Carotenoid and retinoid transport to fish oocytes and eggs: what is the role of retinol binding protein?

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Abstract

Fish eggs contain carotenoids, retinals (retinal and dehydroretinal) and retinols (retinol, dehydoretinol and retinyl-esters) that are utilized during embryonic development, after fertilization. The carotenoids (mainly astaxanthins) are transported in the plasma by the low density lipoproteins, high density lipoproteins, and very high density lipoproteins (VHDL) and were found to be associated also with serum albumin. Retinals were found to be associated vitellogenin (VTG), a component of the plasma VHDL fraction that is internalized by oocytes during vitellogenesis. However, the transport of retinols and retinyl-esters that were located in the oil droplet fraction of homogenized eggs, has yet to be elucidated. Retinols are more abundant in freshwater fish eggs than in eggs of marine fish species. Since retinol is transported in the plasma of vertebrates in association with retinol binding protein (RBP), recent studies on the molecular characterization and expression sites of RBP, could contribute to determining the involvement of RBP in transporting retinol to developing oocytes in vertebrates.

Recently, results from our laboratory show that RBP mRNA levels in the liver and RBP plasma levels did not significantly change with the onset and during vitellogenesis in the Rainbow trout. These results were in contrast with a dramatic elevation in the mRNA levels of VTG in the liver and an increase in VTG plasma levels that was observed in the same females. Moreover, 17β-estradiol treatment of immature fish, resulted in relatively lower mRNA levels of RBP in the liver, concomitantly with an increase in the level of VTG transcripts and the appearance of VTG in the plasma of treated fish. In addition, RBP was localized in the cytosol

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of ovulated oocytes. These results for Rainbow trout are similar to those reported for the chicken but differ from those of Xenopus, where an increase in RBP mRNA was reported in the liver and higher levels of retinal and retinol were found in the plasma of 17β-estradiol treated animals.

The results, reported here for the first time in Rainbow trout, showing RBP transcripts in the ovary, oviduct (the ovarian tissue adjacent to the gonopore) and oocytes, suggest a modulating role for RBP in follicular development, as has been suggested for the bovine ovary.

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1. Introduction

Normal development of embryos in oviparous animals requires the coordinated transport of nutrients to the growing oocytes. The embryos and early life stages that develop within the eggs, after spawning and fertilization, depend on nutrients stored in the eggs as yolk, until commencement of exogenous feeding. Developing embryos crucially require retinoids for the proper development of a whole range of their organ systems as revealed in early deprivation studies (reviewed in Maden, 2001). Retinoids are a family of low molecular weight hydrophobic molecules that are derived from, or structurally related to retinol, known also as vitamin A. Vitamin A or retinoids are not synthesized de novo by vertebrates and the main sources for vitamin A are dietary and derived from vegetable provitamin A carotenoids, retinyl-esters from animal sources or dietary supplements. In general, vitamin A is stored as retinyl-esters, mainly in the liver, but also in the lungs and bone marrow. Cells of the body that require retinol receive it from the bloodstream, where it circulates bound to retinol binding protein (RBP). Inside the cell, retinol is converted by enzymatic oxidation first to the aldehyde retinal (the same molecule that is used in vision) and then by further oxidation of the aldehyde to carboxylic acid into retinoic acid (RA) (reviewed in Duester, 1996, 2000). RA is the biological active metabolite of retinol, acting at the level of the nucleus and functioning as a ligand, regulating transcription by binding to RA receptors or retinoid X receptors. These serve as DNA sequence-specific transcription factors (reviewed in Gudas et al., 1994; Mangelsdorf et al., 1994; Maden, 2001).

Retinol is essential for normal reproductive processes in males and females of mammalian species (Thompson et al., 1964; Schweigert et al., 1999; Brown et al., 2003) and retinoids may function as important regulators of oogenesis and oocyte survival (Eberhardt et al., 1999; Morita and Tilly, 1999; Livera et al., 2000; Whaley et al., 2000). Retinoids also function as important regulatory signaling molecules in cell division, growth and differentiation of tissues of ectodermal, endodermal and mesodermal origin, during embryogenesis and therefore should be contained within the yolk-laden eggs of non-mammalian vertebrates (Morriss-Kay and Ward, 1999). The extreme sensitivity of embryonic development to vitamin A has been known for
several decades and both hypovitaminosis and hypervitaminosis A can lead to abortion and embryonic malformation (reviewed in Gudas, 1994; Curley and Robarge, 1997; Redfern, 1997; Smith et al., 1998; Zile, 1998, 2001; Morriss-Kay and Ward, 1999; Newcomer and Ong, 2000). Administration of teratogenic doses of RA results in craniofacial, cardiac, limb, thymic and central system malformations in mammalian embryos, zebrafish embryos and flounder larvae (Hermann, 1995; Takeuchi et al., 1998; Zile, 1998). Pollution reducing vitamin A stores in the livers resulted in fewer spawns, indicating the dependence of egg formation on vitamin A (Branchaud et al., 1995).

