of the total fatty acids of total egg lipid, whereas 22:6(n-3) in eggs possessing oil globules in general accounts for a smaller proportion of total fatty acids (Table 2). In addition to 20:5(n-3) and 22:6(n-3), 16:0 and 18:1(n-9) are the major fatty acids in total egg lipids of most investigated fish. Other major fatty acids present in fish eggs include 16:1(n-7), 18:0, 18:1(n-7), 20:1(n-9) and 22:5(n-3).

The fatty acid composition of a total lipid extract is generally influenced by that of its constituent lipid classes (Henderson & Tocher, 1987). This may explain, at least in part, the interspecific differences in the fatty acid profiles of total lipids from eggs. Phospholipids of fish eggs, as phospholipids in general, generally contain greater proportions of

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PUFA compared to neutral lipids. Phospholipids also contain relatively more saturated fatty acids and less monounsaturated fatty acids (Henderson & Tocher, 1987; Sargent *et al.*, 1989). The greater proportion of PUFA and saturated fatty acids in phospholipids is a consequence of the non-random arrangement of saturated and unsaturated fatty acids in these molecules. A saturated fatty acid is usually present on the first carbon of the glycerol backbone, and an unsaturated fatty acid, mainly PUFA, at the second carbon (Miller *et al.*, 1976; Bell *et al.*, 1986). However, several species, including burbot, perch, sand eel and whitefish, appear to differ somewhat from the generalised picture given above, since the eggs of these species are dominated by neutral lipids and still have strikingly high PUFA content.

The generally high content of (n-3) PUFA, especially 20:5(n-3) and 22:6(n-3), in fish eggs imply that the developing embryo and emerging larvae has a requirement for high levels of these fatty acids. The exact reasons underlying the preponderance of (n-3) PUFA in eggs is not known. The preferential incorporation of PUFA into phospholipids of fish eggs, however, indicates a specific requirement for these fatty acids for the formation of biomembranes during embryogenesis. The low proportion of (n-6) PUFA in egg lipids of most species indicates that these are not involved in structural functions to any larger extent in fish embryos.

## *Comments on previous studies on egg lipids in fish*

Although numerous analyses of lipids of fish eggs have been reported in the literature, the data are fragmentary and far from extensive. Comprehensive studies where detailed lipid class and fatty acid analyses are presented have only been conducted on relatively few species (Table 2). Egg lipid composition is reported to be influenced by a number of factors, including developmental stages, egg quality, lipid composition of the broodstock diet and methodologies employed for analyses. In most of the studies listed in Table 2, either ovaries and follicles sampled during recrudescence, or fertilized eggs, have been analysed, rather than ovulated eggs. Two circumstances have to be considered when evaluating data on gonadal tissue sampled before spawning, *i.e.* ovaries and follicles. (1) The lipid composition of oocytes is dependent upon the stage of development. The proportion of polar lipids is reported to increase during ovary development in rainbow trout, Atlantic and Baltic herring (Kaitaranta & Ackman, 1981; Wiegand & Idler, 1982; Riazi & Frémont, 1988; Henderson & Almatar, 1989). Furthermore, the relative fatty acid composition in ovaries changes during sexual development in capelin (Henderson *et al.*, 1984), rainbow trout (Wiegand & Idler, 1985), Atlantic herring (Henderson & Almatar, 1989) and northern pike (Schwalme *et al.*, 1993). (2) In addition to the changes in lipid composition according to development, analyses of ovaries and follicles imply that non-oocyte tissue, including connective tissue and follicle cells, contributes to the lipid composition.

# Lipid utilization during embryonic and early larval development

Following fertilization, the developing embryo begins to utilize nutrients within the egg for its development. Deduced from the variety in lipid composition of fish eggs, there appears to be a wide difference in the lipid metabolism during embryogenesis between various species. Available literature indicates significant differences in the extent and timing of lipid utilization as an energy source during embryonic and early larval development (Henderson & Tocher, 1987).

## *Energy metabolism*

Lipids are utilized as an energy source throughout the entire period of embryogenesis in several teleost species, including red drum (Vetter *et al.*, 1983), Atlantic salmon (*Salmo salar*; Cowey *et al.*, 1985), Atlantic herring (Tocher *et al.*, 1985a, b), cod (Fraser *et al.*, 1988), dolphin fish (*Coryphaena hippurus*; Ostrowski & Divakaran, 1991) and both Atlantic and Pacific halibut (*Hippoglossus stenolepis*; Falk-Petersen *et al.*, 1989; Whyte *et al.*, 1993). The degree of lipid catabolism vary during the embryonic and larval development, and an increasing consumption of lipid as embryonic development continues has been reported for a number of fish species (Nakagawa & Tsuchiya, 1972; Hayes *et al.*, 1973; Atchinson, 1975; Boulekbache, 1981; Cetta & Capuzzo, 1982; Dadrowski & Luczynski, 1984; Tocher *et al.*, 1985a, b; Fraser *et al.*, 1988; Wiegand *et al.*, 1991). The maximum utilization of lipids for energetic purposes generally occurs after hatching, *i.e* during larval stages, although the opposite was reported for the dolphin fish (Ostrowski & Divakaran, 1991). Lipids are quantitatively the most important energy source in dolphin fish, Pacific halibut and red drum (Ostrowski & Divakaran, 1991; Vetter *et al.*, 1983; Whyte *et al.*, 1993). In developing red drum eggs, carbohydrate was the second most important energy source (Vetter *et al.*, 1983). Carbohydrates have been reported to be an important energetic source during the early stages of embryogenesis, *i.e* just after fertilization (Boulekbache, 1981; Cetta & Capuzzo, 1982). Protein did not have an important energetic role in the red drum. In contrast, protein and carbohydrate were reported to be the principal energy sources utilized during embryogenesis in winter flounder, and lipid was only significantly utilized after hatching (Cetta & Capuzzo, 1982). Protein was also indicated to be the main energy source in two freshwater coregonid species (Dadrowski & Luczynski, 1984). Free amino acids are considered a major energy source in the embryogenesis of the cod (Fyhn & Serigstad, 1987), the Atlantic halibut (Finn *et al.*, 1991) and the turbot (Rønnestad *et al.*, 1992), and lipids are the principal energy source first after hatching.

Vetter *et al.* (1983) established that both polar and neutral lipids were catabolized during embryogenesis in red drum, although neutral lipid was reported to provide 98 % of the energy consumed during development. Similarly, neutral lipids were the primary form of lipids catabolized in Atlantic salmon, but also appreciable amounts of phosphatidylcholine were used as an energy source (Cowey *et al.*, 1985). In contrast, studies on the changes in

lipid class composition of developing eggs and larvae of cod, Atlantic herring and Atlantic halibut showed that phosphatidylcholine was the principal lipid class to be consumed during embryogenesis (Tocher *et al.*, 1985b; Fraser *et al.*, 1988; Falk-Petersen *et al.*, 1989). No notable changes in the absolute quantities of neutral lipid classes occurred during this period, and only after hatching the larvae started to catabolize neutral lipids such as triacylglycerols, cholesterol and cholesterol ester (Tocher *et al.*, 1985b; Fraser *et al.*, 1988). Obviously, phosphatidylcholine is not exclusively utilized for structural purposes in fish eggs during development, but is also an important source of energy in some species. The reason for the preferential catabolism of polar lipids in these species is currently not known. However, it has been suggested that in addition to being an energy reserve, phosphatidylcholine may also act as source of substances essential for the embryo, including PUFA, phosphate or choline (Tocher *et al.*, 1985b; Fraser *et al.*, 1988). The preferential net consumption of phosphatidylcholine, rather than neutral lipid which is conventionally regarded as energy reserve, may also be related to the fact that polar lipids are more easily mobilized than neutral lipids like triacylglycerols in eggs. This may be a critical factor during early developmental stages due to a lack of a well developed alimentary system, *e.g.* presence of suitable lipases and bile salts.

*Fatty acid metabolism*

Fatty acid analysis of developing eggs and yolk-sac larvae of a number of teleost species shows a preferential catabolism of saturated and monounsaturated fatty acids with a retention of (n-3) PUFA, mainly 22:6(n-3) and 20:4(n-6) (Hayes *et al.*, 1973; Atchison, 1975; Cowey *et al.*, 1985; Tocher *et al.*, 1985a; Fraser *et al.*, 1988; Wiegand *et al.*, 1991). The conservation of 22:6(n-3) during embryogenesis has generally been considered indicative for its structural involvement, whereas the retention of 20:4(n-6) is suggested to be related to the formation of certain ecosanoids (Sargent, 1995). The preferential consumption of the PUFA-rich phosphatidylcholine during embryonic and early larval development in Atlantic herring and cod (Tocher *et al.*, 1985a; Fraser *et al.*, 1988), implies that relatively large proportions of (n-3) PUFA are consumed during energy metabolism. However, all PUFA released by the hydrolysis of phosphatidylcholine during catabolism are not oxidised, but are rather selectively retained in the neutral lipids in exchange for monounsaturated fatty acids mobilized from neutral lipid (Tocher *et al.*, 1985a; Fraser *et al.*, 1988). In the cod, approximately one third of the 22:6(n-3) released from phosphatidylcholine during catabolism was incorporated into neutral lipid. Thus, despite the specific utilization of phosphatidylcholine during embryonic and early larval development, the relative PUFA content is largely conserved. Interestingly, a specific role of (n-3) PUFA in very early embryogenesis is indicated by the considerable loss of these fatty acids immediately after fertilization (Tocher *et al.*, 1985a).

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*Oil globule utilization*

The oil globules in some teleost eggs persist for extended periods throughout the embryonic development, *i.e.* the oil globule remains constant in size until hatching (Brind *et al.*, 1982; Rønnestad et al., 1992). Since the oil globules contain no polar lipids, these can be considered as a source of energy rather than as precursors for membrane construction. Although the exact function of the oil globule in fish eggs is unknown the available information indicates that it serves as a special source of nourishment at a phase when the yolk is depleted and the emerging larvae turns from endogenous to exogenous feeding. Finally, one interesting and unsolved question is whether the origin of lipids of yolk and oil globules differs. A divergent source is indicated by the totally different lipid composition between these two compartments.

## Accumulation of egg lipids in teleosts

Although egg lipids are of major importance to embryo and yolk-sac larvae, the process of lipid accumulation in developing oocytes has received very little research attention. So far, three pathways for the accumulation of lipids in growing oocytes of teleosts have been suggested. These are: (1) the oocyte uptake of lipids as components of vitellogenin, (2) the uptake of other circulating lipoproteins and/or free fatty acids bound to albumin, and (3) the endogenous synthesis of lipids. A fourth pathway may be the bioconversion of incorporated lipids. It is, however, important to recall that none of the above mentioned pathways for egg lipid accumulation has been clearly identified and the relevance of each of them is still speculative.

*Oocyte uptake of lipids as components of vitellogenin*

Vitellogenin has been proposed to represent the major source of lipids for teleost eggs, based on the findings that teleost vitellogenin contains about 20 % lipid by mass, and that large amounts of vitellogenin is incorporated into growing oocytes of fish, (Léger *et al.* 1981; Wallace 1985; Frémont & Riazi 1988; Mommsen & Walsh, 1988; March, 1993; Sargent, 1995). However, the specific uptake of lipids via vitellogenin into growing oocytes, as well as the proportion of egg lipids derived directly from vitellogenin, have never been demonstrated for fish. The information available on the lipid moiety of teleost vitellogenin is limited, and restricted to a few species. The limited number of teleosts investigated, together with the notable interspecific differences in egg lipid composition among fish, indicate that comprehensive and detailed analyses of the lipid composition of vitellogenin and eggs from a broader range of teleost species should contribute significantly to the understanding of the process of egg lipid accumulation. It would be particularly interesting to characterise the fatty acid composition of vitellogenin from marine teleost species, in order to evaluate if the

great proportion of (n-3) PUFA found in eggs of many marine teleosts is deposited as components of vitellogenin.

## Oocyte uptake of lipoproteins other than vitellogenin

For species accumulating large amounts of neutral lipids in their eggs it has been suggested that sources other than vitellogenin may supply the egg with lipids, mainly neutral lipids. This opinion is largely based on the finding that teleost vitellogenin appears to primarily transport polar lipids (Hori *et al.*, 1979; Norberg & Haux, 1985; Norberg, 1995; Matsubara & Sawano, 1995). In rainbow trout, circulating lipoproteins besides vitellogenin exhibit seasonal variations that appear to be related to the reproductive cycle of females. The plasma level of VLDL is increased significantly in rainbow trout during vitellogenesis (Frémont *et al.*, 1984; Black & Skinner, 1987; Riazi & Frémont, 1988; Wallaert & Babin, 1994a). These observations have lead to the speculation that other lipoproteins, in addition to vitellogenin, can be taken up by growing oocytes. The hypothesis that the increased plasma level of VLDL is actually related to the process of lipid accumulation in oocytes is favoured by the finding that in rainbow trout, as in domestic hen, the plasma level of VLDL is controlled by estrogens (Wallace, 1985; Wallaert & Babin, 1992). It is well established that the oocytes of the domestic hen incorporate large amounts of VLDL in addition to vitellogenin, and that VLDL is the main carrier of lipids to the growing oocytes (Wallace, 1995). Furthermore, the same receptor on the oocyte plasma membrane appears to be responsible for the incorporation of both VLDL and vitellogenin in hen (Stifani *et al.*, 1990a). In analogy to the processes in domestic hen, circulating VLDL may be deposited in growing oocytes of some teleost species. Bearing in mind that vitellogenin is incorporated by receptor-mediated endocytosis in teleosts, similar to that in birds, non-vitellogenin lipoproteins may be incorporated by the same mechanism as described for birds.

In addition to changes in plasma lipoprotein profiles during the reproductive cycle in rainbow trout, Black & Skinner (1987) reported that the activities of both lipoprotein lipases and tissue lipases of rainbow trout undergo marked changes during the sexual maturation. A massive increase in the activities of these lipases in the ovaries during vitellogenesis was observed, indicating that in rainbow trout, the developing ovary is able to take up lipid through lipolysis of circulating lipoproteins.

Evidence for a direct ovarian uptake of lipoproteins other than vitellogenin has only been reported for one teleost species, the winter flounder (Nagler & Idler, 1990). In winter flounder, another VHDL, besides vitellogenin, termed VHDL II appears to be taken up by the ovaries of vitellogenic females. Quantitative studies on the oocyte uptake of vitellogenin and VHDL II, indicated that these two lipoproteins contribute equally to the ovarian yolk proteins (Nagler & Idler, 1990). However, the relative contribution is uncertain and in a later study it was suggested that VHDL II makes only a modest contribution to ovarian yolk protein (Nagler *et al.*, 1991). By the use of immunoelectron microscopy techniques it was also shown that vitellogenin was specifically localised in yolk spheres, whereas no

specific localisation for VHDL II within the follicle was found, indicating that VHDL II is not a true yolk component. Furthermore, although VHDL has been identified as a lipoprotein, the lipid content and composition remains to be demonstrated.

*Endogenous synthesis of lipids within the oocyte*

While it has been suggested in some studies that circulating lipoproteins may complement vitellogenin in the process of oocyte lipid accumulation in rainbow trout, Wiegand & Idler (1982), have determined some capacity for endogenous synthesis of lipids from acetate in the ovary of this species. Wiegand & Idler (1982) reported that ovarian tissue of rainbow trout synthesised lipids *de novo* from acetate well into the vitellogenic phase of development. The high proportion of labelled acetate recovered in fatty acids of triacylglycerols in vitellogenic fish suggests that the ovary may make some contribution to its triacylglycerol reserve in addition to triacylglycerols sequestered from vitellogenin or other lipoproteins. It is important to recall that saturated and unsaturated fatty acids of the (n-9) series are the only products of fatty acid synthesis *de novo* in fish (further discussed in the section "Essential fatty acids and dietary requirements in teleost fish") (Henderson & Tocher, 1987). In opaline gourami (*Trichogaster cosby*), a species that accumulate large amounts of wax esters in the egg, Sand *et al.* (1969) showed that wax esters were synthesised *de novo* from acetate or from fatty acids and alcohols. Endogenous synthesis of lipids during oocyte growth in fish was initially proposed in early morphological studies (Chopra, 1958; Guraya, 1963; Beams & Kessel, 1973; Shackley & King, 1977). According to these studies, lipid inclusions begin to accumulate in the cytoplasm of oocytes in most teleosts about the same time as cortical alveoli appears, *i.e.* in previtellogenic oocytes. Although endogenous synthesis was originally suggested for these lipid inclusions, these interpretations were based on ultrastructural criteria. As mentioned previously, under "Definitions and general aspects of vitellogenesis in teleost fish", the origin and function of these lipid inclusions are far from understood. Furthermore, even if the previtellogenic lipid inclusions are endogenously synthesised and will sustain until fertilization of the egg, the previtellogenic oocytes are still very small and it is therefore not likely that these lipids will contribute to any larger extent to the lipid composition of ovulated eggs.

