Reconstitution of muscle F-actin from Atlantic salmon (*Salmo salar* L.) with carotenoids – binding characteristics of astaxanthin and canthaxanthin.

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Running Head: Astaxanthin binding to actin

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Abstract

The binding of carotenoids to the myofibrillar protein F-actin purified from the white muscle of Atlantic salmon (*Salmo salar* L.) was studied using *in vitro* reconstitution. The binding of astaxanthin and canthaxanthin was saturable, and analysis revealed the presence of a single carotenoid-binding site. The dissociation constants (K_d) for actin prepared from 2.5 Kg FW fish were 1.04 ± 0.13 μg carotenoid mg^{-1} actin and 0.54 ± 0.11 $\mu g mg^{-1}$ for astaxanthin and canthaxanthin, respectively. The saturation binding level (B_{max}) for astaxanthin was 1.39 ± 0.07 $\mu g mg^{-1}$ and 1.04 ± 0.08 $\mu g mg^{-1}$ for canthaxanthin. These values were higher for F-actin prepared from organic and small (~0.5 Kg FW) salmon than for non-organic and larger, mature fish. The structural specificity of carotenoid binding revealed a preference for carotenoids that possess a keto group at C-4 on the β end-group of the molecule, but the presence of hydroxyl groups at C-3 or C-4 reduced overall binding efficiency. The study suggests that the ability of myofibrillar proteins to bind carotenoids is not a limiting factor governing the deposition of carotenoids in the muscle of salmonids.

Introduction

Global Atlantic salmon production was 2.0 million tonnes gutted weight equivalent in 2014 (Kontali analysis). The pigmentation of all salmon species is a key quality parameter in the commercial production of these species in seawater cages. Carotenoids are responsible for the pigmentation of the muscle and skin of a number of wild and farmed salmonids. The two most abundant pigments that are deposited in the white muscle of salmonids such as Atlantic salmon (*Salmo salar*) and Rainbow trout (*Oncorhynchus mykiss*) are astaxanthin (3,3′-dihydroxy-ß,ß-carotene-4,4′-dione) and canthaxanthin (ß,ß-carotene-4,4′-dione). The extent of deposition of these carotenoids in muscle varies amongst different fish species. For example, canthaxanthin is preferentially bound to the muscle of *S. salar* compared to astaxanthin (Kiessling *et al.* 2003). However, in *O. mykiss*, the situation is reversed with studies by Choubert & Storebakken (1989) and Storebakken & Choubert (1991), showing that the mean retention for astaxanthin was 1.3-1.5x higher than that for canthaxanthin. In addition a range of other dietary carotenoids and a small number of carotenoid metabolites are found at smaller concentrations in such muscle tissue including lutein (β,ε-carotene-3,3′-diol), zeaxanthin (β,β-carotene-3,3′-diol) and idoxanthin (β,ε-carotene-3,3′-diol; Schiedt 1998). These carotenoids are associated with the muscle myofibrils and were originally thought to be loosly bound to the actomyosin complex (Henmi *et al*. 1987; 1989).

Actin is a single chain polypeptide (Mr = 43,000) and exists as two inter-changeable forms, namely the filamentous form and the monomeric globular form, termed F-and G-actin, respectively. F-actin is the major protein of muscule thin filaments. Although a number of isoforms of actin are known to exist the behaviour and properties of actin isolated from a wide range of animals (including fish) appear to be similar (Swezey & Somero 1982). The 3D structure of actin filaments reveals the presence of two strings of actin globules wound around each other in a double helix structure (Kabasch *et al*. 1990; Lorenz *et al*. 1993; von der Ecken *et al*. 2015). Even though the atomic structure of F-actin is not yet fully resolved, it is clear that a series of hydrophobic grooves or pockets are created between the F-actin subunits (Dominguez & Holmes 2011; von der Ecken *et al*. 2015).