All-trans-retinol (retinol) is found in the liver of marine fish and several species of freshwater fish, but 3,4-didehydroretinol (dehydroretinol) is the abundant form in some freshwater fish species. In most fish species, β-carotene and other structurally related carotenoids, are transformed into retinol, via retinal, in the intestine. In freshwater fish, dehydroretinol may be synthesized from β-carotene, as in the goldfish (Hata et al., 1973), or from lutein (3,3′ Dihydroxy α-carotene), as reported for the liver of the freshwater Sacchobranchus fossilis (Barua and Das, 1975; Barua et al., 1977; Goswami and Barua, 1981; summarized in Al-Khalifa and Simpson, 1988).

The mode of retinoid transport to developing oocytes in oviparous vertebrates, has therefore gained significant attention in studies performed in the chicken (Vieira and Schneider, 1993; Vieira et al., 1995a,b), Xenopus (McKearin et al., 1987; McKearin and Shapiro, 1988; Irie et al., 1991; Azuma et al., 1993a,b) and in fish (Sammar et al., 2001; Irie and Seki, 2002). In the present study we aim at examining the mode of transport of carotenoids and retinoids into fish eggs and compare these events with those occurring in other vertebrate species forming oviparous eggs.

2. Carotenoids, retinal, retinol and retinyl-esters in oviparous eggs

The bright yellow or orange color eggs of salmonids and of other fish, is due to the presence of carotenoids and their composition has been studied for several decades (Ando, 1986; summarized in Ando and Hatano, 1991; Torrissen and Christiansen, 1995; Christiansen and Torrissen, 1997). Among the carotenoids identified within the fish ovaries or eggs are: astaxanthins, tunaxanthins, luteins, zeaxanthin and ido-xanthins and several others that are found in trace amounts. The relative composition of the various carotenoids and their isomers depends on the food consumed, with astaxanthins usually being the most abundant ones (Miki et al., 1982; Kitahara, 1984; Ando and Hatano, 1986; Ando et al., 1989; Østerlie et al., 1999; Bjerkeng, 2000). The amount of carotenoids in eggs varies (Table 1) between species living in the same habitat but consuming different types of food (e.g. plankton, fish or shellfish; Miki et al., 1982). The inter-dependence of ovarian or egg carotenoid content on the food is also reflected in variation within species, especially when comparing cultured fish to those caught from natural resources (Ando, 1986; Ando and Hatano, 1991). Carotenoids, specifically astaxanthin that constitutes about
80% of the egg carotenoids, were found to be transported from the yolk sac to the developing Chum salmon pro-larva, where they were deposited mainly in the skin but also utilized by other organs (Kitahara, 1984). However, no relationship was found between the astaxanthin concentration of the eggs and fertilization rates or survival to hatching stage in the Atlantic salmon eggs, in contrast to several earlier studies (Christiansen and Torrissen, 1997). In freshwater fish, where dehydroretinol is the usual form of vitamin A stored in the liver, zeaxanthin and lutein (64.6% and 15.8%, respectively) were found as the main carotenoids in the eggs (e.g. the goldfish; Hata and Hata, 1971). In mature Chum salmon eggs, carotenoids were located in almost equal amounts in the oily fraction (chylomicra particles) and in the aqueous fractions that were obtained, after centrifugation. Most of the carotenoids in the aqueous fraction were associated with lipovitellin, the main protein component of yolk (Ando and Hatano, 1986, 1991).