Lipid class composition of eggs may also be related to metabolic processes within the oocyte, *i.e.* bioconversion of lipids incorporated from the blood. In opaline gourami, wax esters constitute the major lipid class of the egg, whereas maternal depot lipids contain hardly no wax esters, but are in instead dominated by triacylglycerols (Sand & Schlenk, 1969; Sand *et al.*, 1969). Thus, the wax esters may be synthesised endogenously in the oocyte from fatty acids delivered as part of lipoproteins or bound to serum albumin. Finally, considering the limited capacity of teleost fish, especially of marine species, to *de novo* synthesise and bioconvert fatty acids, endogenous synthesis of lipids within fish oocytes, particularly in marine species, is unlikely to be an important or major contributor to egg lipid composition.

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# Lipid metabolism in teleosts during reproduction

## Functional role of polyunsaturated fatty acids in teleost fish

The functional role of long-chain highly unsaturated fatty acids in fish, as well as in all other animals, is poorly understood (Sargent *et al.*, 1993). The physicochemical aspects of PUFA in animals in general have been reviewed by Brenner (1984) and Cullis & Hope (1991), and in fish by Bell *et al.*, (1986). The function of specific fatty acids in animals appears to be related to both biochemical and biophysical mechanisms, and two principal functions of PUFA, in addition to their role as an energy source, have been proposed (Brenner, 1984; Bell *et al.*, 1986; Cullis & Hope, 1991). One of them is the role of certain PUFA, principally 20:4(n-6) and 20:5(n-3), as precursors of eicosanoids, such as prostaglandins, thromboxanes and leukotrienes. The second function is less specific and is related to the role of PUFA as essential components of biological cell membranes. The fatty acid moiety of membrane lipids contributes to the physicochemical properties of biological membranes, and certain PUFA are believed to be critically important for an extensive array of membrane-associated functions. The fatty acid composition of membrane lipids influence structure, fluidity, permeability and lipid-protein interaction of cellular membranes, although in ways that are not completely understood at present (Brenner, 1984; Bell *et al.*, 1986; Cullis & Hope, 1991). It is, however, well recognised that membranes with low metabolic activity, such as nerve myelin, have a relatively high content of saturated and monounsaturated fatty acids, whereas metabolically active membranes, such as those of mitochondria and nervous cells, tend to contain greater proportions of highly unsaturated fatty acids (Cullis & Hope, 1991; Sargent *et al.*, 1993).

The physical and chemical properties of a fatty acid is largely dependent on the length and number of double bonds (Bell *et al.*, 1986; Cullis & Hope, 1991). If a fatty acid is fully saturated, it is a straight carbon chain. Straight carbon chains interact favourably with adjacent chains and cluster or pack in ordered structures. A double bond, however, produces a bend in the hydrocarbon chain, which interferes with the highly ordered packing of acyl chains and profoundly influence the physical properties of the fatty acids. Saturated fatty acids with 10 or more carbon atoms are solid at room temperature, but the insertion of one single double bond make these fatty acids fluid. The melting point, also referred to as the phase transition point, of the fatty acid 16:0 is 63 °C, whereas the same fatty acid with one double bond, *i.e.* 16:1(n-7), has a phase transition point around 0 °C (Hadley, 1985). The presence of additional double bonds further depresses the phase transition points, and the transition temperature for the PUFA commonly found in natural fish lipids have transition temperatures far below 0 °C, *i.e.* -50 °C, -54 °C and -45 °C for 20:4(n-6), 20:5(n-3) and 22:6(n-3), respectively (Fasman, 1975).

Since the physicochemical properties of lipids built from fatty acids to a large extent are determined by its constituent fatty acids, fatty acid containing membrane lipids follow the same phase transition behaviour as described for free fatty acids. The feature that fatty acids

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and lipids derived from them have variable physical properties under different physiological conditions, *e.g.* at different temperatures, have important implications for biological cell membranes. When a biological membrane is cooled under its transition temperature, the membrane changes from a fluid liquid-crystalline stage to a very viscous gel phase (equivalent to solid). This changes the physical properties of the membrane drastically and the lateral mobility of the membrane compounds is considerably reduced (Cullis & Hope, 1991). According to the fluid-mosaic model by Singer & Nicholson (1972), functional biomembranes must be in a "fluid" state or liquid-crystalline state to function properly, *i.e.* the lipids and proteins of membranes shall move relatively freely in the plane of the double layer. The lateral mobility of membrane components is commonly described by the term "membrane fluidity" and is largely determined by the lipid composition of the membrane. Thus, introducing lipid classes containing fatty acids with higher degree of unsaturation into membranes prevents close packing of the membrane lipids and making the membrane more open and fluid. However, the physicochemical properties of biomembranes are not only determined by the length of the fatty acid residues and their degree of unsaturation, the properties are also influenced in a complex manner by the interactions of these lipids with cholesterol and membrane proteins. In addition, the physical properties of cell membranes are determined by the individual phospholipids therein.

In accordance with the temperature dependent phase behaviour of membrane lipids, the particularly great proportion of (n-3) PUFA in phospholipids of teleosts is hypothesised to be an adaptation to maintain normal cellular functions at low temperatures (Hazel, 1984; Bell *et al.*, 1986). Fish are poikilothermic animals, and the noticeably high degree of fatty acid unsaturation in fish, especially in marine cold water species, is suggested to allow structure, fluidity, permeability and lipid-protein interaction of biomembranes to be preserved even at low temperatures. Long-chain PUFA may also have important functions in maintaining the structural and functional role of lipoproteins (Wallaert & Babin, 1993, 1994b) and other compartmentalisations of lipid, including stored lipids. Although the exact function of specific fatty acids are unclear, the precise lipid composition of cellular membranes appears to be biologically important, since poikilothermic organisms like fish change the fatty acid composition of their membranes when the water temperature change, so as to maintain relatively constant membrane properties (Hazel, 1984; Wallaert & Babin, 1994b).

It has to be emphasised that the functional roles of PUFA in fish may be related to other and more complex mechanisms than just membrane fluidity. This is supported by the fact that all PUFA have a phase transition temperature far below that of the body of any fish. In other words, there is no physico-chemical basis for believing that membrane fluidity at low temperatures is enhanced either by replacing 20:4(n-6) by 20:5(n-3) or by replacing 20:5(n-3) with 22:6(n-3). In fact, 22:6(n-3) melts some 10 °C higher than 20:5(n-3). Various PUFA have conformations very different from saturated and monounsaturated fatty acids. Long-chain highly unsaturated fatty acids, such as 22:6(n-3), form compact conformations and can assume a U-shape or be nearly circular as well as adopt highly ordered helical stuctures

(Cullis & Hope, 1991; Sargent *et al*., 1993). These complex structures may result in markedly different properties of the molecules which can have a strong implication on structuring cell membranes, which in turn will influence a variety of membrane functions such as permeability, ion-channels and transport, the activity of membrane-associated enzymes and receptor functions. In addition to the sheetlike lipid bilayer (Singer & Nicholson, 1972), lipids can also adopt entirely different structures on hydration, at least in isolation. These are referred to as mesomorphic phases and some of these are believed to be of major physiological importance (Cullis & Hope, 1991).

## Essential fatty acids and dietary requirements in teleost fish

Although the precise role of (n-3) PUFA in fish is relatively unclear, it is well recognised that these fatty acids are required by fish for normal growth, development and survival (reviewed by Castell, 1978; Watanabe, 1982; Henderson & Tocher, 1987; Sargent *et al*., 1989). Extensive studies have shown that PUFA are essential in fish as well as in most other animals, *i.e.* they cannot be synthesised *de novo* by the animal yet is required for the well-being. The essentiality of certain fatty acids in animals was first suggested by Burr & Burr in 1929, who demonstrated that exclusion of lipid from the diet of rats resulted in severe deficiency symptoms. These symptoms could be prevented by adding 18:2(n-6) to the diet (Burr & Burr, 1930).

Lipid requirements for fish, mainly salmonid species, came under investigation in the 1960s. In accordance with knowledge from studies on terrestrial vertebrates, it was assumed that also fish required (n-6) fatty acids. Formulated fish diets were therefore supplemented with vegetable oils like corn, peanut, or sunflower oil, which are rich in 18:2(n-6) and were popular oil components in diets for avian and mammalian species. However, the use of vegetable oils produced a number of adverse effects on the growth and condition of fish, including fin erosions, swollen pale livers, acute local myocarditis, shock or fainting syndrome and reproductive disturbances. Instead it was found that dietary fish oil was superior to vegetable oil in promoting growth and preventing nutritional pathologies of fish. Pioneering nutritional studies by Castell *et al*. (1972a, b) conclusively proved that PUFA of the (n-3) series, rather than (n-6), played the role of essential fatty acids for rainbow trout. This was later demonstrated to be true for other teleosts as well, with the exception of some freshwater species living in warm water, including *Tilapia zillii* and *Oreochromis nilotica*, which appear to require (n-6) fatty acids rather than (n-3) fatty acids (for rewiews see Castell, 1978; Watanabe, 1982; Henderson & Tocher, 1987; Sargent *et al*., 1989). However, fish in general appear to have an essential requirement for (n-6) PUFA as well, albeit in lower concentrations, especially 20:4(n-6), as a precursor for eicosanoids (Sargent *et al*., 1995).

The dietary requirement of fish and other animals for PUFA results from limitations in the capacity in *de novo* synthesis and bioconversion of fatty acids. The pathway of fatty acid

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biosynthesis in fish is assumed to be basically similar to that operating in mammals (Henderson & Tocher, 1987). In contrast to mammals, where lipids are synthesised both in the liver and adipose tissue, the major site for lipid synthesis in fish is the liver. Animals can synthesise saturated fatty acids and (n-9) series unsaturated fatty acids *de novo* from acetyl-CoA. Briefly, the two-carbon acetyl-CoA unit is carboxylated to malonyl-CoA, which is subsequently converted to fatty acids by the fatty acid synthetase complex. The product of fatty acid synthetases is saturated fatty acids, mainly 16:0 and lesser amounts of 18:0 and 14:0. These saturated fatty acids, together with dietary saturated fatty acids, can then be modified by processes of desaturation and elongation to synthesise unsaturated fatty acids of the (n-9) configuration. The first double bond introduced to a saturated fatty acid in animals is inserted between the 9 and 10 carbon by a $\Delta9$ desaturase enzyme (the position where desaturation occurs, and the respective desaturase enzyme, is designated numerically by the carbon from carboxyl carbon). In common with all other vertebrates, fish lack the $\Delta12$ and $\Delta15$ desaturase enzymes necessary for the synthesis of (n-6) and (n-3) fatty acids. These series of fatty acids must therefore be obtained in the diet (Sargent *et al*., 1989). Plants, on the other hand, posses these enzymes and thereby the capacity to desaturate fatty acids between the already existing double bond and the methyl terminus, *i.e.* capable of converting (n-9) fatty acids to (n-6) and (n-3) fatty acids. Consequently, all (n-3) and (n-6) PUFA in fish ultimately originate from (n-3) and (n-6) PUFA formed in plants and ingested by the diet (Sargent *et al*., 1989). However, once 18:2(n-6) and 18:3(n-6) are obtained from the diet, these fatty acids can be further desaturated (by $\Delta6$, $\Delta5$ and $\Delta4$ desaturases) and elongated to longer chain and more unsaturated fatty acids in most vertebrates (Sargent *et al*., 1989).

Metabolism of PUFA in fish has been carefully investigated by both radioactive tracers and nutritional balance experiments and the pathways of desaturation and elongation of fatty acids that are possible in fish is outlined in Figure 6. Although animals in general can insert further double bonds into dietary (n-3) and (n-6) PUFA between existing double bonds and the carboxyl terminus, the ability of fish to desaturate and elongate fatty acids varies among species (Henderson & Tocher, 1987; Sargent *et al*., 1989). The overall situation is that freshwater and euryhaline teleost species can desaturate and elongate C18 PUFA of the (n-3) and (n-6) series by the pathways outlined in Figure 6, *i.e.* similar to that of birds and mammals. This means that the essential fatty acid requirements of most freshwater fish can be met by supplying 18:2(n-6) and 18:3(n-3) in their diet. Marine fish, however, differ from most other vertebrates by lacking or having a low capacity for the conversion of C18 PUFA to C20 and C22 PUFA due to an impairment of one or more of the enzymes in the desaturation/elongation pathways for fatty acids (Sargent *et al*., 1989). This poor ability leads marine fish to require long-chain PUFA, particularly 20:5(n-3) and 22:6(n-3), and certainly also 20:4(n-6) in their diet.
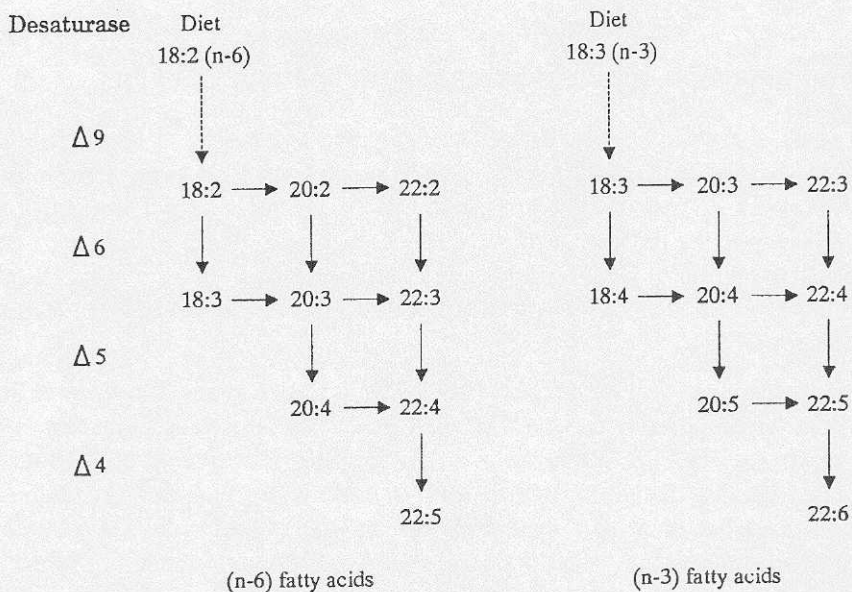
Desaturase    Diet                                    Diet
              18:2 (n-6)                               18:3 (n-3)

Δ9                │                                        │
                  ▼                                        ▼
              18:2 ⟶ 20:2 ⟶ 22:2                 18:3 ⟶ 20:3 ⟶ 22:3

Δ6              │      │      │                     │      │      │
                ▼      ▼      ▼                     ▼      ▼      ▼
              18:3 ⟶ 20:3 ⟶ 22:3                 18:4 ⟶ 20:4 ⟶ 22:4

Δ5                     │      │                            │      │
                       ▼      ▼                            ▼      ▼
                     20:4 ⟶ 22:4                         20:5 ⟶ 22:5

Δ4                            │                                   │
                              ▼                                   ▼
                            22:5                                 22:6

              (n-6) fatty acids                        (n-3) fatty acids

**Fig. 6.** Schematic representation of the possible pathways for desaturation and elongation of fatty acids in teleost fish. Horizontal lines represent elongation by the addition of a two-carbon unit, and vertical lines represent desaturation steps.

Although teleosts living in the marine environment appear to differ in their ability to modify dietary fatty acids compared to freshwater species, it must be emphasised that this apparent difference is based on detailed studies on a few species. Most of the information on PUFA metabolism in marine fish is based on studies on carnivorous species such as plaice (Owen *et al.*, 1972), gilt-head sea bream (*Sparus aurata*, Koven *et al.*, 1989; Mourente & Tocher, 1993) and turbot (Owen *et al.*, 1975; Cowey *et al.*, 1976a, b; Tocker & Mackinlay, 1990; Linares & Henderson, 1991). The dietary lipids of these marine species are rich in PUFA of the (n-3) series, especially 20:5(n-3) and 22:6(n-3) (Sargent *et al.*, 1989). Consequently, marine carnivorous fish receive copious amounts of C20 and C22 PUFA in their natural diet and therefore seldom need to form these fatty acids. This can be the explanation for the impaired capacity of marine teleost species to modify dietary fatty acids such as 18:2(n-6) and 18:3(n-3) (Henderson & Tocher, 1987). In contrast, the majority of the freshwater and euryhaline species studied, including ayu (*Plecoglossus altivelis*), carp (*Cyprinus carpio*), eel (*Anguilla japonica*) and rainbow trout, are more omnivorous in their feeding habits and the diets are mainly based on invertebrates and plants which are rich in 18:3(n-3) and less rich in 20:5(n-3) and 22:6(n-3) (Watanabe, 1982; Sargent *et al.*, 1989). The discrepancy in the capacity of conversion of fatty acids in fish may thus be related to feeding habits rather than to environmental salinity (Sargent, 1995). This hypothesis is actualised by the recent finding that an extreme freshwater carnivore species, pike (*Esox lucius*), appears to be unable to synthesise long chain PUFA from shorter chain precursors due to a deficiency of Δ5-desaturase (Henderson *et al.*, 1995).