Both *in vivo* and *in vitro* F-actin exists in filaments that can reach several μm in length (Schoenenberger *et al*. 1999). *In vivo* F-actin combines with mysosin to form an actomyosin complex in the myofbrils. This is the major contractile protein complex in the white muscle

and in species such as Atlantic salmon and Rainbow trout is pigmented with dietary carotenoids and their metabolites. Previous studies on the isolated actomyosin complex by Henmi and colleagues (1989, 1990a, 1990b, 1991) have explored some of the properties of the complex in relation to pigment binding and have demonstrated that carotenoids can bind to this complex *in vitro*. Subsequently, Saha *et al*. (2005, 2006) and Matthews *et al*. (2006) have explored aspects of astaxanthin binding to fish muscle proteins. The latter study tentatively identified that that carotenoids are specifically bound to α-actinin, a rod-like protein belonging to the spectrin family. This particular protein serves an important role in the organisation of muscles (and the cytoskelton) by providing cross-linking resulting in the bundling together of F-actin filaments (Sjöblom *et al*. 2008).

The aim of this study was to determine the binding characteristics of the two principle carotenoids used in the pigmentation of farmed fish, namely astaxanthin and canthaxanthin, to a preparation of muscle actin filaments purified from Atlantic salmon. To achieve this we adopted an *in vitro* reconstitution approach whereby exogenous purified carotenoids are added to the F-actin apo-protein to produce a caroteno-protein complex. A number of carotenoids were tested in order to determine whether F-actin displayed and specific structural preferences for the binding of carotenoids.

Materials and methods

Isolation of actin and myosin

Fresh fish were obtained from EWOS Innovation research facilities at Dirdral, Norway (Atlantic salmon) and at Colaco, Chile (Rainbow trout and Coho salmon). The procedures used for the isolation and purification of actomyosin, actin and myosin from the white muscle of Atlantic salmon and other fish was that of Martone *et al*. (1986) as modified by Park & Lanier (1989). Unless otherwise stated all chemicals were obtained from Sigma (Poole, UK). All procedures were carried out at 4^{o}C. Muscle proteins were prepared from white muscle (fresh and not frozen fillets were used throughout). The muscle was ground in 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M KCl and 0.02% (w/v) NaN_3 and left to stand for 15 min before centrifugation for 10 min at 1,000 g (Sigma 6K15). The supernatant was discarded and the pellet ('myofibrilar proteins') was resuspended in 20 mM Tris-maleate buffer (pH 6.8) containing 0.45 M KCl, 5.0 mM beta-mercaptoethanol, 0.2 M $Mg(CH_3COO)_2$ and 1.0 mM ethylene glycol-bis N,N,N',N'-tetraacetic acid. To this, ATP was added to a final concentration

of 5.0 mM, mixed and left for 1 hr, after which it was centrifuged at 10,000 g for 15 min. The resulting supernatant ('crude myosin') was used for the further purification of myosin (see below). The pellet, containing 'crude actin', was resuspended in 0.8 M KCl, 5.0 mM beta-mercaptoethanol and 0.2 M $Mg(CH_3COO)_2$, stirred for 5 min and centrifuged at 10,000 g for 15 min. The supernatant was discarded and the pellet ('actin') was further extracted with 2.0 mM $KHCO_3$ for 1 hr, after which it was centrifuged at 20,000 g for 1 hr. The pellet consisted of insoluble F-actin and the supernatant contained purified soluble G-actin.

Myosin was purified from the 'very crude myosin' supernatant (see above) by dilution of the supernatant with 1.0 mM $KHCO_3$ for 15 min, followed by centrifugation at 12,000 g for 10 min. The supernatant was discarded and the pellet (containing 'crude myosin') was resuspended in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 M KCl and 5.0 mM beta-mercaptoethanol with gentle homogenisation. After 10 min, 1.0 mM $KHCO_3$ and $MgCl_2$ (to a final concentration of 10.0 mM) were added. After incubation for 12 hr, the preparation was centrifuged at 20,000 g for 15 min and the resulting supernatant was discarded. The pellet (containing 'myosin') was resuspended in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 M KCl and 5.0 mM beta-mercaptoethanol with gentle homogenisation to yield a fraction termed 'purified myosin'. SDS-PAGE was used to identify and confirm the purity of each fraction obtained.