Plack and colleagues showed the occurrence of all-trans-retinal (retinal) and 3,4-didehydroretinal (dehydroretinal) in eggs of various species of fish, a frog, turtles and birds (Plack et al., 1959; Plack, 1960; Plack and Kon, 1961; Plack, 1964; Plack and Pritchard, 1968). This was the first demonstration of the occurrence of retinal not in the association with the visual organs. Studies also showed, that in marine fish, retinal and dehydroretinal were the predominant form of vitamin A, constituting between 74% and 100% of the retinoids in the egg or ovaries (Table 2). In freshwater fish and in salmonids, however, a relative higher abundance of retinol, dehydroretinol and their esters (31.1–56% of retinoids) was found (Plack, 1964; Irie and Seki, 2002). The retinals were found to be associated with lipovitellin in *Xenopus* and in fish, while the retinols and retinyl-esters were located in the oil droplets obtained from eggs. A low molecular mass protein band (14–21 kDa) was identified in oil droplets (Irie and Seki, 2002; Fig. 4). The specific function or advantage of the oc-
The relative abundance of retinals (retinal and dehydroretinal), retinols (retinol and dehydroretinol) and retinyl-esters in eggs of oviparous vertebrates (1, 2, 3, 5 and 8, 9, 10, 11 and 12 after Plack, 1964; 4, 6, 7 and 13, from Irie and Seki, 2002)

<table>
<thead>
<tr>
<th>Species</th>
<th>Total (µg/g dry weight)</th>
<th>% Retinal + dehydroretinal</th>
<th>% Retinol + dehydroretinol and their esters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Marine teleosts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Cod (Gadus callarias L.)</td>
<td>4.3</td>
<td>91</td>
<td>9</td>
</tr>
<tr>
<td>2. Herring (Clupea harengus L.)</td>
<td>5.0</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>3. Plaice (Pleuronectes platessa L.)</td>
<td>5.8</td>
<td>91</td>
<td>9</td>
</tr>
<tr>
<td>4. Marbled flounder (Pleuronectes yokohamae)</td>
<td>~100</td>
<td>Not detected</td>
<td></td>
</tr>
<tr>
<td>5. Red gurnard (Trigla cuculas L.)</td>
<td>4.3</td>
<td>79</td>
<td>21</td>
</tr>
<tr>
<td>6. Stingfish (Inimicus japonicus)</td>
<td>~100</td>
<td>Not detected</td>
<td></td>
</tr>
<tr>
<td>7. Black porgy (Acatopagrus schlegelii)</td>
<td>93.3</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td><strong>Freshwater teleosts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. Char (Salvinus alpinus willughbii Gunther)</td>
<td>5.3</td>
<td>66</td>
<td>34</td>
</tr>
<tr>
<td>9. Perch (Percia fluvais L.)</td>
<td>9.4</td>
<td>63</td>
<td>37</td>
</tr>
<tr>
<td>10. Pike (Esox lucius L.)</td>
<td>6.2</td>
<td>61.3</td>
<td>38.7</td>
</tr>
<tr>
<td>11. Trout (Salmo truta L.)</td>
<td>6.6</td>
<td>44</td>
<td>56</td>
</tr>
<tr>
<td>12. Salmon (Salmo solar L.)</td>
<td>5.7</td>
<td>64.9</td>
<td>35.1</td>
</tr>
<tr>
<td>13. Chum salmon (Oncorhynchus keta)</td>
<td>68.9</td>
<td>31.1</td>
<td></td>
</tr>
</tbody>
</table>

currence of retinal and dehydroretinal in vertebrate eggs is not clear. It could be speculated, at least for the pelagic eggs of marine fish species, that there may be of importance in preventing predation by other planktonic organisms by reflecting light at a range that differs from that of retinols, especially as the retinal is bound by a Schiff base linkage to vitellogenin (VTG).