## Dietary influence on egg lipid composition and egg viability

In recent years, factors influencing female fecundity and egg quality in teleost fish have received considerable attention, mainly due to an increasing interest in aquaculture and wild stock enhancement of various teleost species. One of the most critical aspects in fish aquaculture today is the production of viable eggs (Bromage, 1995). This appears to be largely related to nutritional factors, particularly in marine species, and special attention has been paid to the metabolism of lipids with emphasis on (n-3) PUFA (Watanabe & Kiron, 1994; Sargent, 1995).

It is well recognized that the fatty acid composition of fish tissues and lipoproteins is altered within certain limits by the composition of the dietary fatty acids (Watanabe, 1982; Greene & Selivonchick, 1987; Henderson & Tocher, 1987; Sargent et al., 1989). The dietary influence on the distribution of fatty acids in tissue is largely dictated by the limited capacity of teleost fish to de novo synthesise and convert fatty acids. In this context, the impact of dietary fatty acids is larger in marine teleost species compared to freshwater and euryhaline fish. Several studies on cultured fish ave shown that the fatty acid profile of teleost eggs is markedly influenced by the fatty acid composition of the diet supplied to the maternal fish (Léger et al., 1981; Watanabe et al., 1984b; Leray et al., 1985; Mourente & Odriozola, 1990; Chou et al., 1993; Harel et al., 1994). Inadequate diet, especially with respect to the essential (n-3) PUFA, fed to maternal broodstock has, in addition to affecting the distribution of fatty acids within the eggs, also been found to adversely affect fecundity and egg viability in fish (Léger et al., 1981; Watanabe et al., 1984a; Leray et al., 1985; Chou et al., 1993; Harel et al., 1994). A decrease in the number and size of eggs produced, a lower rate of fertilization, the appearance of embryonic abnormalities, an increased incidence of early embryonic mortality, a lowered hatching rate and subsequent physiological dysfunctions of the emerging larvae have been reported for broodstock fish fed (n-3) PUFA deficient diet. Furthermore, when rainbow trout were fed an (n-3) fatty acid-deficient diet for several months prior to spawning, a reduced concentration of vitellogenin in serum was observed, together with a lower content of (n-3) PUFA in both eggs and vitellogenin (Léger et al., 1981; Frémont et al., 1984).

Despite intensive work in evaluating nutritional requirements of a wide range of teleost species, there is a lack of knowledge concerning the interactions between maternal broodstock nutrition and reproduction, as well as the relationship between egg lipid composition and egg quality. Considering that the ovaries of many sexually mature teleosts account for more than 20 % of the body weight, together with the notably high content of (n-3) PUFA in fish eggs, it is obvious that female fish require large amounts of (n-3) PUFA in the diet prior to or during sexual maturation.

Despite the knowledge that the composition of the diet affects the fatty acid composition of eggs, very few studies have compared the fatty acid profile of eggs from cultured fish with the profile of eggs from wild stock. Ashton et al. (1993) reported that the fatty acid composition of total lipids from eggs of cultured chinook salmon fed a formulated

diet was significantly different than those from eggs of wild fish of the same stock. The mean total percentage of (n-3) PUFA in total lipid of eggs from wild and cultured fish were 40 and 29 %, and in polar lipid 46 and 39 %, respectively. The lower level of (n-3) fatty acids in cultured fish was mainly due to lower proportions of 20:5(n-3) and 22:5(n-3). However, 22:6(n-3) was highly conserved in eggs of chinook salmon in spite of the apparently differentiated diet composition. Significantly higher levels of (n-3) PUFA and 20:5(n-3), and 22:6(n-3) as well, were also observed for eggs from wild caught females of striped bass compared to eggs from cultured individuals (Harrell & Woods III, 1995).

Formulated diets generally contain higher levels of 18:2(n-6) (linoleic acid), 20:1(n-9) and 22:1(n-11) compared to natural diets. This is caused by the oil constituents of the formulated food, which usually is based on a mixture of vegetable oils (rich in (n-6) fatty acids) and commercial fish oil extracted from capelin, herring and sprat (rich in the monounsaturated fatty acids, 20:1n-9 and 22:1n-11) (Ackman, 1980; Sargent et al., 1989). The high content of 20:1(n-9) and 22:1(n-11) in neutral lipids of capelin, herring and sprat is due to the abundance of these fatty acids in their natural diet, i.e. calanoid copepods. A high content of these fatty acids is characteristic of virtually all commercial fish oils produced from northern Atlantic fisheries (Ackman, 1980; Sargent, 1995).

## Mobilisation of lipids during oocyte growth

Teleost fish exhibit seasonal variations in their body lipid levels that are related to the reproductive cycle (Sargent, 1995). Lipids accumulated in growing oocytes during gonadal recrudescence in female fish, may have a direct alimentary origin, or be mobilized from body-lipid reserves such as muscle, subcutaneous adipose tissue, liver and mesenteric lipids (Sheridan, 1988).

In spring spawning populations of capelin (Henderson et al., 1984) and Atlantic herring (Henderson & Almatar, 1989), an appreciable amounts of lipids are accumulated in the body, i.e. 10 to 20 % of their wet body weight, during the summer when there is plentiful supply of diet from the plankton blooms of spring. Capelin and herring store most of their depot lipids in the muscles and subcutaneous tissue in the form of triacylglycerols, which for Atlantic herring accounted for > 90 % of total lipid in the carcass. Henderson et al. (1984) examined the seasonal changes in the lipid content and composition of capelin from January when gonadal development became noticeable, until May when the fish spawned. During this period a considerable amount of lipids was mobilised from the muscles and subcutaneous tissue (76 % of total lipid) (Henderson et al., 1984). Approximately 40 % of the lipid mobilised could be accounted for by lipid accumulated in the ovary, while the remaining 60 % was utilised for energy production (Henderson et al., 1984). Shatonovskiy (1971) reported similar values for cod studied over a period of 4 months when gonadal maturation occurred in the absence of food intake.

In capelin there was a selective mobilisation of fatty acids from muscle lipid with discrimination against 20:1 and 22:1 (Henderson *et al.*, 1984). No evidence for a selective mobilisation of depot fatty acids was observed for Atlantic herring (Henderson & Almatar, 1989). However, in both capelin and herring, 20:1(n-9) and 22:1(n-11) were only minor components of the ovary lipid, despite the preponderance of these fatty acids in the body-lipid reserves. This suggests that these long-chain monounsaturated fatty acids are specifically oxidised for energy production rather than being utilised in the production of gonadal lipid. A selective mobilisation of certain fatty acids during vitellogenesis has also been reported for northern pike (Schwalme *et al.*, 1993) and cod (Jangaard *et al.*, 1967; Takama *et al.*, 1985). Finally, selectivity during the mobilisation of depot lipids for oocyte development is further confirmed by the observation that triacylglycerol was the major lipid class (> 90 %) in the carcass lipid of Atlantic herring, while approximately 70 % of ovary lipids from the same species were polar lipids (Henderson & Almatar, 1989).

# AIMS OF THE STUDY

The overall objective of the present thesis was to gain information about lipid metabolism in female fish during reproduction, with emphasis on the processes occurring during vitellogenesis. Special attention has been paid to the egg yolk precursor, vitellogenin, and the role this lipoprotein plays in the process of lipid accumulation in growing oocytes.

Specific aims were:

- To further develop a simple and reproducible isolation method that yields a pure and intact fraction of teleost vitellogenin.
- To evaluate methods for the analysis of fish lipids, including lipid extraction, thin-layer chromatography (TLC), gas-liquid chromatography (GLC) and high performance liquid chromatography (HPLC) with light scattering detection.
- To partially characterise the apolipoprotein of teleost vitellogenin, and to raise homologous antisera directed against vitellogenin from four teleost species.
- To examine the total lipid content, and the lipid class and fatty acid composition of teleost vitellogenin.
- To characterise the lipid class and fatty acid composition of ovulated eggs from wild and cultured turbot (*Scophthalmus maximus*), in order to estimate the nutritional requirements during embryonic and early larval development.
- To provide an insight into the interrelationship between the lipid moiety of ovulated eggs and that of vitellogenin from cod (*Gadus morhua*) and turbot, by comparing the distribution of lipid classes and fatty acids in eggs and vitellogenin.

# RESULTS AND DISCUSSION

The most important results of the papers included in the present thesis will be presented and discussed below. This section will begin with a description and evaluation of the methods that have been developed and used in this study. A more detailed description of materials and methods used, is presented in the individual papers (I-V).

## Induction and isolation of teleost vitellogenin

In order to study the biochemical composition of teleost vitellogenin, as well as various events of vitellogenesis, a pure preparation of intact vitellogenin is required. This project was originally initiated to examine the lipid moiety of teleost vitellogenin. To enable such studies, the first aim was to develop an effective and reproducible isolation method that yielded a pure fraction of undegraded vitellogenin. As the obtained vitellogenin preparation was going to be used for further studies of the lipid composition, special care had to be taken to avoid loss the of noncovalently associated lipid molecules.

Successful isolation of pure and intact vitellogenin from teleosts has proved difficult due to the chemical characteristics of fish vitellogenin. Vitellogenin is susceptible to proteolysis, which appears to be particularly true for vitellogenin of various teleost species (Hickey & Wallace, 1974; deVlaming et al., 1980; Selman & Wallace, 1983; Norberg & Haux, 1985; Tyler & Sumpter, 1990a; Goodwin et al., 1992). In addition, methods for the purification of vitellogenin that are based on surface charge are not generally applicable to teleost vitellogenin, due to their lower degree of phosphorylation (deVlaming et al., 1980; Selman & Wallace, 1983; Norberg & Haux, 1985; Tyler & Sumpter, 1990a). Further, teleost vitellogenin, appears to be biochemically more heterogeneous compared with vitellogenin of other vertebrates, and this may complicate the purification procedure (Norberg & Haux, 1985). In the present thesis, vitellogenin was purified from plasma of cod, rainbow trout, turbot and wolffish *(Anarhichas lupus)* by precipitation with EDTA:$Mg^{2+}$ and distilled water, and by high-performance anion-exchange chromatography (I; II). Although vitellogenin from several teleost species, including rainbow trout, had been isolated and partially characterised earlier, vitellogenin of cod, turbot and wolffish had not been previously isolated, and hence, the properties of these vitellogenins were not known.

### Induction of vitellogenin by injections of estradiol-17β

Vitellogenin synthesis was induced by repeated intraperitoneal injections of estradiol-17β. Administration of estradiol-17β to cod, rainbow trout, turbot and wolffish caused a rapid

increase in total plasma protein concentration (I; II). The plasma protein concentrations for the four species investigated increased from 28-40 mg/ml to 100-115 mg/ml after treatment. The increase in plasma protein levels was principally due to the appearance of vitellogenin, as shown by chromatography and gel electrophoresis (I; II).

In previous studies, mainly two intraperitoneal injection methods have been used for the administration of estradiol-17β to fish. Estradiol-17β was either dispersed in vegetable oil, usually peanut oil, or dissolved in anhydrous ethanol. The use of vegetable oil as a vehicle for estradiol-17β is the most commonly employed method, mainly due to an apparently slower and more stable rate of release of estradiol-17β from the intraperitoneal cavity to the circulation (Pankhurst et al., 1986). A particularly slow rate of release is obtained when cocoa butter is used (Pankhurst et al., 1986), as cocoa butter has a relatively high melting point and becomes solid at fish body temperatures, due to a great proportion of saturated fatty acids (Christie, 1987). Furthermore, ethanol may cause toxic effects on fish tissues. On the other hand, vegetable oils have a fatty acid composition very different from that of fish lipids (Sargent et al., 1989), and there is a risk that the fatty acid composition of the oil used as injection vehicle may influence the fatty acid composition of vitellogenin and tissue lipids of the experimental fish.

In order to examine if the injection technique influenced the fatty acid composition of fish liver and vitellogenin, groups of cod (488±100 g) were injected with 10 mg estradiol-17β·kg$^{-1}$, either dispersed in peanut oil (n=5) or dissolved in ethanol (n=5) according to the method described in paper II. The water temperature was 10-12 °C. Both injection methods resulted in high plasma protein concentration, 68±12 mg/ml and 80±3 mg/ml for fish injected with estradiol-17β in peanut oil and ethanol, respectively. The simplified relative fatty acid composition of total lipid extracted from liver and vitellogenin of the two groups of cod is shown in Table 3, along with the fatty acid composition of peanut oil. The peanut oil was particularly rich in 18:1(n-9) and 18:2(n-6), and differed drastically from the fatty acid composition of total lipid from liver and vitellogenin (Table 3). However, no effect of peanut oil on the relative fatty acid composition of total lipids from cod liver and vitellogenin could be discerned, except for a somewhat larger variation between individuals in the group injected with peanut oil. A larger variation was also observed for the total plasma protein concentration indicating a less consistent induction of vitellogenin with peanut oil as carrier compared to ethanol. Based on these results, estradiol was injected with ethanol in the subsequent studies, except for paper I. It is important to recall that the solution of ethanol-estradiol-17β was diluted with at least four volumes of 0.9 % NaCl prior to injection to reduce the risk of local toxic effects on the intraperitoneal organs (II).

**Table 3.** Fatty acid composition of peanut oil and of total lipid extracted from liver and vitellogenin of Atlantic cod injected with estadiol-17ß dispersed in peanut oil or dissolved in ethanol.

| Fatty acids | Peanut oil | Liver Oil | Liver Ethanol | Vitellogenin Oil | Vitellogenin Ethanol |
|---|---|---|---|---|---|
| 14:0 | — | 2.6 ± 1.3 | 2.7 ± 0.5 | 1.4 ± 0.6 | 1.4 ± 0.3 |
| 16:0 | 10.5 | 18.1 ± 3.5 | 16.1 ± 0.6 | 21.2 ± 1.5 | 20.4 ± 0.5 |
| 16:1 (n-9) | — | 1.0 ± 0.2 | 1.0 ± 0.1 | — | — |
| 16:1 (n-7) | — | 6.7 ± 2.4 | 7.1 ± 1.2 | 2.8 ± 0.4 | 2.8 ± 0.3 |
| 18:0 | 2.6 | 3.9 ± 1.0 | 3.7 ± 0.7 | 2.8 ± 0.5 | 2.7 ± 0.3 |
| 18:1 (n-9) | 48.2 | 17.9 ± 2.7 | 15.1 ± 2.3 | 13.4 ± 0.9 | 12.1 ± 0.9 |
| 18:1 (n-7) | — | 5.6 ± 1.8 | 6.7 ± 1.4 | 3.9 ± 1.2 | 4.6 ± 0.5 |
| 18:2 (n-6) | 29.5 | 1.6 ± 0.6 | 1.2 ± 0.2 | — | — |
| 20:0 | 1.4 | — | — | — | — |
| 20:1 (n-11) | — | 1.0 ± 0.4 | 1.2 ± 0.2 | — | — |
| 20:1 (n-9) | 1.5 | 1.5 ± 0.6 | 1.8 ± 0.5 | — | — |
| 20:1 (n-7) | — | 1.1 ± 0.5 | 1.4 ± 0.3 | — | — |
| 20:4 (n-6) | — | 2.1 ± 0.8 | 2.3 ± 0.4 | 3.7 ± 0.8 | 4.1 ± 0.3 |
| 20:5 (n-3) | — | 8.3 ± 2.6 | 8.0 ± 1.6 | 10.6 ± 2.1 | 11.1 ± 1.0 |
| 22:1 (n-11) | 3.0 | — | — | — | — |
| 22:5 (n-3) | — | 2.9 ± 1.6 | 4.3 ± 0.9 | 3.0 ± 1.2 | 3.7 ± 0.4 |
| 22:6 (n-3) | — | 15.7 ± 6.3 | 16.5 ± 4.0 | 28.6 ± 4.2 | 28.2 ± 1.3 |
| 24:1 (n-9) | 2.1 | — | — | — | — |
| Total saturated | 14.5 | 26.9 ± 2.5 | 24.9 ± 1.4 | 26.9 ± 0.9 | 26.0 ± 0.4 |
| Total monounsaturated | 54.8 | 37.0 ± 7.0 | 36.9 ± 2.9 | 22.8 ± 1.8 | 22.6 ± 0.9 |
| Total polyunsaturated | 29.5 | 36.1 ± 4.5 | 38.1 ± 3.9 | 50.2 ± 1.2 | 51.4 ± 0.5 |
| Total (n-3) | — | 31.0 ± 4.4 | 33.1 ± 3.7 | 44.6 ± 1.8 | 45.4 ± 0.6 |
| Total (n-6) | 29.5 | 4.9 ± 0.6 | 4.9 ± 0.5 | 5.4 ± 1.1 | 5.7 ± 0.4 |
| Total (n-3)/(n-6) | — | 6.3 ± 0.9 | 6.8 ± 0.6 | 8.3 ± 1.7 | 8.0 ± 0.5 |

Data are are expressed as weight % of total identified fatty acids. Fatty acids accounting for less than 1.0 % of total fatty acids are not presented (—). Data for peanut oil are the average of two samples and data on liver and vitellogenin represent means ± SD of five fish. The cod were injected intraperitoneally every fifth day 10 mg E-17ß/kg. Totally the fish recieved three injections and the blood was sampled 15 days after first hormone-injection. Vitellogenin was isolated by selective precipitation according to paper II.