Proteins were prepared from previously pigmented fresh fish of 2.5 Kg FW, unless otherwise stated. Purified F-actin was stored at $4^{o}C$ in complete darkness and used for reconstitution studies usually within 48 hours of preparation.

Protein determination

The total protein content in the isolated muscle fractions and in reconstituted actin-carotenoid complexes were determined using the Bradford method (Bioquant Kit, Merck Ltd, Poole, UK).

In vitro *reconstitution of muscle proteins*

The methods employed are based on those described by Zagalsky (1985). The structures of the carotenoids used in the reconstitution studies are shown in Fig. 1. All procedures were carried out at $4^{o}C$. F-actin was purified as described above. The bound carotenoids were stripped from the protein by adding acetone (1:1, v/v) while votex mixing.

Three volumes of diethyl ether were added and the sample inverted three times. The resulting upper solvent layer was carefully removed and the extraction procedure repeated until the no more colour could be extracted from the protein. Any remaining solvent was removed from the carotenoid-free protein by gentle flushing with oxygen-free nitrogen.

Purified carotenoid (1 $mgmL^{-1}$) was dissolved in acetone (0.42mL) then added, with vigorous mixing, to 1.00 mL of the carotenoid-free apoprotein (typically $1mgmL^{-1}$). Sodium phosphate buffer (3.00mL; 50mM, pH 7) was added and the sample inverted. Acetone was removed from the resulting caroteno-protein by either rotary evaporation or by flushing the vial with oxygen-free nitrogen. The sample was then centrifuged for 10 min at 2000g, the pellet washed in buffer and re-centrifuged. The final pellet was resuspended in sodium phosphate buffer.

In order to remove any non-specifically bound carotenoid present in the sample following reconstitution, the resulting caroteno-protein was gently washed with *n*-heptane. The level of free (i.e. unbound) carotenoid was always less than 0.1% (w/w) of total carotenoid at this stage. The efficiency of binding of carotenoids was expressed as: binding efficiency (%) = $\left[\left(\frac{0.5\ x\ Bmax)}{Kd}\right] x\ 100\right.$

Chromatography and spectroscopy

Carotenoids were extracted from reconstituted proteins as follows. In a separating funnel, acetone (3.00mL) was added to the reassembled caroteno-protein complex (3.00mL) and thoroughly mixed. Diethyl ether (1.00mL) was then added and the solution inverted three times to ensure good mixing. The lower aqueous layer was removed and the upper, pigmented, layer reserved. The diethyl ether layer was then washed with distilled water at least three times, before the sample was dried under a steady stream of oxygen-free nitrogen. The extraction was repeated at least once and extracts pooled. The carotenoid content and composition was determined by normal-phase HPLC using a modification of the method developed by Schüep & Schierle (1995). A Lichrosorb silica 60 rapid analysis column (50 x 4.6mm, 5µm particles; Phenomenex, Macclesfield, U.K.) and security guard cartridge were first acidified with 1% (v/v) H_3PO_4 in methanol (Kirkland & Dilks, 1973) to prevent peak tailing. The solvent system (*n*-hexane: acetone, 86:14, v/v) was delivered at a flow rate of 1.2 $mLmin^{-1}$ at 20^{o}C using an Agilent (Stockport, U.K.) 1100 series Binary pump. Samples were injected in

20µL of the eluting solvent using an Agilent 1100 series autosampler. Carotenoids were detected using an Agilent 1100 series diode-array detector and integrated at their λ_{max} using Chemstation 6.1 software (Agilent). The t_R of canthaxanthin and all-*trans* astaxanthin were 1.54 and 4.26 min, respectively.