3. The mode of transport of carotenoids and retinoids into oviparous eggs

The carotenoids are not synthesized in animals and are absorbed through the food. They are carried in the circulatory system in association with lipoproteins (Ando et al., 1985; Nakamura et al., 1985; Ando, 1986; Ando et al., 1986a,b; Ando and Hatano, 1987, 1988). Three types of carotenoid-carrying lipoproteins (CCLs) were identified in Chum salmon (Oncorhynchus keta): low density lipoprotein (LDL), high density lipoprotein (HDL) and very high density lipoprotein (VHDL). These LDL, HDL and VHDL fractions contained 5%, 32% and 34% of the total serum carotenoids, respectively (Ando et al., 1985). In addition, carotenoids were found to be prominently associated with serum albumin in the plasma of Atlantic salmon (Aas et al., 1999). A CCL of the HDL fraction was identified in male and female plasma in association with carotenoid transport from the muscle to the integument and its carotenoid content is especially conspicuous during spawning migration. The
CCL from the VHDL fraction was identified as VTG, the precursor of the egg yolk lipovitellin, synthesized in the liver of oviparous vertebrates and transported via the circulatory system to the oocytes, where it is sequestered by endocytosis with specific receptors (Byrne et al., 1989). VTG was suggested to function in the transport of carotenoids, mainly astaxanthins, from the muscles to the ovary during spawning migration of Chum salmon (Ando et al., 1986a,b; Ando and Hatano, 1987).

VTG, the main protein transporting carotenoids from the liver to the developing oocytes in most fish species, is also involved with carrying retinals into the oocytes during vitellogenesis. Early studies showed the occurrence of retinals in fish ovaries (Plack and Kon, 1961; Plack et al., 1959, 1961) and associated their appearance in the plasma, with egg development and with estradiol treatment of immature fish (Plack and Pritchard, 1968). Plack and colleagues, noticed that the retinal was associated with a lipid and probably also with a protein and speculated that retinal is carried by a phosphor-protein in the plasma of fish, as it was more abundant in the aqueous fraction prepared from eggs. It was only several years later that retinal was shown to be associated with an egg yolk protein, displaying the apparent molecular mass of lipovitellin (Irie and Seki, 2002). The apparent association of retinal with lipovitellin was also reported for eggs of *Xenopus* (Seki et al., 1987; Irie et al., 1991). Moreover, retinal was found to be associated with VTG in estradiol treated male *Xenopus* (Azuma et al., 1993a,b). However, similar direct evidence for the association of retinal with VTG has not been yet shown for avian or other species, where VTG is also the main protein component of eggs.

The mature oocyte also contains retinols and retinyl-esters and the mode of their transport from the liver has not been fully elucidated in oviparous species. In the chicken and gull retinol comprises 70%–72% of the total egg retinoids, in contrast to its lower content in the eggs of other avian species (Plack, 1964; Joshi et al., 1973; Surai et al., 2001). In the eggs of goose and duck, retinol and retinal content ranges from 27% to 50% and 24% to 45%, respectively (Plack, 1964). In the frog, and in several reptile species, the abundance of retinols was equal or slightly higher than that of retinals and substantial amounts of retinyl-esters were also identified (Plack and Kon, 1961; Plack, 1964). However, only trace amounts of retinol and retinyl-esters were found in *Xenopus* eggs (Irie et al., 1991; Azuma et al., 1993a,b; Irie et al., 2002). As mentioned before, relatively higher amounts of retinol and retinyl-esters were found in the eggs of freshwater fish and in particular in the trout and Chum salmon (Table 2). In the chicken, retinol in the egg originates from the serum RBP (Vieira and Schneider, 1993; Vieira et al., 1995a). In summary, retinals are the main form of retinoids in fish eggs and their relative abundance is reduced through evolution, whilst retinols that are almost undetected in some marine fish species, form the major retinoid component in avian eggs (Fig. 1).

4. Retinol binding protein and its role in transporting retinol to developing oocytes

Serum RBP, is a ~21 kDa protein synthesized by hepatocytes and a member of the lipocalin family proteins that transport lipophilic molecules (Pervaiz and Brew,
The liver is one of the major sites for gene expression of RBP and protein synthesis, but transcripts for RBP and protein synthesis were also detected in other tissues including the retinal pigment epithelium, kidney cells and the seminal vesicle epithelium (Blaner, 1989; Redfern, 1997). In birds and mammals, RBP circulates in the plasma complexed with transthyretin (TTR), a protein carrier of thyroxine (Heller, 1976; Kopelman et al., 1976; Blaner, 1989; Blomhoff et al., 1990). Three-dimensional studies of human RBP demonstrated that one tetramer of TTR binds two molecules of RBP (Monaco et al., 1995). The association of RBP with TTR increases the stability of the retinol–RBP complex, and it was suggested, to prevent ready filtration in the renal glomerulus of both the RBP and the bound retinol (Blaner, 1989). While RBP may be the major carrier of retinol in the blood, retinol may also be transported by serum albumin. Although retinol has a lower affinity for serum albumin, the relatively higher concentration of albumin potentially enables the transport of significant amounts of retinol. In this case, the transfer of retinol will lack the specificity afforded by cell surface receptors for retinol bound to RBP (Noy and Xu, 1990). As mentioned before, an association of astaxanthins with serum albumin was reported in Atlantic salmon plasma (Aas et al., 1999).