## High performance anion-exchange chromatography

Teleost vitellogenin can be successfully isolated from plasma of estradiol-17β-treated fish (I; II), as well as from plasma of naturally vitellogenic fish (Silversand et al., 1996, data not shown), by means of high performance anion-exchange chromatography. A Mono Q anion-exchange column connected to a fast protein liquid chromatography (FPLC) system was employed for the purification. The plasma preparation obtained was loaded directly onto the Mono Q column or first processed by selective precipitation of vitellogenin. The method was evaluated for the purification of vitellogenin from estradiol-17β-treated turbot (I), and later adopted for the isolation of vitellogenin from other teleost species (II). When plasma from estradiol-17β-treated fish was analysed by anion-exchange chromatography on a

Mono Q column and eluted with a linear salt gradient, vitellogenin emerged as a large absorbance peak that eluted as the last component for all species. This peak was absent in the plasma of control fish. The observation that vitellogenin eluted at the highest salt concentration from the ion-exchange column, verifies the high negative surface charge of the vitellogenin molecule, and corroborates other studies using anion-exchange chromatography for the purification of vitellogenin (Wiley *et al*., 1979; deVlaming *et al*., 1980; Selman and Wallace, 1983; Norberg & Haux, 1985; Tyler & Sumpter, 1990a; Kishida *et al*., 1992; Tao *et al*., 1993; Mañanós *et al*., 1994).

To monitor the presence of vitellogenin in chromatography, estradiol-17β-treated turbot were injected with carrier free [$^{32}$P]orthophosphate 24 h prior to blood sampling. [$^{32}$P]-orthophosphate is commonly used as a specific marker for vitellogenin, because vitellogenin is the only highly phosphorylated protein in the plasma of oviparous vertebrates and will incorporate [$^{32}$P]orthophosphate during synthesis (Wallace & Jared, 1968; deVlaming *et al*., 1980; Selman & Wallace, 1983). [$^{32}$P]orthophosphate was associated only with the major absorbance peak when plasma from estradiol-17β-treated turbot was analysed by anion-exchange chromatography, showing that this peak consisted of vitellogenin (I). The observation that no other chromatographic fraction contained [$^{32}$P]orthophosphate verified that there were no other proteins rich in protein-bound phosphorus. This was critical since earlier studies have shown that highly phosphorylated fragments of vitellogenin, possibly phosvitin, can dissociate from the vitellogenin molecule during the isolation procedure (deVlaming *et al*., 1980; Norberg & Haux, 1985). The low absorbance at 280 nm, due to a general lack of aromatic amino acids, makes phosvitin difficult to detect spectrophoto-metrically (Wallace, 1985). The identification of vitellogenin has also been based on several other criteria: that the protein is induced by estradiol-17β, that it precipitate in the presence of EDTA:Mg$^{2+}$, that the chromatographic and electrophoretic characteristics are similar to that of vitellogenin from other studies and, finally, its biochemical characteristics, such as molecular mass and amino acid composition (deVlaming *et al*., 1980; Selman & Wallace, 1983; Norberg & Haux, 1985; Babin, 1987).

Vitellogenin eluted as a single and symmetrical peak for rainbow trout, turbot and wolffish, when chromatographed on a Mono Q column at 4 °C in the presence of the proteolytic enzyme inhibitor, aprotinin (I; II). The symmetrical appearance of the vitellogenin peak for these three species indicated that vitellogenin was undegraded. The purity and the integrity of vitellogenin from the different species obtained by chromatography were confirmed by native PAGE and SDS-PAGE as well as by Western blot using a homologous antiserum directed against vitellogenin. For cod, the vitellogenin peak dissociated into two partly separated peaks when subjected to anion-exchange chromatography (II). These results were consistent, even when the chromatographic procedure was performed at low temperature in the presence of aprotinin. Interestingly, the level of dissociation was dependent on the time elapsed between aprotinin injection and blood sampling. When blood was sampled 10 minutes after aprotinin injection, the vitellogenin peak eluted as two completely separated peaks, but if blood was sampled after

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40 minutes, a partly dissociated peak was observed. Both peaks were identified as vitellogenin since they exhibited identical migration patterns on SDS-PAGE and both peaks cross-reacted with the homologous antiserum against cod vitellogenin, as determined by Western blot (II). It is not known why cod vitellogenin separated into two peaks upon anion-exchange chromatography. Possibly, the surface charge of the two peaks is different, as if small fragments were lost from the vitellogenin molecule. However, the fact that the two peaks demonstrated similar migration pattern on SDS-PAGE, makes it more likely that the two peaks consist of the same polypeptides with different posttranslational modifications. Another explanation may be the appearance of dimeric and monomeric forms of vitellogenin. The presence of both dimeric and monomeric forms under chromatographic conditions is not unusual phenomenon for vitellogenin (deVlaming *et al.*, 1980; I).

*Selective precipitation*

An alternative and simple method to obtain large quantities of relatively pure vitellogenin is to use selective precipitation with EDTA and $MgCl_2$, followed by precipitation with distilled water (Wiley et al., 1979). Vitellogenin was precipitated by mixing 2.0 ml plasma from estradiol-17β-treated fish with 8.0 ml of 20 mM EDTA, pH 7.7, and by subsequently adding 0.2-0.5 ml of 0.50 M $MgCl_2$. The method was initially developed and evaluated for the purification of vitellogenin from plasma of Xenopus laevis, and was shown to give a relatively pure vitellogenin fraction. However, the original method by Wiley et al. (1979) failed to precipitate vitellogenin in all four species in the present study, unless the molar ratio between EDTA and $Mg^{2+}$ was modified (II). Vitellogenin from cod, rainbow trout, turbot and wolffish precipitated when the molar ratio between EDTA and $MgCl_2$ was lowered, which was achieved by reducing the amount of $MgCl_2$ added to the mixture of plasma and EDTA. This is consistent with earlier findings on teleost vitellogenin. EDTA:$Mg^{2+}$ did not precipitate vitellogenin from plasma of estradiol-17β-treated goldfish, even when the EDTA and $Mg^{2+}$ ratio was altered (deVlaming et al., 1980). It was suggested that fish vitellogenin was not sufficiently rich in protein-phosphorus to permit cross-linking by EDTA and $Mg^{2+}$. Similar results have been reported or discussed by others (Selman & Wallace, 1983; Kanungo et al., 1990; Tyler & Sumpter, 1990a), suggesting that fish vitellogenin cannot be precipitated by EDTA and $MgCl_2$. However, Norberg & Haux (1985) found that vitellogenin from rainbow trout and sea trout did precipitate by the method of Wiley et al. (1979), but only when the total plasma protein levels were above 70-80 mg/ml. This indicates that the plasma concentration of vitellogenin is critical for the precipitation. Thus, Norberg & Haux (1985) proposed that the absence of precipitation of goldfish vitellogenin (deVlaming et al., 1980) may have been related to the plasma concentration of vitellogenin rather than to the low content of protein-bound phosphorus, since the protein-bound phosphorus content of goldfish, rainbow trout and sea trout vitellogenin is very similar.

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During the precipitation steps in the present study, all solutions were kept at 0-2 °C (I; II). When precipitation was performed at room temperature, the recovery increased, i.e. more vitellogenin precipitated. However, precipitation of vitellogenin from plasma at room temperature always resulted in less pure preparations compared to when vitellogenin was precipitated at 0-2 °C. In order to achieve maximal purity, it is thus vital that precipitation is carried out at low temperature. Low temperature also reduces degradation of vitellogenin by proteolytic enzymes during the precipitation procedure. To summarise, when vitellogenin was precipitated from plasma of estradiol-17β-treated fish, only small amounts of other plasma proteins co-precipitated, and a highly enriched vitellogenin preparation was obtained, as evaluated by anion-exchange chromatography. However, the minor contamination still found in the precipitated vitellogenin, shows that the precipitation procedure alone is not sufficient to provide vitellogenin of highest purity.

## Instability of teleost vitellogenin

The present study confirms earlier findings that teleost vitellogenin can be highly susceptible to proteolysis, and will easily degrade during its preparation and isolation (I; II). Precautions to prevent proteolytic activity are therefore essential. During the efforts to purify vitellogenin from the four species, it became apparent that vitellogenin disintegrated even when the previously described precautions against proteolysis were taken. Thus, the purification procedures had to be re-evaluated and further improved.

The instability of vitellogenin demanded consistent low temperature during all preparative procedures, including anion-exchange chromatography. Any attempt to perform chromatography at room temperature resulted in both irregular and dissociated vitellogenin peaks, although vitellogenin from rainbow trout differed from the others by being less degraded by chromatography at higher temperatures. Protease inhibitors, such as aprotinin ($10^4$ KIU/ml), are necessary ingredients in all reagents used during preparative and analytical work with vitellogenin. This has been suggested previously (deVlaming et al., 1980; Norberg & Haux, 1985), but was accentuated in the present study due to the particular lability of cod vitellogenin. The use of aprotinin was found to be most effective if the fish were injected at least 30 min before sampling. The frequently used procedure to add aprotinin to the blood after sampling was not adequate for the species investigated.

The degree of degradation was also dependent on the storage conditions prior to separation (I; II). It was observed that repeated freezing and thawing of plasma samples before isolation of vitellogenin facilitated degradation of vitellogenin. This finding is consistent with other studies which have shown that vitellogenin, as well as other plasma proteins including circulating lipoproteins and vitelline envelope proteins, are degraded by repeated freezing and thawing of plasma samples (Norberg & Haux, 1985, 1988; Hyllner et al., 1994a; Wallaert & Babin, 1995). However, vitellogenin appeared largely unaffected by a single freezing - thawing step. From these data, I propose that vitellogenin is best stored in plasma compared to in isolated form. Furthermore, vitellogenin should only be

isolated from freshly sampled plasma or from plasma that has been frozen once. This is particularly important when working with species like the cod.

It is clear that vitellogenin from different teleost species vary in their stability during purification, as well as in their properties upon precipitation (II). Cod vitellogenin was more sensitive compared with turbot and wolffish vitellogenin, which in turn were more sensitive than vitellogenin of rainbow trout. The observation that rainbow trout vitellogenin is relatively insensitive to proteolysis, corroborates previous reports on vitellogenin (Norberg & Haux, 1985; Tyler & Sumpter, 1990a). The reason for the pronounced lability of cod vitellogenin is not known. Vitellogenin from Atlantic halibut has recently been isolated from plasma and found to be highly susceptible to proteolytic degradation (Norberg, 1995), and exhibited similar instability when processed as cod vitellogenin. Norberg (1995) proposed that the great sensitivity to degradation of cod and halibut vitellogenin can be a common feature to marine cold-water species.

It is possible that the method used for the isolation of vitellogenin alters the biochemical composition of this molecule. The lipid content and fatty acid composition of vitellogenin obtained by double precipitation and anion-exchange chromatography were therefore analysed and compared (III). The vitellogenin preparations obtained by these two methods were identical in terms of both lipid content and fatty acid composition. This was true for all four species. Thus, the lipid composition of vitellogenin was apparently not influenced by the isolation procedures used. As mentioned, the vitellogenin fraction obtained by selective precipitation was not entirely pure. A minor contamination was present in precipitated vitellogenin fraction. However, this minor contamination did not affect the fatty acid composition.

Combining the results from the present study, it is concluded that a pure preparation of vitellogenin can be obtained from teleost fish plasma. Vitellogenin from the species investigated was sufficiently negatively charged to enable vitellogenin to be separated from other plasma proteins by anion-exchange chromatography as well as to be precipitated by EDTA:$Mg^{2+}$. Thus, anion-exchange chromatography on a Mono Q column was sufficient to isolate a pure fraction of vitellogenin from all four species studied. Vitellogenin of high purity was obtained by precipitation followed by chromatography.

Anion-exchange chromatography is preferable for the isolation of vitellogenin when present in low concentrations in the blood. However, precipitation is a simple method which yields large amounts of relatively pure vitellogenin, although the success depends on the plasma concentration of the vitellogenin as well as the chemical characteristics of the vitellogenin molecule. Considering that the minor contamination of the vitellogenin fraction obtained by double precipitation did not influence the lipid content and composition, this method was chosen as the most suitable method for the preparation of vitellogenin aimed at further lipid analyses (III; V). However, the double precipitation had to be followed by anion-exchange chromatography to obtain a highly purified vitellogenin preparation to be used for the preparation of antisera.

## Lipid analysis

To accomplish detailed analyses of lipid compositions, various lipid chromatographic techniques have to be employed, including thin-layer chromatography (TLC), gas-liquid chromatography (GLC) and high performance liquid chromatography (HPLC) with light scattering detection. The principles and theory for most of these methods have been the subject of a number excellent textbooks (Christie, 1982, 1987) and will not be reviewed in detail here. However, some newly developed methods, as well as modifications and evaluations of importance for the results in this study will be discussed below.

### *Total lipid extraction*

Before the lipid composition of a tissue sample can be analysed, the lipids must be isolated from non-lipid contaminants, *e.g.* proteins and carbohydrates. This is referred to as lipid extraction and should always be carried out as soon as possible after a fresh tissue sample has been obtained. If this is not possible, the tissue should be frozen quickly in liquid nitrogen or on dry ice and stored frozen until lipid extraction. Throughout, precautions must be taken to minimise the risk of oxidation of PUFA or hydrolysis of lipids. The presence of excessive amounts of free fatty acids is usually indicative of improper storage or extraction. With some modifications, the extraction procedure in the present study followed the method described described by Bligh & Dyer (1959) (III; IV; V).

### *Fatty acid analysis*

Fatty acid methyl esters were analysed on a gas-liquid chromatograph equipped with a split injector, a flame ionisation detector (FID) and a silica capillary column (III; IV). Before fatty acid s can be analysed by GLC, it is necessary to convert them to methyl esters, which are sufficiently non-polar and volatile to be separated by GLC. Fatty acid methyl esters were prepared from total lipid extracts by acid-catalysed transmethylation in methanol containing 5 % (wt/wt) anhydrous HCl at 70 °C for one hour (Christie, 1982). After transmethylation the crude fatty acid methyl esters fraction contain compounds other than fatty acid methyl esters, including cholesterol, alcohols and non-lipid contaminants. These must be removed before fatty acid methyl esters are analysed, which is easily achieved by TLC (IV).

Individual fatty acid methyl esters in the samples were identified by comparison of their retention time with the retention time of known a standard. Further evidence of identity was obtained by positive identification by gas-liquid chromatography/mass spectrometry of fatty acid methyl esters on a gas chromatograph equipped with two detectors: a flame ionisation detector and a mass selective detector (5890 A, 5970 quadrupole mass selective detector working with electron impact ionisation at an electron energy of 70 eV, Hewlett Packard, U.S.A.) (Nilsson & Liljenberg, 1991). Mass spectrometry was also used to identify fatty alcohols when present. A detailed study of the relative responses of various fatty acid

methyl esters and the linearity and reproducibility of the GLC analysis was confirmed (**III**). Response factors for pure 14:0, 16:0, 18:0, 16:1(n-7) and 22:6(n-3) relative to 18:1(n-9) varied between 0.97 to 1.03. As the variation in response factor for the fatty acid methyl esters was relatively small, all fatty acid methyl esters were assumed to have the same response factor and the area of each peak detected was therefore converted to weight.