RBP was purified from the plasma of several non-mammalian vertebrates including chicken, snapping turtle, bullfrogs and fish [eel (Anguilla japonica), carp (Cyprinus carpio), blue shark (Pronace glauca) yellowtail (Seriola quinqueradia) and trout (Oncorhynchus mykiss), Mokady and Tal, 1974; Kopelman et al., 1976; Shidoji and Muto, 1977; Hayashi et al., 1990; Berni et al., 1992; Vieira and Schneider, 1993]. Trout RBP was purified as a ~21 kDa protein and its amino acid sequence was determined by Edman degradation (Berni et al., 1992; Zapponi et al., 1992). RBP from carp, blue shark and yellowtail showed a low abundance in the plasma and its relatively low molecular mass suggested that it occurs as a monomer, not associated with TTR (Shidoji and Muto, 1977). This “fish type” RBP was identified also in tadpole frogs, while the adult frogs exhibited the “human type” RBP that binds TTR (Shidoji et al., 1979).

Recent studies on the molecular characterization of RBP cDNA have been published for carp, the Gilthead seabream and trout (Bellovino et al., 2001, 2002;
Funkenstein, 2001; Sammar et al., 2001). Additional sequences of RBP cDNA were obtained in our laboratory (Fig. 2) for the eel (*Anguilla anguilla*), the White grouper (*Epinephelus aenus*) and from the Gilthead seabream (*Sparus aurata*). Comparison of these sequences with those of Medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), Puffer fish (Fugu; *Sphoeroides maculates*), *Xenopus*, crocodile and several mammalian species revealed that fish RBPs show conserved characteristics in parallel to

![Figure 2](image-url)

**Fig. 2.** Alignment of human RBP (Accession #PO2753) with the RBP of the following fish species: Gilthead seabream [BF; #AF083556 (Funkenstein, 2001), SM; #AF257327 (Sammar et al., 2001)], Medaka (#AU179388), Grouper (#AF538328), eel (#AF538330), Rainbow trout [Trout I; #P24775, Trout II; #AAB24973; MS; #AF257326 (Sammar et al., 2001)], carp (#AJ277123), zebrafish (#NM130920), Fugu 2665 (#26658) and Fugu (#28751). The consensus sequence was obtained after additional comparison with RBP of *Xenopus* (#A30013), crocodile (#A011392), rabbit (#VARB), rat (PO4916) and mouse (U63146). Sequence numbers follow those available at http://www.ncbi.nlm.nih.gov. Sequence alignment was formed with SeqWeb (version 2.0) software. Database searches and multiple local alignment were performed with the BLAST suit of programs (http://www.ncbi.nlm.nih.gov/BLAST). Sequence signal prediction was performed according to Nielsen et al. (1997) (http://genome.cbs.dtu.dk/services/SignalP). The signal peptide is underlined. Cysteines conserved residues are highlighted by green. Lipocalin domains are highlighted by yellow and amino acids involved in mammalian species with TTR are shown in blue. The amino acids binding to retinol are underlined by a double line (=). An arrow indicates the position of introns in human RBP.
unique ones. The conserved characteristics in the primary structure include: (a) the conserved amino acid residues comprising the signal peptide that were found in some of the fish species, but are not conserved in mammalian species; (b) the six cysteines that are involved in disulfide bond formation; (c) the amino acids comprising the lipocalin motifs show some differences between fish species and in comparison to the human RBP sequence. The lipocalin motifs (Flower, 1996) show the following replacements between fish and human RBP sequences (Fig. 2): In Motif 1, F-61 and M-68 are replaced by Y and V, respectively. In Motif 2 and Motif 3, consistent differences between all the fish species and the human sequence include the following replacements: V-148 is replaced by I, T-154 is replaced by N, V-157 is replaced by I, Q-158 is replaced by H, D-181 is replaced by H (except for carp), P-186 is replaced by R, A-189 is replaced by D. There are additional replacements between the various fish species within these motifs; (d) the amino acids associated with binding to retinol are conserved in most species with some exceptions. All fish species show the following replacements of amino acid residues: F-61 is replaced by Y, L-104 is replaced by I, Q-158 is replaced by H (this amino acid is also part of the lipocalin Motif 2). In grouper, trout, eel and carp, I-82 is replaced by V. In the Trout I and Trout II, M-94 is replaced by V. The most noticeable species specific replacement is of G-116 by A in seahream and Medaka. It is not known whether these replacements affect the affinity.
of the RBP for retinol; (e) only four out of the eight amino acid residues associated with the interaction of human RBP with human TTR are conserved in all fish RBP sequences. Two amino acids are replaced in all species; K-130 is replaced by R in seabream, grouper, eel and trout, S-136 is replaced by A in Trout II, carp and zebrafish and K-140 is replaced by T in most species except Trout II where it is replaced by S. These observations support previous studies on the low affinity of fish RBP for human TTR and the monomeric appearance of fish holoRBP in the plasma (Shidoji and Muto, 1977). The two consensus glycosylation sites near the NH2-terminus located in carp (Bellovino et al., 2001, 2002) were not found in the primary structure of other fish species. One putative glycosylation site was found for zebrafish RBP (Bellovino, personal communication). Therefore, it remains to be explained how RBP in most fish species escapes glomerular filtration as most are neither glycosylated nor bound to TTR; (f) all fish species showed the absence of the COOH-terminal tetrapeptide, RNL(S)L, that is found in the primary structure of mammalian RBP and is presumed to stabilize the association of RBP with TTR. The sequences available for Fugu RBPs show large divergence from those of other species.