Gas-liquid chromatography of the fatty acid methyl esters revealed that 34 fatty acids could be identified in the sampled studied in in this thesis. However, relatively few species made up the bulk of the fatty acids present. A representative gas-liquid chromatogram of the fatty acid methyl esters of total lipid extracted from cod vitellogenin is shown in Figure. 7. This chromatogram demonstrates the separation capacity of the GLC system from which the relative composition has been calculated (**III; IV**). The chromatogram shows that few non-identified peaks are present. The proportion of unidentified peaks never exceeded 4 % of the total fatty acids.

All solvents used for lipid extraction and fatty acid analysis contained BHT (50 mg/l) as an antioxidant to protect the PUFA. In order to examine possible oxidation of the fatty acids during preparation, particularly during freeze-drying of vitellogenin and eggs, lipid was extracted from eggs and vitellogenin at different stages: from freshly isolated vitellogenin, from dialysed and freeze-dried vitellogenin, from freeze-dried eggs, and from freshly sampled eggs. The fatty acid composition of lipid extracted from eggs and vitellogenin that had been dialysed and freeze-dried prior to extraction was identical to the composition obtained when lipids of eggs and vitellogenin was extracted directly.
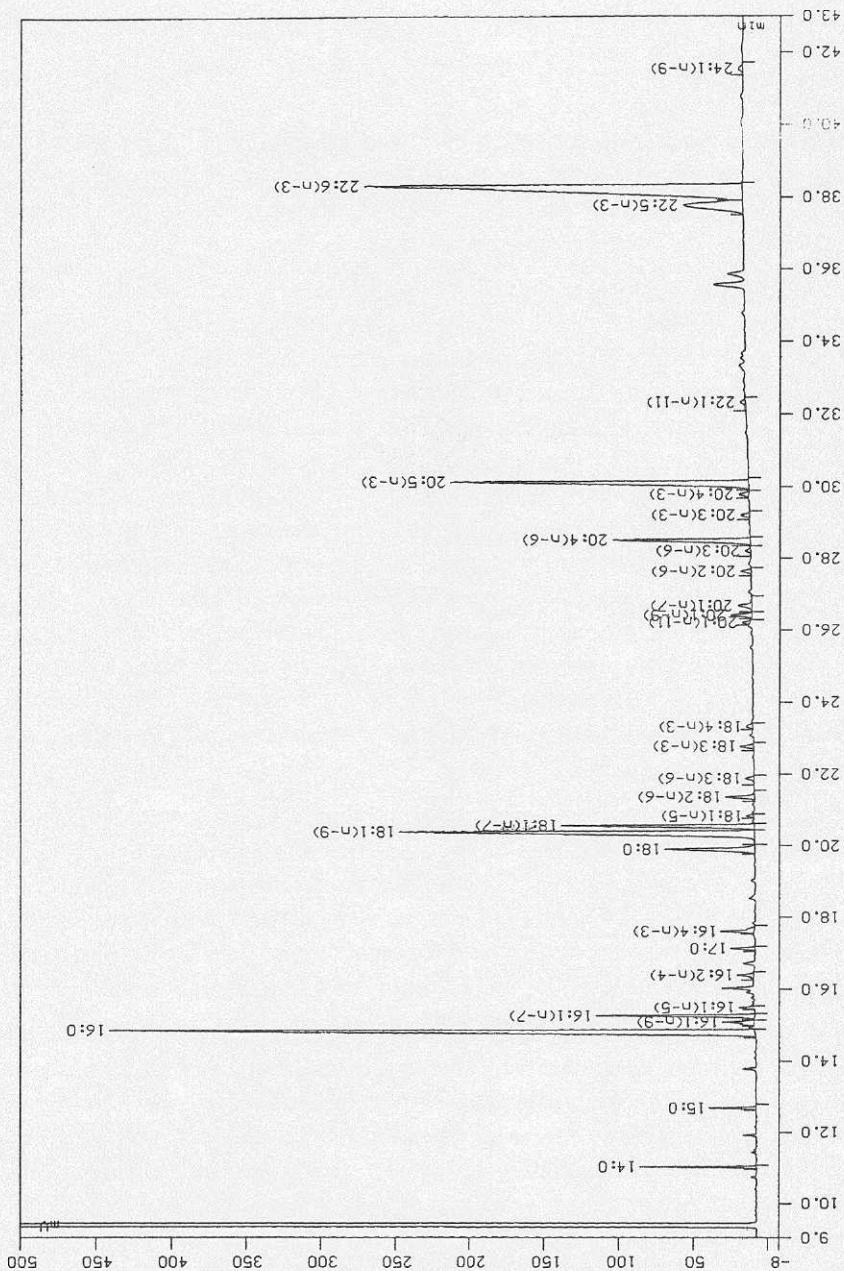
**Fig. 7.** Gas-liquid chromatogram of fatty acid methyl esters derived from total lipid extracted from vitellogenin of estradiol-17β-treated cod. Fatty acid methyl esters were isolated by thin-layer chromatography prior to gas-liquid chromatography. The analysis was carried out on a chromatographic system containing a split injector, a fused silica capillary column (DB-225, 30 m x 0.25 mm I.D.) and a flame ionisation detector.

## Lipid class analysis

Separation of lipid classes was performed by TLC or HPLC (**IV; V**). TLC was utilised for the preparative separation of lipid classes for further analysis of fatty acids of specific classes or groups of lipids (**III**). For quantitative lipid analysis, HPLC combined with light scattering detection was developed and used in this study (**V**).

One of the major problems in studies of lipids has been to find an accurate method for the separation and quantification of lipid classes. In recent years, HPLC has become a very useful tool for the separation and quantification of various compounds. However, HPLC has not become commonly used until recently for the analysis of lipid classes, due to a lack of a satisfactory detection method universal to all lipid classes. A relatively new method is the light scattering detection. HPLC combined with light scattering detection has proved to be a useful technique for the separation and quantification of lipids (Christie, 1985, 1986, 1987). Several applications of HPLC with light scattering detection for the separation and quantification of lipid classes, mainly phospholipid classes, have been described (Christie, 1985; Breton *et al.*, 1989; Lutzke & Braughler, 1990; Redden & Huang, 1991; Letter, 1992; Melton, 1992). With very few exceptions, these HPLC techniques have been developed for the analysis of lipids from mammals and plants. The aim was to further develop these systems for analysis of lipid classes present in lipid extracts from fish. When this was achieved, the HPLC methods were used for a detailed study of the lipid class composition of eggs and vitellogenin from turbot.

Due to the wide range in polarity of various lipid classes present in natural lipid extracts, two different solvent systems for the separation of lipid classes were used: one for polar and one for neutral lipids (**V**). Total lipids were fractionated into neutral and polar lipids before the analysis of neutral lipid classes. Neutral lipids were isolated from polar lipids by HPLC and the solvent system used for separation of polar lipids. The recovery of neutral lipids, when isolated by HPLC and solvent system for polar lipids, was > 98 %.

In total, the resolution for 15 lipid classes was investigated and most of the classes were base-line separated from each other, provided that triethylamine was added to the solvent system of polar lipids. However, sphingomyelin and phosphatidylserine were not entirely resolved when applied at high concentrations and the cholesterol- and wax esters were not separated. All lipid classes eluted as single peaks, except sphingomyelin and phosphatidylinositiol, which eluted as two and three peaks, respectively. Lipid classes were identified by comparison of their retention time with the retention time of known standards. The different lipid class fractions obtained by HPLC were collected and analysed by TLC, to asses the purity of each peak.

The appearance and elution position of separated peaks were highly reproducible for most lipid classes, although phosphatidic acid and diphosphatidylglycerol showed some variation in the elution pattern. Lipid standard curves were generated in order to study the linearity of the detection method and to quantify lipid classes in unknown samples. Calibration curves were prepared for both neutral and polar lipid classes. Correlation

($r^2$) was determined for all curves by linear regression analysis. Strong linear correlations between peak areas and concentrations of lipids were observed for all lipid classes within certain concentration ranges, with $r^2$ between 0.98 and 1.00 for all lipid classes, except for phosphatidic acid for which $r^2$ was 0.92. The detector response varied for different lipid classes, and neutral lipids exhibited greater detector response compared with polar lipids. The fact that the response is different for various lipid classes means that an accurate quantitative analysis of lipids requires individual response factors for each lipid class to be studied.

In summary, the HPLC system described can be applied to the separation and quantification of a wide range of lipid classes from various origin. Application of the HPLC methods has demonstrated that the methods are excellent for quantitative analysis of the major lipid classes present in lipids from turbot eggs and vitellogenin. This study shows that HPLC with light scattering detection is an attractive alternative to TLC methods for the analysis of fish lipids.

## Biochemistry of the vitellogenin polypeptide

*Molecular mass*

When plasma from estradiol-17β-treated turbot was subjected to native PAGE and stained with Coomassie Brilliant Blue, vitellogenin appeared as two bands (I). Both bands were absent in plasma from untreated turbot. The apparent molecular mass of these two bands was estimated to be 530 kDa and 275 kDa, respectively. Both bands were identified as vitellogenin since they were radiolabelled after injection with [$^{32}$P]orthophosphate, as detected by autoradiography using an X-ray film. The estimated molecular mass of the first band was about twice that of the second band, suggesting that they represent vitellogenin in a dimeric and a monomeric form, respectively. This was further supported by the observation that both bands exhibited the same migration pattern upon SDS-PAGE in the presence of mercaptoethanol (I; II). The finding that turbot vitellogenin exists in both dimeric and monomeric forms on native PAGE agrees with other observations on teleosts (Hori *et al.*, 1979; deVlaming *et al.*, 1980; Matsubara *et al.*, 1994). In the blood, vitellogenin is assumed to be present mainly as a dimer (Wallace, 1985).

The estimated molecular mass of native dimeric turbot vitellogenin is similar to what has been reported for native vitellogenin of other flatfishes, *e.g.* 550 kDa for flounder (Korsgaard-Emmersen & Petersen, 1976) and 520 kDa for barfin flounder (Matsubara & Sawano, 1995), as well as vitellogenin of other teleost species, such as 440 kDa for brown trout and rainbow trout (Norberg & Haux, 1985), 495-520 kDa for Atlantic salmon (So *et al.*, 1985), 420 kDa for medaka (*Oryzias latipes*; Hamazaki *et al.*, 1987), 500 kDa for *Oreochromis aureus* (Ding *et al.* 1989), 540 kDa for coho salmon (Hara *et al.*, 1993), 445 kDa for sea bass (*Dicentrarchus labrax*, Mañanós *et al.*, 1994), and 450 kDa for Japanese

sardin (*Sardinops melanostictus*, Matsubara *et al.*, 1994). It is important to recall that molecular mass determination of native glycolipophosphoproteins results in approximative estimates, due to the anomalous behaviour of these proteins compared with the more globular standard proteins typically used during gel filtration and PAGE (Rodbard, 1976). Thus, the discrepancies in native molecular mass between different species may to a large extent be related to the method employed for the estimation of molecular mass. This is indicated by the different molecular masses reported for the same species in different studies. For instance, the reported molecular mass for native rainbow trout vitellogenin varies between 440 and 600 kDa (Hara & Hirai, 1978; Campbell & Idler, 1980; Norberg & Haux, 1985; Shibata *et al.*, 1993).

When plasma from cod, rainbow trout, turbot and wolffish was subjected to PAGE in the presence of SDS and mercaptoethanol, one major protein with an apparent molecular weight just below 200 kDa appeared after estradiol-17β-treatment (I; II). This protein was not detected in control plasma. The SDS-PAGE migration patterns of plasma from untreated and estradiol-17β-treated rainbow trout are shown in Figure 8. The relative molecular mass of vitellogenin under denaturing and reducing conditions was estimated to be 167±5 kDa for cod, 170±6 kDa for rainbow trout, 175±6 kDa for turbot and 176±6 kDa for wolffish (II). These apparent molecular masses are similar to those reported for vitellogenin from other teleost species analysed by SDS-PAGE, *e.g.* 140-147 kDa for goldfish (Hori *et al.*, 1979; deVlaming et al., 1980), 200 kDa for killifish (Selman & Wallace, 1983), 176 kDa for Atlantic salmon (So *et al.*, 1985), 200 kDa for medaka (Hamazaki *et al.*, 1987), 170 kDa for striped bass (Kishida *et al.*, 1992), 165 for coho salmon (Hara *et al.*, 1993), 180 kDa for sea bass (Mañanós *et al.*, 1994) and 160 kDa for Atlantic halibut (Norberg, 1995). In addition, the molecular mass of rainbow trout vitellogenin estimated by SDS-PAGE in the present study is in line with previously reported values for this species; 170 kDa (Chen, 1983; Fremónt & Riazi, 1988) and 175 kDa (Babin, 1987a).



**Fig. 8.** Coomassie Brilliant Blue stained SDS-PAGE using discontinuous polyacrylamide gels with a 4 % stacking gel and a 9 % separating gel (Olsson, Kling, Petterson & Silversand (1995) Biochem. J. 307, 197-203). Lanes 1 and 2, blood plasma from two control rainbow trout; lanes 3 and 4, blood plasma from two estradiol-17β-treated rainbow trout. 0.2 µl of plasma was added to each lane. The treated fish were given an intraperitoneal injection of 10 mg estradiol-17β/kg body weight and control fish were injected with vehicle only. Blood was sampled from each fish five days after the injections.
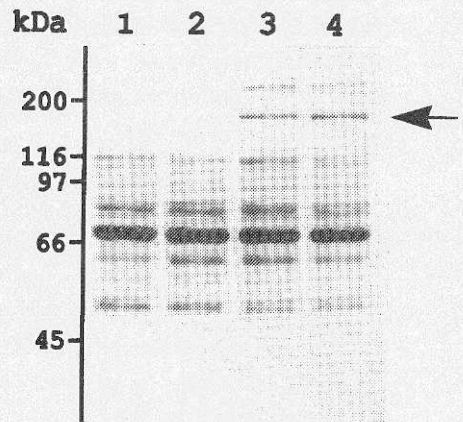
## Multiple vitellogenins

The number of genes and circulating forms of vitellogenin in fish are still unknown. The presence of a single vitellogenin band on SDS-PAGE of highly diluted plasma samples in the present study indicates the existence of a single form of circulating vitellogenin in cod, rainbow trout, turbot and wolffish (**II**). This is in accordance with most other SDS-PAGE studies of teleost vitellogenin, although *Cichlidae* are reported to differ by having two forms of circulating vitellogenin (Ding *et al.*, 1989; Lim *et al.*, 1991; Lee *et al.*, 1992; Kishida & Specker, 1993). However, it must be emphasised that according to Wiley & Wallace (1981), the molecular mass of the different forms of vitellogenin monomers from *Xenopus* are very similar (197, 188 and 182 kDa) and exhibited homogenous behaviour on ion-exchange columns. Altogether, this indicates that the isolation of different forms of vitellogenin is a difficult task, and the methods employed in the present study may not be sufficiently to resolve multiple forms of vitellogenin. Hence, more conclusive evidence is required to determine whether teleost vitellogenin occur in multiple forms.

## Amino acid composition

Amino acid composition of turbot vitellogenin together with the amino acid composition of vitellogenin from several other teleost species, as well as from *Xenopus laevis* and domestic hen, are given in Table 4. The table shows that the amino acid composition of vitellogenin in different teleost species are remarkably similar. Compared with vitellogenin from *Xenopus laevis* and domestic hen, teleost vitellogenins contain a lower proportion of serine. As serine is the amino acid which is almost exclusively phosphorylated in phosphoproteins such as vitellogenin (Wallace, 1985; Byrne *et al.*, 1989), the data in Table 4 corroborate the data on protein-bound phosphorus, which is reported to be lower in vitellogenin of teleosts (Hori *et al.*, 1979; Campbell & Idler, 1980; deVlaming *et al.*, 1980; Norberg & Haux, 1985, Norberg, 1995) compared with vitellogenin of domestic hen (Deeley *et al.*, 1975) and *Xenopus laevis* (Wallace, 1970).

In contrast, the non-polar amino acids alanine, isoleucine and leucine are relatively more abundant in vitellogenin of teleosts than in vitellogenin of *Xenopus laevis* and domestic hen. It can be speculated that the consistently greater proportion of non-polar amino acids in fish vitellogenin is functionally related to the higher content of lipid compared with *Xenopus laevis* and domestic hen vitellogenin. It is possible that teleost vitellogenins contain a greater proportion of non-polar amino acids to accommodate the higher content of lipids, for example by increasing the hydrophobic core of the molecule.