The phylogenetic analyses (Fig. 3) of the putative amino acid sequences of fish RBP indicates close association of RBP from the gilthead seabream (Sparidae; Perciformes) with that of Medaka (Adrianichthidae; Beloniformes) although they are from two distantly related two taxonomic groups. On the other hand, seabream RBP is more distantly related with the RBP of grouper (Serranidae, Perciformes) belonging to the same taxonomic group. The position of the eel RBP reflects its evolutionary position in the phylogenic tree. It should be considered that some of the variation outlined above may not be due to species specific differences but to intra-specific variation or even to variation between multiple genes within one genome, as the number of replicate RBP genes in fish genomes remains unknown at this stage.

The fish RBP was found to be conspicuously expressed in the liver and intestine, with low levels of expression in the spleen, kidney, ovary and brain (Bellovino et al., 2001, 2002; Funkenstein, 2001; Sammar et al., 2001). No differences were found in the plasma RBP relative levels between males, previtellogenic, vitellogenic or post-vitellogenic Rainbow trout females and in 17β-estradiol treated immature fish. This result was in contrast with the dramatic elevation of VTG in these plasma samples. Northern-blot analyses of hepatic mRNA, did not reveal dramatic changes in the level of RBP transcripts in the liver of females at the various stages of vitellogenesis but showed a significant increase of VTG mRNA levels in the livers of vitellogenic females. The amount of RBP mRNA was reduced after 17β-estradiol treatment in immature fish (Sammar et al., 2001, submitted for publication).

While these results are similar to those reported for chicken (Vieira et al., 1995a,b), it is not clear whether they show similar functional implications. In the chicken, retinol is the main retinoid stored in the oocytes. The lack of detectable RBP mRNA in ovarian follicles, indicated that no significant amounts of RBP are synthesized by the oocytes or ovary. The relatively large quantities of retinol loaded RBP originate from the serum and are taken up by the oocytes in association with TTR, during vitellogenesis. However, estrogen decreased RBP transcription in re-
lation to other hepatically synthesized proteins that are components of the yolk, such as VTG and the VLDL. In parallel, estrogen treatment did not affect RBP plasma levels but an increase in VTG and VLDL levels, was found (Vieira and Schneider, 1993; Vieira et al., 1995a). In contrast to the chicken, estrogen treatment increased hepatic RBP mRNA transcription (McKearin et al., 1987; McKearin and Shapiro, 1988) in *Xenopus laevis*. Moreover, estrogen treatment resulted in a significant increase in plasma levels of retinol and retinal (Azuma et al., 1993a,b). The increased retinal plasma levels were associated with VTG, but the allocation of retinol remains obscure, especially as retinol was not located within the *Xenopus laevis* oocyte (Azuma et al., 1993a,b). Resolving the mode of regulation of RBP expression in amphibian requires additional studies.
5. Retinol binding protein in fish ovaries