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**Table 4.** Amino acid composition of vitellogenin from different species

| Amino acid | Mol % of total amino acid | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Turbot | Barfin[a] flounder | Carp[b] | Goldfish[c] | Rainbow[d] trout | Sea[e] bass | Striped[f] bass | *Xenopus*[c] *laevis* | Domestic[g] hen |
| Aspartic acid | 7.8 | 7.4 | 6.7 | 6.5 | 8.4 | 6.3 | 7.6 | 8.7 | 9.0 |
| Threonine | 5.2 | 4.5 | 5.4 | 5.5 | 5.0 | 4.9 | 5.2 | 5.2 | 5.0 |
| Serine | 7.5 | 5.7 | 7.6 | 6.9 | 7.5 | 8.4 | 7.2 | 10.1 | 19.0 |
| Glutamic acid | 10.1 | 10.0 | 11.8 | 11.9 | 11.5 | 11.1 | 8.3 | 13.5 | 10.3 |
| Proline | 4.5 | 4.5 | 5.9 | 5.5 | 5.2 | 6.0 | 4.3 | 4.9 | 4.9 |
| Glycine | 4.6 | 4.2 | 5.1 | 4.6 | 4.2 | 4.8 | 4.2 | 4.9 | 4.9 |
| Alanine | 12.0 | 11.6 | 12.6 | 12.8 | 11.7 | 13.2 | 11.9 | 8.0 | 7.4 |
| Valine | 7.6 | 6.9 | 6.3 | 6.9 | 7.1 | 7.2 | 7.8 | 6.0 | 6.3 |
| Methionine | 2.8 | 2.4 | 1.9 | 2.0 | 2.6 | 0.9 | 2.8 | 2.5 | 2.2 |
| Isoleucine | 6.3 | 5.9 | 5.4 | 6.6 | 5.5 | 6.3 | 7.0 | 4.8 | 5.3 |
| Leucine | 10.2 | 10.6 | 10.5 | 10.8 | 9.5 | 10.2 | 10.8 | 8.3 | 8.0 |
| Tyrosine | 2.9 | 2.6 | 2.8 | 2.6 | 3.0 | 3.1 | 3.4 | 3.0 | 3.0 |
| Phenylalanine | 3.0 | 2.9 | 2.8 | 2.9 | 4.0 | 3.6 | 3.4 | 3.6 | 3.1 |
| Histidin | 2.2 | 9.7 | 3.4 | 2.3 | 2.1 | 1.5 | 3.1 | 3.5 | 3.2 |
| Lysine | 7.1 | 6.8 | 6.3 | 7.0 | 7.1 | 6.9 | 7.3 | 7.8 | 6.7 |
| Arginine | 4.4 | 3.7 | 5.0 | 4.9 | 4.5 | 5.4 | 4.9 | 5.1 | 5.5 |

The results for turbot are from paper **I**, [a] Matsubara & Sawano (1995), [b] Tyler & Sumpter (1990a), [c] deVlaming *et al.* (1980), [d] Hara & Hirai (1978), [e] Mañanós *et al.* (1994), [f] Tao *et al.* (1993) and [g] Christmann *et al.* (1977).

## Antisera directed against vitellogenin

Western blot demonstrated that vitellogenin from each species was strongly immunoreactive to the homologous antiserum (**II**). There was no immunoreactivity between antisera and plasma from control fish. Below the major protein band, vitellogenin, several smaller protein bands were also immunoreactive. These are probably degradation fragments of vitellogenin.

Another group of smaller proteins, present only in plasma of estradiol-17β-treated fish, and with a molecular mass around 50 kDa, was observed when the plasma was subjected to SDS-PAGE in the presence of mercaptoethanol. These smaller proteins were distinct from vitellogenin, as they showed no immunoreactivity with homologous antisera directed against vitellogenin. These proteins are probably the major vitelline envelope proteins which have recently been demonstrated to be induced by estradiol-17β in rainbow trout and turbot (Hyllner *et al.*, 1991). The appearance of other proteins than vitellogenin in plasma after estradiol-17β-treatment excludes the possibility to use estradiol induction as the only criterion for the identification of vitellogenin.

## Lipid composition of teleost vitellogenin

Despite the apparent importance of vitellogenin in supplying lipid to growing oocytes in teleosts, little is known about the lipid moiety of this molecule. The present study was undertaken to examine the lipid content, lipid class and fatty acid composition of vitellogenin from cod, rainbow trout, turbot and wolffish, and to evaluate the degree of individual variation in order to estimate if the distribution of fatty acids in vitellogenin is defined or variable (III; V). Several species were studied in parallel in order to elucidate if vitellogenins exhibit species-specific fatty acid patterns and if general patterns common to all species can be discerned. The lipid composition and variation of vitellogenin from wild fish, rather than cultured, were examined for two reasons. Firstly, the fatty acid composition of lipids from tissues and lipoproteins in fish, including lipids of egg and vitellogenin, is influenced by the dietary composition (Léger *et al*., 1981; Watanabe *et al*., 1984b; Leray *et al*., 1985; Mourente & Odriozola, 1990; Chou *et al*., 1993; Harel *et al*., 1994). Secondly, this was the first study to present comprehensive data on the lipid composition of teleost vitellogenin. The lipid composition of vitellogenin from wild-caught fish that have fed in their natural habitats can then be used as a reference for future studies on fish living under cultured and experimental conditions.

*Lipid content and class composition*

The present study shows that administration of estradiol-17β markedly increased the lipid content in plasma in cod, rainbow trout, turbot and wolfish (III). Hyperlipidemia associated with the administration of estrogens has been demonstrated in several groups of oviparous vertebrates, including teleosts, amphibians, reptiles and birds (Hillyard *et al*., 1956; Bailey, 1957; Follett & Redshaw, 1967; Hahn, 1967; Wiegand & Peter, 1980). The increased plasma lipid content caused by estradiol-17β-stimulation in the present study appeared concurrently with the accumulation of large amounts of vitellogenin in plasma. This indicates that the increased plasma lipid content was mainly due to lipids associated to vitellogenin. However, other plasma lipoproteins, as well as free fatty acids associated to albumin, may also contribute to the increased lipid content, as has been demonstrated for domestic hen and rainbow trout (Luskey *et al*., 1974; Wallaert & Babin, 1992). Notably, the plasma lipid level of estradiol-17β-treated rainbow trout was considerably more elevated compared with the other three species investigated (III). This indicates that other lipoproteins are involved in the increased plasma lipid content following estradiol-17β-treatment in rainbow trout in the present study, which corroborates the earlier study by Wallaert & Babin (1992). Although little is known about the role of ovarian steroids in the mobilisation of body lipids in teleosts, estrogens are suggested to be involved not only in the induction of vitellogenin and other circulating lipoproteins, but also in the mobilisation of the body lipid depots (Wiegand & Peter, 1980).

Determination of the total lipid content of vitellogenin from cod, rainbow trout, turbot and wolffish, revealed that vitellogenin from these species was a lipoprotein carrying similar amount of lipids (III). The total lipid content of vitellogenin was determined to be 18.1±0.8 % for cod, 17.6±0.3 % for rainbow trout, 15.8±0.3 % for turbot and 16.8±0.9 % for wolffish, calculated on dry weight basis. Lipid class analysis of turbot vitellogenin, as determined by HPLC and light scattering detection, shows that turbot vitellogenin mainly transports polar lipids, preferentially phosphatidylcholine (V). Polar lipids accounted for 73 % of the total lipids in turbot vitellogenin and the most abundant lipid class was phosphatidylcholine, which comprised 60 % of total lipid classes and 81 % of total polar lipid classes. The second most abundant lipid class in turbot vitellogenin was cholesterol followed by triacylglycerol, which comprised 16 % and 9 % of total lipids, respectively. Lipid class analysis of lipid extracts from turbot vitellogenin further revealed the presence of significant amounts of phosphatidylethanolamine and phosphatidylinositol, as well as minor amounts of sphingomyelin, phosphatidylserine and cholesterol ester. As mentioned previously the GLC-system used in the present study for the analysis of fatty acid methyl esters (III) was capable of detecting alcohols derived from wax esters. Such alcohols were not establishing that no wax esters are present in vitellogenin of the species investigated.

In a preliminary study (Silversand & Haux, 1991), the lipid class composition of vitellogenin from cod, rainbow trout, turbot and wolffish was estimated. Briefly, polar lipids, triacylglycerols and cholesterol were isolated from total lipid extracts by TLC, using 20 x 20 cm glass plates coated with 0.25 mm silica gel 60 H and a solvent system of hexane:diethyl ether:acetic acid (80:20:2, v/v/v). After fractionation, the separated lipids were scraped off the TLC plate and quantified by their fatty acid moiety on GLC. The fatty acid 19:0 was used as an internal standard and the lipids were converted to fatty acid methyl esters by acid-catalysed transmethylation (III). In all these species, polar lipids were the major constituents and accounted for 75 %, 68 %, 71 % and 71 % of the total lipid classes in cod, rainbow trout, turbot and wolffish, respectively. The reliability of these values is supported by the fact that polar lipids of turbot vitellogenin was found to be 73 % by HPLC and light scattering detection (V).

The lipid content and lipid class composition of vitellogenin obtained for the four teleost species confirm other studies showing that fish vitellogenin has a total lipid content of about 20 % lipid (wt/wt) of which about two thirds are polar lipids and the reminder being triacylglycerol and cholesterol (Hori *et al.*, 1979; Norberg & Haux, 1985; Norberg, 1995; Matsubara & Sawano, 1995).


*Fatty acid composition*

The relative fatty acid composition, in percent by weight, of total lipid extracted from vitellogenin of cod, rainbow trout, turbot and wolffish was also examined (III). The results show that the fatty acid composition of total lipids from vitellogenin of all four species can fairly accurately be described by nine fatty acids, which together make up 89 to 91% of

the total fatty acids. These major fatty acids are 16:0, 16:1(n-7), 18:0, 18:1(n-9), 18:1(n-7), 20:4(n-6), 20:5(n-3), 22:5(n-3) and 22:6(n-3). About 20 of the totally 34 identified fatty acids in lipids of vitellogenin represent less than 1.0 % of the total fatty acids. The lipids of vitellogenin were characterised by a high content of PUFA, with considerably more (n-3) than (n-6) fatty acids. In all species, PUFA accounted for about 50% of total fatty acids. The dominating (n-3) fatty acids were 20:5(n-3) and 22:6(n-3). Since polar lipids are known to be particularly rich in PUFA (Henderson & Tocher, 1987; Sargent et al., 1989), the high content of PUFA in vitellogenin is in line with the abundance of polar lipids in vitellogenin.

The proportions of saturated, monounsaturated and polyunsaturated fatty acids (approximately 1:1:2) were similar in vitellogenin from all four species (III). Turbot vitellogenin differed slightly by containing a somewhat higher proportion of saturated fatty acids, especially 16:0 and a lower proportion of PUFA, particularly 20:5(n-3). The similar proportion of total saturated, monounsaturated, polyunsaturated and (n-3) fatty acids in vitellogenin of all four species points to a specific requirement for these groups of fatty acids in functions common to all the species. Teleost vitellogenin is a vital precursor to the egg yolk, and special compositional demands of vitellogenin may be related to nutritional requirements for developing embryos and larvae. Although the fatty acid composition of total lipid bound to vitellogenin of the species studied shared several common characteristics, it is important to emphasise that the relative content of specific fatty acids differed between species. The most notable difference was observed for wolffish vitellogenin, in which the most abundant (n-3) PUFA was 20:5(n-3) instead of 22:6(n-3) for vitellogenin from cod, rainbow trout and turbot. Wolffish vitellogenin also contained a greater proportion of (n-6) fatty acids, due mainly to a higher level of 20:4(n-6). The different fatty acid composition of lipid from wolffish vitellogenin and liver, compared with the other species, may be attributed to an adaptation to a different feeding habit.

A detailed comparison of the total fatty acid composition of vitellogenin in the present study with previous findings (Léger et al. 1981; Frémont et al. 1984) is complicated to conduct as the previous studies have only presented the relative content of a few selected fatty acids or groups of fatty acids in total lipid of vitellogenin. In the studies by Léger et al. (1981) and Frémont et al., (1984), the simplified fatty acid composition, i.e. the combined percentages of groups of fatty acids such as saturates, (n-3) fatty acids, (n-6) fatty acids and the percentage of 20:5(n-3) and 22:6(n-3), for rainbow trout vitellogenin were reported. In general, the total lipids of rainbow trout vitellogenin in the present study contained more PUFA, (n-3) fatty acids, 20:5(n-3) and 22:6(n-3) relative to the lipids of vitellogenin from previous studies. The differences in fatty acid composition may reflect differences in the fatty acid composition of the diet. However, the apparent differences between the present and earlier studies can also be caused by a number of other factors, including different methodologies used for the isolation of vitellogenin and the analysis of fatty acids. In the studies by Léger et al. (1981) and Frémont et al. (1984), vitellogenin was isolated by density ultracentrifugation and no evidence for an identification of vitellogenin or any evaluation of the purity was presented. Imprecision in density ultracentrifugal separation of plasma

protein subclasses, including vitellogenin, may result in contamination of the different density subclasses. In the present study, the vitellogenin analysed for fatty acid composition was identified and the purity and structural integrity of the isolated molecule were confirmed by ion-exchange chromatography and SDS-PAGE (**I**; **II**).

To evaluate if the fatty acid composition of vitellogenin was a reflection of the composition of the tissue in which vitellogenin is synthesised, or if vitellogenin is selectively incorporated during vitellogenin synthesis, the fatty acid composition of the liver from the experimental fish was analysed for all four species. Several differences were apparent when the total fatty acid composition of vitellogenin was compared with the fatty acid composition of liver from estradiol-17β-treated fish of the same species. In general, the liver lipid contained lower levels of PUFA and fatty acids of the (n-3) series than the corresponding vitellogenin lipid. The livers also exhibited a relatively large variation in fatty acid composition both within and between species. The generally larger variation in the fatty acid composition of liver, compared with vitellogenin, in all four species, can be explained by the physiological function of this tissue. The liver of teleost species is the main organ for lipid metabolism and the lipid content and composition is therefore subjected to variations associated with diet and metabolism (Greene & Selivonchick, 1987; Sheridan 1988; Sargent *et al.*, 1989).

Taken together, analysis of several individuals of each species revealed that the lipid content and the relative fatty acid composition of vitellogenin were highly consistent among individual females of each species. The cod, turbot and wolffish used in the present study were all captured in the wild. They had consumed a natural diet assumed to be of a more or less heterogeneous composition, as indicated by the comparatively large individual variation in the liver fatty acid composition. Thus, the homogenous fatty acid pattern of vitellogenin within each species is most probably not directly correlated the composition of the diet. Instead, these findings indicate that vitellogenin is synthesised with a defined and species-specific lipid content and fatty acid composition. An observation that is further supported by the differences in the fatty acid composition of vitellogenin and liver from each species. The finding that teleost vitellogenin is highly consistent among individual females is further strengthened by comparing the relative fatty acid profile of vitellogenin from two groups of cod captured in the wild at during different years, 1991 (**III**) and 1992 (Table 5, unpublished data).

**Table 5.** Fatty acid composition of total lipid extracted from vitellogenin of estradiol-17ß-treated cod captured in the wild during two different years.

| Fatty acids | Cod (1991)* | Cod (1992) |
|---|---|---|
| 14:0 | 1.8 ± 0.1 | 1.4 ± 0.3 |
| 15:0 | 0.6 ± 0.1 | 0.7 ± 0.1 |
| 16:0 | 20.2 ± 1.3 | 20.4 ± 0.5 |
| 16:1 (n-9) | 1.0 ± 0.1 | 0.8 ± 0.1 |
| 16:1 (n-7) | 2.8 ± 0.4 | 2.8 ± 0.3 |
| 16:1 (n-5) | 0.3 ± 0.0 | 0.3 ± 0.0 |
| 16:2 (n-4) | — | 0.3 ± 0.1 |
| 16:4 (n-3) | 0.5 ± 0.1 | 0.7 ± 0.1 |
| 17:0 | 0.4 ± 0.0 | 0.5 ± 0.1 |
| 18:0 | 2.4 ± 0.1 | 2.7 ± 0.3 |
| 18:1 (n-9) | 12.4 ± 0.7 | 12.1 ± 0.9 |
| 18:1 (n-7) | 4.1 ± 0.5 | 4.6 ± 0.5 |
| 18:1 (n-5) | 0.2 ± 0.0 | 0.2 ± 0.0 |
| 18:2 (n-6) | 1.0 ± 0.2 | 0.7 ± 0.1 |
| 18:3 (n-6) | 0.2 ± 0.0 | 0.2 ± 0.0 |
| 18:3 (n-3) | 0.5 ± 0.1 | 0.4 ± 0.1 |
| 18:4 (n-3) | 0.4 ± 0.1 | 0.3 ± 0.1 |
| 20:0 | — | — |
| 20:1 (n-11) | 0.4 ± 0.2 | 0.3 ± 0.1 |
| 20:1 (n-9) | 0.9 ± 0.5 | 0.6 ± 0.2 |
| 20:1 (n-7) | 0.4 ± 0.1 | 0.4 ± 0.1 |
| 20:2 (n-6) | 0.3 ± 0.1 | 0.3 ± 0.1 |
| 20:3 (n-6) | 0.1 ± 0.0 | 0.2 ± 0.0 |
| 20:3 (n-3) | 0.2 ± 0.0 | 0.3 ± 0.0 |
| 20:4 (n-6) | 2.9 ± 0.4 | 4.1 ± 0.3 |
| 20:4 (n-3) | 0.5 ± 0.1 | 0.3 ± 0.0 |
| 20:5 (n-3) | 10.0 ± 0.9 | 11.1 ± 1.0 |
| 22:0 | — | — |
| 22:1 (n-11) | 0.6 ± 0.6 | 0.9 ± 0.1 |
| 22:1 (n-9) | 0.1 ± 0.1 | 0.3 ± 0.2 |
| 22:1 (n-7) | — | — |
| 22:5 (n-3) | 3.1 ± 0.7 | 3.7 ± 0.4 |
| 22:6 (n-3) | 31.4 ± 2.9 | 28.2 ± 1.3 |
| 24:1 (n-9) | 0.3 ± 0.1 | 0.2 ± 0.0 |
| Total | 100.0 | 100.0 |
| Total saturated | 25.4 ± 1.3 | 25.7 ± 0.4 |
| Total monounsaturated | 23.5 ± 1.1 | 22.3 ± 0.9 |
| Total polyunsaturated | 51.1 ± 2.1 | 52.0 ± 0.5 |
| Total (n-3) | 46.5 ± 2.4 | 45.2 ± 0.6 |
| Total (n-6) | 4.6 ± 0.4 | 6.5 ± 0.4 |

* Data from paper IV. Data are expressed as weight % of total fatty acids and represent means ± SD of five fish. A dash indicates not detected or trace amounts (< 0.1 %)

## Lipid composition of turbot eggs

The nutritional requirements for turbot have been extensively investigated and it has been shown that turbot has an absolute dietary requirement for long-chain PUFA, particularly 20:5(n-3) and 22:6(n-3), (Owen *et al* 1975; Cowey *et al*. 1976a, b; Tocher and Mackinlay 1990; Linares & Henderson 1991). However, little is known about the lipid reserves available to the developing embryos and the fatty acid requirements during embryonic development. Turbot eggs are approximately 1.0 mm in diameter, transparent and with a single oil globule well separated from the yolk. Turbot embryos develop rapidly, from fertilization to hatch in 4.5 days at 14 °C (**IV**). After hatching, the yolk lasts for approximately three days before it is depleted and the larva begins to feed exogenously. Within eight days, the egg components are converted into a self-supporting larva, a feature that puts large demands, both quantitatively and qualitatively, on the biochemical composition of the initial nutritive reserves present in the turbot egg. In order to estimate the lipid requirements during embryonic and early larval development, the lipid content, lipid class and fatty acid composition of total lipids, phospholipids, triacylglycerols, cholesterol esters and wax esters from ovulated eggs of wild and cultured turbot were examined (**IV**; **V**).

Only freshly ovulated eggs of apparently good quality were used. Egg quality was determined by visual characteristics, and batches containing eggs with loss of spherical shape, dimpled egg surface, diminished egg transparency, and/or collapsed plasma membrane, were excluded. In addition, the viability of sampled eggs from cultured turbot was further assessed by measuring the fertilization and the hatching rate after artificial fertilization of each batch. The average fertilization and hatching rate of the batches sampled for the present study were 96±4 % and 88±7 %, respectively.

### *Lipid content and class composition*

The total lipid content of ovulated turbot eggs was 13.8 ±0.5 % (n=5) and 13.2 ± 0.7 % (n=7) of the egg dry weight in wild and cultured turbot, respectively. Lipid class analysis, revealed that turbot eggs contained 52 % polar lipids and 48 % neutral lipids (**V**). Phosphatidylcholine was the major polar lipid class, accounting for 34 % of total egg lipids. Turbot eggs also contained 27 % cholesterol- and wax esters and 14 % triacylglycerols. The neutral lipids of turbot eggs were mainly confined to the oil globule, which consisted exclusively of neutral lipid classes. The content of polar lipids measured in the present study confirms previous work showing that turbot eggs contain 40 to 50 % phospholipids (Devauchelle *et al*., 1988; McEvoy *et al*., 1993). Furthermore, the lipid class composition is in line with the composition of eggs from other teleost species that contain oil globules, *i.e.* species rich in neutral lipid classes (Nakagawa & Tsuchiya, 1976; Léger *et al*., 1981; Brind *et al*., 1982; Eldridge *et al*., 1983; Moodie *et al*., 1989; Ando & Hatano, 1991).

## Fatty acid composition

Fatty acid analysis reveals that the total lipids extracted from turbot eggs are rich in PUFA, predominantly the (n-3) series (IV). (n-3) PUFA accounted for 39 % of the total fatty acids in eggs of both wild and cultured turbot. The major PUFA was 22:6(n-3), which comprised 24 and 23 % of the fatty acids in wild and cultured turbot, respectively, and was thereby also the most abundant fatty acid in wild turbot eggs. The proportion of 22:6(n-3) was three times that of 20:5*(n-3)*, which constituted the second most abundant PUFA. Monounsaturated fatty acids, mainly 18:1(n-9), and saturated fatty acids, mainly 16:0, comprised approximately 30 and 20 %, respectively, of the total fatty acids in both groups of turbot. Analyses of eggs from several females of both wild and cultured turbot showed that the individual variation in fatty acid composition was small. The richness of PUFA, particularly 20:5(n-3) and 22:6(n-3), indicates that turbot embryos and yolk-sac larvae have a nutritional requirement for these fatty acids.

Phospholipids, triacylglycerols and cholesterol- wax esters of wild turbot eggs all exhibited a specific fatty acid profile distinctly different from that of total lipid (IV). Thus, phospholipids contained the highest level of PUFA (56 %), mainly due to the preponderance of 22:6(n-3), which accounted for 33 % of the fatty acids. Triacylglycerols, compared with phospholipids, contained considerably less PUFA and was instead dominated by 16:0 and 18:1(n-9). The cholesterol- wax ester fraction was unique among the lipid classes in that saturated fatty acids were almost absent.

The observation that each lipid class had its own specific fatty acid composition is a well documented feature and is related to the diverse physiological functions of different lipid classes (Henderson & Tocher, 1987; Sargent *et al.* 1989). As mentioned previously, the exact reasons underlying the great proportion of (n-3) PUFA in phospholipids of marine teleost species are currently not known. Phospholipids are mainly considered to be structural lipids used for the synthesis of biomembranes, and the richness in (n-3) PUFA has been hypothesised to be an adaptation to maintain normal cellular membrane functions at low temperatures (Hazel 1984; Bell *et al.* 1986). Since the properties of fatty acid containing lipids are to a large extent determined by the fatty acid moiety, the distribution of fatty acids in polar lipids is likely to have important implications for the structure and function of cellular membranes (Brenner, 1984; Bell *et al.*, 1986; Cullis & Hope, 1991). On the other hand, several studies have reported that phosphatidylcholine is catabolised during embryogenesis in various teleost species, indicating that phospholipids are not exclusively utilised for structural purposes (Tocher *et al.* 1985b; Fraser *et al.* 1988; Falk-Petersen *et al.*, 1989).

The great proportion of 22:6(n-3) in lipids, predominantly in phospholipids, of turbot eggs (IV) is consistent with earlier data on several marine fish eggs (Tocher and Sargent 1984; Falk-Petersen *et al.* 1986) and could be related to a specific requirement for 22:6(n-3) during the development of nervous tissues, such as brain and retina (Sargent 1995).

Nervous tissues are highly enriched in 22:6(n-3) (Tocher & Harvie, 1988; Mourente *et al.*, 1991), and during the early developmental stages in fish, a considerable growth and development of neural tissue, such as brain and eyes, occurs. This is reflected in the great proportion these tissues make up of the total body mass of the embryo and yolk-sac larva (IV).

## Eggs of wild versus cultured turbot

Comparison of eggs from wild and cultured turbot show that the general pattern of the fatty acid distribution is rather similar between the two groups (IV). However, several differences in the relative amounts of specific fatty acids can be discerned. Principal component analysis (PCA) was used to show that the fatty acid composition was similar between individuals within each group of turbot, *i.e.* wild and cultured, and that these groups were separated from each other. These differences were extended to all lipid classes. The most prominent difference was the greater proportion of linoleic acid (18:2n-6) in the cultured turbot eggs. Linoleic acid is scarce in the marine environment and an unnatural deposition of this fatty acid in body lipids, including egg lipids, is typical of fish given a formulated diet containing vegetable oils (Watanabe *et al.* 1984b; Sargent *et al.* 1989; Harel *et al.* 1994). The high content of linoleic acid in lipids of cultured turbot eggs in the present study was most likely caused by the wheat flour in the formulated diet. The linoleic acid appeared to be deposited unaltered in the eggs, *i.e.* was not converted to longer fatty acids of the (n-6) series such as arachidonic acid (20:4n-6). In fact, eggs from cultured turbot contained lower proportions of arachidonic acid compared with eggs of wild turbot. The lack of bioconversion of linoleic acid to arachidonic acid in turbot is in good agreement with earlier observations showing that turbot possesses little enzyme capacity for conversion of PUFA (Owen *et al* 1975; Cowey *et al.* 1976a, b; Tocher & Mackinlay 1990; Linares & Henderson 1991).

In addition to elevated levels of linoleic acid, lipids of cultured turbot eggs also contained higher levels of the long-chain monounsaturated fatty acids, 20:1(n-9) and 22:1(n-11), compared with eggs from the wild stock. The higher percentage of these fatty acids indicates the presence of commercial fish oils in the formulated diet (Sargent 1995). It is reasonable to assume that the disparity in the fatty acid compositions between eggs of wild and cultured turbot in the present study was caused by differences in the dietary fatty acid composition. However, neither the exact fatty acid composition of the natural diet consumed by the wild fish, nor the fatty acid composition of the formulated diet given to the cultured turbot was known, and it has therefore to be emphasised that the differences observed may not be entirely due to the diet.

Although the fatty acid profile of eggs from wild and cultured turbot differed, total lipid, phospholipids and triacylglycerols of eggs from both groups of turbot contained a similar great proportion of (n-3) PUFA, mainly 22:6(n-3). Such results have also been obtained for wild and cultured chinook salmon (Ashton *et al.* 1993). Thus, the apparent conservation

of (n-3) PUFA, particularly 22:6(n-3), by turbot females during the accumulation of egg lipids may be indicative of the essentiality of these fatty acids during early ontogeny. Several studies have concluded that the over-all fatty acid composition of fish roe is generally conserved in comparison with body lipids (Sargent *et al.*, 1989). There appears to be a strong selection pressure to maintain the proportions of (n-3) PUFA, especially 22:6(n-3), in egg lipids within a narrow range.

Comparison of the data in the present study with previous findings on turbot eggs is complicated by the differences in the approaches taken. Planas *et al.* (1993a) reported the fatty acid composition of total lipid extracted from cultured turbot eggs one day after fertilization, which may partly explain the greater proportion of 18:0 and 18:1(n-9) and the lower level of 22:6(n-3) compared with both groups of turbot in the present study. McEvoy *et al.* (1993) fractionated the lipids extracted from freshly ovulated eggs into total neutral lipids and phospholipids prior to fatty acid analysis and observed a markedly greater proportion of 18:1(n-9) in phospholipids. Turbot eggs in earlier studies exhibited a much larger individual variation in total lipid content (Devauchelle *et al.* 1988; McEvoy *et al.* 1993) and the fatty acid profile was also much less consistent among individual females (McEvoy *et al.* 1993). The apparent discrepancies between the present and previous studies may be caused by differences in dietary fatty acid composition, but other factors such as developmental stage of the eggs, egg quality, and analytical methodologies used may also contribute to these differences.

When GLC of the fatty acid methyl esters from total lipids of turbot eggs was performed without prior isolation by TLC, several unknown peaks appeared. These peaks were positively identified by GLC/MS as alcohols. Unfortunately, several of the wax ester derived alcohols co-eluted with the fatty acid methyl esters, and it was therefore obligatory to purify the fatty acid methyl esters prior to GLC. However, these alcohols cause no major problem as they can easily be removed by TLC. The alcohols derived from wax esters were particularly abundant in the oil globule. No such peaks were obtained when fatty acid methyl esters from PL and TAG were analysed. It is not clear if this isolation procedure was conducted in earlier studies, and this may be one further explanation for the observed differences in fatty acid composition between this and earlier studies.

## Oil globule

The lipids of turbot eggs were present in two distinct forms: the yolk lipids and the lipids confined to the oil globule. The the oil globule was isolated and its lipid composition analysed in order to evaluate the role of the oil globule during embryonic development (IV; V). The relatively high content of neutral lipids in turbot eggs was largely related to the presence of the oil globule, which contained exclusively neutral lipids in the form of triacylglycerols, cholesterol- and wax esters, as determined by TLC (IV) and HPLC (V). The cholesterol- and wax ester fraction was estimated to make up about 2/3 of the oil globule, the rest being triacylglycerols. No trace of polar lipids could be detected in the

oil globule. The lipid class composition of the oil globule present in turbot egg is consistent with earlier findings on other teleosts, showing that oil globules of eggs are comprised of non-polar lipids (Nakagawa and Tsuchiya 1976; Léger *et al.* 1981; Brind *et al.* 1982, Eldridge *et al.* 1983; Moodie *et al.* 1989; Ando & Hatano, 1991).

Based on calculations from light microscopical observations in the present study, it could be concluded that the oil globule makes up a considerable proportion of the total egg lipids in turbot. More than 50 % of the total egg lipids were estimated to be confined to the oil globule. Considering that phospholipids account for 52 % of the total lipids present in turbot eggs (V) and that no phospholipids are present in the oil globule, it is reasonable to suggest that the yolk lipids of turbot eggs principally consist of phospholipids. This is in good agreement with earlier studies showing that phospholipids are dominating the yolk lipids in both eggs with oil globules (Nakagawa & Tsuchiya 1976; Léger *et al.*, 1981; Eldridge *et al.* 1983; Moodie *et al.* 1989; Ando & Hatano, 1991) and in eggs without oil globule (Tocher & Sargent 1984; Falk-Petersen *et al.* 1986). Unfortunately, lipids of turbot egg yolk were not analysed in the present study, because the yolk could not be completely separated from the oil globule. Previously presented centrifugation methods for the separation of yolk and oil globules from fish eggs were not applicable to turbot (Léger *et al.* 1981; Eldridge *et al.* 1983; Moodie *et al.* 1989). Thus, after homogenisation and centrifugation of the eggs, numerous lipid droplets, *i.e.* an emulsion, were found in the yolk fraction, even when the fraction was repeatedly stirred and recentrifuged.

Eggs and larvae from turbot were collected at different stages of development and investigated by light microscopy in order to follow the morphological development of the embryos with special emphasis on the oil globule. This study shows that the size of the oil globule in turbot eggs remains constant during embryogenesis, and a reduction in size occurs first after hatching and mainly after yolk depletion (IV). These findings confirm earlier reports on turbot (Rønnestad *et al.* 1992) and other teleosts (Brind *et al.* 1982; Eldridge *et al.* 1983; Moodie *et al.* 1989), and suggests that the oil globule in turbot eggs is not catabolised during embryonic development.

The fact that the oil globule is not metabolised during embryogenesis in turbot implies that the total amount of lipids utilised during the embryonic development is considerably less than the total lipid content present in ovulated eggs. Thus, despite the substantial amounts of neutral lipids in the egg, only yolk lipids are metabolised to any larger extent during embryogenesis. Altogether, the assumption that the fatty acid requirements of fish embryos are indicated by the fatty acid composition of the initial lipid reserves present in ovulated eggs is not valid for turbot. The fatty acid requirements for the turbot embryo are more likely to match the composition of the phospholipids of the egg. This means that the requirement of (n-3) PUFA, mainly 22:6(n-3), is considerably greater than previously indicated by analysis of total lipids in turbot eggs.