In the chicken oocytes, retinol was associated with RBP and was localized in the electron-lucent phase by immunohistochemistry, as mentioned before (Vieira et al., 1995a). Recent results of Western-blot analysis indicated the occurrence of RBP in the Rainbow trout oocytes (Sammar et al., submitted for publication). In view of the results that RBP plasma levels did not change during vitellogenesis (Sammar et al., submitted for publication) and as RBP synthesis was located in the ovine oviduct (Eberhardt et al., 1999), we decided to examine the possibility that RBP in Rainbow trout oocytes originates from the ovarian tissues. RBP transcripts were amplified by RT-PCR, in immature, previtellogenic, vitellogenic and ovulating ovaries and the oviduct (a term used here to indicate the ovarian tissue close to the gonopore in vitellogenic or ovulating ovaries and not containing visible vitellogenic oocytes) and in oocytes removed from vitellogenic and ovulating ovaries (Fig. 4). The sequences of the RT-PCR transcripts from the ovary, oviduct and oocytes were verified as RBP (data not shown). The relative abundance of transcripts varied between samples, with almost undetectable transcripts for the oviduct of ovulating ovaries. More studies will be needed, however, to verify the relative abundance of these transcripts in the ovary, oviduct and oocytes. Furthermore, Northern-blot analyses (Fig. 5)

![Fig. 4. RT-PCR analysis of total RNA extracted from trout liver, ovaries, oviduct and oocytes. Total RNA (10 μg) was mixed with 0.1 μg of oligo-dT and H2O (up to 17 μl), and incubated for 5 min at 70 °C and immediately cooled on ice for 5 min. Then were added 5 μl of MMLV 5× buffer (Promega), 2 μM dNTPs, 25 U of rNasIn and 200 U of MMLV Reverse Transcriptase (Promega), to reach a final volume of 25 μl. After an incubation of 1 h at 42 °C, 75 μl of H2O were added to the reaction. PCR amplification was carried out with 1 μl of the cDNA in 25 μl of reaction mixture with two primers, (1) RBP-7: 5'-GCA GAA CTT CGA TAG GAG CAG G-3' and (2) RBP-8: 5'-CGC GTC TGT ACT TGC CGA GG-3'. The thermal profile for RT-PCR consisted of initial denaturation at 94 °C for 2 min followed by 47 cycles of denaturation (94 °C for 30 s), annealing (52 °C for 1 min), and extension (72 °C for 1 min). Amplified products were separated by 1.2% agarose gel electrophoresis. Amplified 462-bp RBP the respective 18S rDNA amplification products, are shown in the upper and lower panels, respectively.](image-url)
indicated a similar size for the transcripts from the examined tissues. Numerous studies, in a variety of species have indicated that retinoids play important roles in the development and regulation of ovarian function. More recently (Brown et al., 2003), it was demonstrated that RBP was expressed and synthesized by theca and granulose cells of the ovarian follicle and the luteal cells of the corpus luteum in the bovine ovary. The results reported here for the first time in fish, suggest that the modulating role of RBP in follicular and oocyte development and ovarian steroidogenesis encountered in mammalian, has been conserved through vertebrate evolution.

6. Summary and conclusions

Studies spanning over several decades are starting to unveil the mechanisms involved in transport of retinoids to oviparous eggs in vertebrates. While the most systematic studies were performed on the chicken egg, this model may not fully apply to other species as retinol is more abundant in the eggs of chicken, in contrast to retinal in fish and frog eggs. The functional significance of storage of retinals vs. retinols in oviparous eggs is not known at this stage but it was speculated that it maybe associated with defense against predation of the planktonic eggs that are produced by pelagic marine fish.

While VTG is suspected as the main carrier of carotenoids and retinal to fish eggs, the origin of retinols and retinyl-esters has yet to be resolved. It is not clear whether plasma RBP contributes retinols to oocytes, as the plasma levels do not increase during vitellogenesis, in contrast to other hepatically synthesized proteins (e.g. VTG) that are incorporated into the developing oocytes. The occurrence of RBP within the oocytes cytosol raises the question on its origin. Recent studies reported here for the
first time, indicate that ovarian expressed RBP may have an important function in follicular development during vitellogenesis.

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References


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