# Interrelationship between vitellogenin and egg lipids

There is a lack of knowledge about the processes of lipid accumulation in growing oocytes in teleost fish. One of the objectives in this study was to examine the interrelationship between egg lipids and the lipid moiety of vitellogenin. This was accomplished by performing detailed comparisons of the lipid composition of eggs and vitellogenin.

Since vitellogenin contains 16-21 % lipids (dry wt), which are characterised by a high content of (n-3) PUFA, it is reasonable to assume that vitellogenin is an important source of these fatty acids for developing oocytes in fish (III; Hori et al., 1979; Norberg & Haux, 1985; Norberg, 1995; Matsubara & Sawano, 1995). Cod vitellogenin contains 75 % polar lipids (dry wt) (Silversand & Haux, 1991), which corroborates the 72 % (dry wt) of polar lipids which have been reported for cod eggs (Tocher & Sargent, 1984). Furthermore, the fatty acid composition of vitellogenin from cod (III) was strikingly similar to the fatty acid composition of total lipid from cod eggs (Ulvund & Grahl-Nielsen 1988; Klungsøyr et al. 1989). This similarity has been further confirmed by a recent study on cultured cod, where the dietary influence on the fatty acid composition of vitellogenin and eggs was examined (Silversand et al., 1996) (Table 6). Vitellogenin and eggs were sampled from cod at the time of ovulation. In addition to vitellogenin from naturally vitellogenic females, vitellogenin was also obtained from estradiol-17β-treated male cod. This experiment provides unique possibilities to compare the lipid and fatty acid composition between vitellogenin and eggs from the same group of fish. Table 6 shows that the fatty acid composition of total lipid from cod vitellogenin was similar to the fatty acid composition of total lipid from cod eggs. Furthermore, the data in Table 6 reveal that the fatty acid profile of vitellogenin induced by estradiol-17β-injections (exogenous induced) was similar to that of naturally-induced (endogenously induced) vitellogenin. In summary, the strong similarity in the lipid class and fatty acid composition of total lipids between vitellogenin and eggs of cod suggests that the lipids of cod eggs to a large extent originate from vitellogenin (Silversand et al., 1996).

In contrast to cod, comparison of the lipid class and fatty acid composition of vitellogenin and eggs from turbot revealed several differences. Turbot vitellogenin is dominated by polar lipids, which account for 73 % of the total lipids, whereas turbot eggs contain equal amounts of polar and neutral lipids (V). Furthermore, egg lipids contain a higher percentage of monounsaturated fatty acids (33 versus 23 %), mainly 18:1(n-9), and a lower percentage of saturated fatty acids (24 versus 32 %), mainly 16:0, compared with lipids of vitellogenin. Turbot eggs also contain wax esters which were not detected in vitellogenin from turbot. Altogether, the observed differences between vitellogenin and eggs of turbot imply that the source of egg lipids is not restricted to lipids supplied by vitellogenin.

**Table 6.** Relative fatty acid composition of vitellogenin and ovulated eggs of cultured cod. Data are expressed as weight % of total identified fatty acids and represent means ± SD of six fish

| Fatty acids | Diet | Vitellogenin Exogenous estradiol | Vitellogenin Endogenous estradiol | Egg |
|---|---|---|---|---|
| 14:0 | 4.5 | 1.5 ± 0.2 | 1.3 ± 0.1 | 1.4 ± 0.1 |
| 15:0 | 0.3 | 0.3 ± 0.0 | 0.3 ± 0.0 | 0.3 ± 0.0 |
| 16:0 | 13.2 | 22.1 ± 0.6 | 23.7 ± 0.8 | 21.6 ± 0.6 |
| 16:1 (n-9) | 0.5 | 0.7 ± 0.1 | 0.9 ± 0.1 | 1.3 ± 0.2 |
| 16:1 (n-7) | 6.0 | 1.9 ± 0.1 | 1.8 ± 0.2 | 2.0 ± 0.1 |
| 16:1 (n-5) | 0.3 | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.3 ± 0.0 |
| 16:2 (n-4) | 0.3 | — | 0.2 ± 0.0 | 0.2 ± 0.0 |
| 16:4 (n-3) | 0.9 | 0.3 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.0 |
| 17:0 | 0.1 | 0.2 ± 0.0 | 0.3 ± 0.0 | 0.2 ± 0.0 |
| 18:0 | 1.6 | 3.1 ± 0.4 | 3.5 ± 0.5 | 3.0 ± 0.2 |
| 18:1 (n-9) | 9.9 | 13.2 ± 0.7 | 13.1 ± 1.2 | 11.3 ± 0.4 |
| 18:1 (n-7) | 2.6 | 2.9 ± 0.2 | 3.9 ± 0.3 | 3.1 ± 0.2 |
| 18:1 (n-5) | 0.4 | 0.4 ± 0.0 | 0.4 ± 0.0 | 0.5 ± 0.0 |
| 18:2 (n-6) | 5.8 | 3.3 ± 0.1 | 3.1 ± 0.2 | 3.3 ± 0.1 |
| 18:3 (n-6) | 0.2 | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.1 ± 0.0 |
| 18:3 (n-3) | 1.2 | 0.6 ± 0.0 | 0.4 ± 0.0 | 0.5 ± 0.0 |
| 18:4 (n-3) | 2.5 | 0.6 ± 0.1 | 0.5 ± 0.1 | 0.6 ± 0.0 |
| 20:0 | 0.1 | — | — | — |
| 20:1 (n-11) | 0.6 | 0.4 ± 0.0 | 0.3 ± 0.1 | 0.4 ± 0.0 |
| 20:1 (n-9) | 12.7 | 2.5 ± 0.2 | 2.3 ± 0.3 | 2.6 ± 0.2 |
| 20:1 (n-7) | 0.5 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.1 ± 0.0 |
| 20:2 (n-6) | 0.2 | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.0 |
| 20:3 (n-6) | — | — | 0.1 ± 0.0 | 0.1 ± 0.0 |
| 20:3 (n-3) | 0.1 | 0.1 ± 0.0 | 0.3 ± 0.0 | — |
| 20:4 (n-6) | 0.4 | 1.4 ± 0.1 | 1.1 ± 0.1 | 1.3 ± 0.1 |
| 20:4 (n-3) | 0.4 | 0.4 ± 0.0 | 0.3 ± 0.0 | 0.3 ± 0.1 |
| 20:5 (n-3) | 7.5 | 11.9 ± 0.4 | 11.6 ± 0.7 | 13.8 ± 0.5 |
| 22:0 | 0.1 | — | 0.2 ± 0.0 | — |
| 22:1 (n-11) | 14.3 | 1.1 ± 0.1 | 0.8 ± 0.1 | 0.8 ± 0.1 |
| 22:1 (n-9) | 1.6 | — | 0.1 ± 0.0 | 0.1 ± 0.0 |
| 22:5 (n-3) | 0.7 | 1.1 ± 0.1 | 1.1 ± 0.1 | 0.9 ± 0.1 |
| 22:6 (n-3) | 9.1 | 28.3 ± 1.2 | 26.3 ± 1.0 | 28.3 ± 0.7 |
| 24:1 (n-9) | 0.9 | 0.3 ± 0.0 | 0.2 ± 0.1 | 0.5 ± 0.1 |
| Total | 100.0 | 100.0 | 100.0 | 100.0 |
| Total saturated | 20.0 | 27.1 ± 0.4 | 29.2 ± 1.0 | 26.4 ± 0.7 |
| Total monounsaturate | 50.6 | 24.6 ± 1.0 | 25.0 ± 0.7 | 23.7 ± 0.6 |
| Total polyunsaturated | 29.5 | 48.3 ± 1.0 | 45.8 ± 1.1 | 49.8 ± 1.0 |
| Total (n-3) | 22.5 | 43.2 ± 1.1 | 40.8 ± 1.0 | 44.5 ± 1.0 |
| Total (n-6) | 6.6 | 5.2 ± 0.2 | 4.8 ± 0.3 | 5.2 ± 0.2 |
| Total (n-3)/(n-6) | 9.0 | 4.9 ± 0.2 | 8.5 ± 0.7 | 8.6 ± 0.4 |

(—), not detected or trace amounts (<0.1%).

In species accumulating appreciable amounts of neutral lipids in their eggs, it has been suggested that sources other than vitellogenin may supply the egg with lipids, mainly neutral lipids. Thus it appears that lipids can accumulate in growing oocytes by the uptake of other circulating lipoproteins such as VLDL (Léger et al. 1981; Frémont et al., 1984; Wallaert & Babin 1992; 1994a) or VHDL (Nagler & Idler 1990). Lipids may also be taken up from blood through lipolysis of circulating lipoproteins (Black & Skinner 1987) or be synthesised endogenously within the oocyte (Wiegand & Idler 1982).

The discrepancies in the relationship between the lipids of vitellogenin and eggs between turbot and cod may be correlated to the fact that turbot eggs contain an oil globule which is not present in cod eggs. As mentioned, lipids of teleost vitellogenin are characterised by a high content of phospholipids (V; Hori et al. 1979; Norberg & Haux 1985; Norberg 1995; Matsubara & Sawano, 1995) and (n-3) PUFA (III), which also has been shown for the egg yolk of turbot in the present study (III). Taken together, it is tempting to hypothesise that yolk lipids are derived from vitellogenin, and the lipids of the oil globule originate from other lipoproteins, such as VLDL, or by autosynthesis in the oocyte. This hypothesis highlights the importance of vitellogenin and the lipid composition of this lipoprotein for teleost embryonic development. Both the oil globules and VLDL in teleost fish consist exclusively of neeutral lipids (IV, V; Babin & Vernier, 1989)

Finally, only yolk lipids are metabolised to any larger extent during embryonic development despite the substantial amounts of neutral lipids in turbot eggs (IV). Thus, although the lipid composition of turbot eggs was different from that of cod eggs, the metabolism of lipid during embryogenesis may not necessarily differ between these two species.

# CONCLUSIONS

• Teleost vitellogenin can be successfully isolated from plasma by precipitation with EDTA:$Mg^{2+}$, distilled water and high performance anion-exchange chromatography. The use of a Mono Q anion-exchange column connected to a fast protein liquid chromatographic system is a highly reproducible one-step procedure which yields a pure and apparently undegraded preparation of vitellogenin in a relatively short processing time. When vitellogenin is selectively precipitated, most other plasma proteins disappear, but a minor contamination is still present in the precipitated fraction.

Although the stability of teleost vitellogenin during isolation appears to differ between species, the general susceptibility of teleost vitellogenin to degradation demands consistent low temperature and the use of a proteolytic enzyme inhibitor in all preparative and chromatographic steps, including the injection of protease inhibitor into the fish before to blood sampling. The observed variation in stability among species suggests that isolation procedures should be modified according to the specific requirements for each species. In a wider context, these findings have implications for experiments where purified vitellogenin is used to study different aspects of vitellogenesis in fish. If possible, the integrity of the isolated vitellogenin should always be evaluated.

Vitellogenin obtained by selective precipitation and anion-exchange chromatography was identical in terms of lipid content and fatty acid composition. Therefore, precipitation has been used for the preparation of vitellogenin for lipid analyses. For the preparation of antiserum, precipitation was followed by anion-exchange chromatography in order to obtain highly purified vitellogenin.


• High performance liquid chromatography combined with light scattering detection has been evaluated, further developed, and applied for the separation and quantification of lipid classes from eggs and vitellogenin of fish. Due to the wide range in polarity of various lipid classes present in natural lipid extracts, two different solvent systems were employed for the separation of lipid classes, one for polar and one for neutral lipids. The methods are excellent for quantitative analysis of the major lipid classes present in fish lipid extracts. The procedures yield high resolution between most lipid classes in a relatively short processing time, exhibit linear dose-response within certain concentration intervals and are highly reproducible.


• The relative molecular mass of the vitellogenin polypeptide of cod, rainbow trout, turbot and wolffish was estimated by SDS-PAGE to be 167±5 kDa for cod, to 170±6 kDa for rainbow trout, 175±6 kDa for turbot and to 176±6 for wolffish. Turbot vitellogenin is present both in dimeric and monomeric forms when analysed by native polyacrylamide

gel electrophoresis, with estimated native molecular masses of 530 and 275 kDa, respectively. The amino acid composition of turbot vitellogenin was very similar to the composition of other species.

•    Vitellogenin of cod, rainbow trout, turbot and wolffish contain 16 to 18 % lipid, by weight, characterised by a high content of polar lipids, preferentially phosphatidylcholine, representing 68 to 75 % of total lipids. The lipids of vitellogenin are rich in polyunsaturated fatty acids, predominantly 20:5(n-3) and 22:6(n-3), which comprise about 50 % of the total fatty acids. The fatty acid composition of vitellogenin is species specific although general fatty acid patterns can be discerned. The lipid content, lipid class composition and distribution of fatty acids in total lipids of vitellogenin are highly consistent among individual females of each species. In contrast, the liver fatty acid composition vary considerably, both within and between species. Thus, the homogenous lipid composition of vitellogenin within each species is presumably not attributable to a homogenous composition of diet and liver composition. Instead, the results indicate that a specific selection of  lipids occurs during the lipidation of vitellogenin and that vitellogenin is synthesised with a well defined and species specific lipid content and composition.

•    Lipid comprise 14 % of the egg dry weight in turbot, and contains approximately equal amounts of polar and neutral lipids. Turbot egg lipids are rich in polyunsaturated fatty acids, with considerably more (n-3) fatty acids than (n-6), and account for about 45 % of total fatty acids. The abundance of (n-3) polyunsaturated fatty acids is mainly due to the presence of 22:6(n-3), which constitute one quarter of the total fatty acids. The polyunsaturated fatty acids are preferentially esterified to polar lipids. Analyses of eggs from several females of both wild and cultured turbot show that the individual variation in fatty acid composition is small. Based on the fatty acid profile, a high (n-3) PUFA requirement during embryogenesis is suggested.

The neutral lipids of turbot eggs are mainly confined to the oil globule, which only contains neutral lipids in the form of triacylglycerol, cholesterol- and wax esters. Calculations based on light microscopical studies show that the oil globule constitutes more than 50 % of the total lipids in cultured turbot eggs. Furthermore, the size of the oil globule remains constant during embryogenesis, indicating that the nutritients consumed by the embryo are chiefly derived from the yolk, whereas the oil globule appears to be metabolised after hatch. This implies that the total amount of lipids utilised during the embryonic development is considerably less than the total lipids present in ovulated turbot eggs. Thus, the assumption that the fatty acid requirements of fish embryos are indicated by the fatty acid composition of the initial lipid reserves present in ovulated eggs is not valid for turbot. The fatty acid requirements for the turbot embryo are more likely to match the composition of the phospholipids of the egg. This means that the requirement of (n-3) PUFA is considerably greater than previously indicated by analysis of total lipids in turbot eggs.

The general pattern of the fatty acid distribution in lipids of eggs from wild and cultured turbot is similar, but the relative amount of 18:2(n-6) is considerably higher and 20:1(n-9) slightly higher in cultured fish. These differences are extended to all lipid classes and probably reflect the dietary intake of certain vegetable and marine fish oils. On the other hand, the data in the present study indicate that there appears to be a selection pressure to maintain the proportions of (n-3) polyunsaturated fatty acids, especially 22:6(n-3), in turbot egg lipids within a narrow range. Altogether, it appears like care must be taken in using data from studies on cultured fish fed formulated diet to explain nutritional requirements. It is recommended that the biochemical composition and nutritional requirements of wild fish is considered to guide future studies of the nutritional requirements during early development. It is likely that eggs from healthy wild fish contain a supply of fatty acids that are optimised nutritionally for development and growth of the embryo and larvae up to the stage of yolk sac absorption.

- From the data in the present study it is reasonable to assume that vitellogenin is an important source of egg lipids in teleost fish, and is most likely the major source of polar lipid classes in fish eggs. In cod, a species without egg oil globules, the accumulation of lipids in growing oocytes is suggested to occur principally via the uptake of vitellogenin. In contrast, the high content of phospholipids, the absence of wax esters and the fatty acid composition in turbot vitellogenin, exclude vitellogenin as the only source for the egg lipids. Since turbot vitellogenin mainly transports polar lipids, it can be hypothesised that vitellogenin deposits lipids in the egg yolk, which primarily consists of polar lipids, whereas lipids of the oil globule originate from other sources, such as the lipoprotein VLDL. It is suggested that the discrepancies in the apparent relationship between the lipids of vitellogenin and eggs in cod and turbot can be a general principle for teleost species with and without oil globules.

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# ACKNOWLEDGEMENTS

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