Carotenoids were quantified in *n*-hexane using their published extinction coefficients ($A\frac{1\%}{1cm}$): all-*E* astaxanthin 2100; canthaxanthin 2200 (Britton, 1995). Roche Vitamins Ltd. (Basel, Switzerland) kindly provided the carotenoid standards used in this study.

Data analysis

The analysis of data from the reconstitution experiments was performed by non-linear regression using the ligand-binding macro of SigmaPlot 7 (SPSS UK Ltd., Woking, UK).

Results

Carotenoid distribution

The isolation of the myobrillar protein fractions from pigmented fillets of both Atlantic salmon (containing astaxanthin and canthaxanthin) and Rainbow trout (astaxanthin only) revealed that the ratio of carotenoid : protein was highest in the fraction containing F-actin (Table 1). These pigments were also present in those other fractions that possess F-actin (e.g., actomyosin), however carotenoids could not be detected in the fractions containing only myosin or G-actin. A similar pattern was seen in Coho salmon (*O. kisutch*; possessing only astaxanthin), but the F-actin fraction in Coho had a much higher ratio of carotenoid : protein than that seen in either Atlantic salmon or Rainbow trout. Myofibrillar protein fractions obtained from an unpigmented fish (Haddock; *Melanogrammus aeglefinus*) were devoid of carotenoids (data not shown).

In Atlantic salmon (the only fish studied which had more than one carotenoid at high concentration in the muscle) the ratio of astaxanthin : canthaxanthin was increased in all fractions (e.g., in F-actin 63:38) compared to the original fillet (55:45), suggesting that canthaxanthin is less tightly bound to muscle proteins than astaxanthin and is preferentially lost from the muscle during the isolation of proteins. In Rainbow Trout both lutein and

zeaxanthin were detected in all protein fractions, in a near-identical ratio to astaxanthin as that seen in the original fillets (data not shown).

Reconstitution of actomyosin and myosin

Actomysin purified from fillets of Atlantic salmon was found to readily bind both astaxathin and canthaxanthin (data not shown). Differences in the binding behaviour for these two carotenoids were only evident at very high doses of carotenoid, well above the *in situ* levels observed in farmed or wild fish. However, the *in vitro* reconstitution of actomyosin with these carotenoids displayed characteristics consistent with non-specific binding of the pigments to the protein (data not shown). As a result the actomyosin fraction was not used in further studies. Neither astaxanthin nor canthaxanthin would bind to myosin isolated from salmonids.

Reconstitution of F-actin

The binding characteristics of astaxanthin and canthaxanthin to F-actin purified from Atlantic salmon are shown in Figs. 2A-C for large (~2.5 Kg FW), organically-reared (~2.5 Kg FW), and small (~0.5 Kg FW), respectively. Both carotenoids display behaviour characteristic of ligand-binding saturation for a single binding site. In contrast to the behaviour seen for actomyosin there is little evidence of non-specific binding of carotenoids to F-actin purified from Atlantic salmon. Saha *et al*. (2005) have proposed that carotenoid aggregates (see Ruban *et al*. 1993) may be present in isolated pigment-protein preparations. However, there was no evidence in this study that these micro-crystalline structures were present.

The saturation binding capacity (B_{max}) and the dissociation constants (K_d) of these two carotenoids are shown in Table 2. For large salmon, both the B_{max} and the K_d are higher for astaxanthin than for canthaxanthin. There were no significant differences seen in binding of astaxanthin and canthaxanthin at low (more physiologically relevant) doses of carotenoid. Similarly, no significant differences in the binding of carotenoids between fish of different sizes at low doses of carotenoid were observed. Where differences are seen in the overall capacity for F-actin from different sized fish to bind carotenoids, the doses are well in excess of those found in farmed fish. However, the binding efficiency of carotenoids (determined over the full dose range) is lowest in smaller, younger fish (~0.5 Kg FW). The capacity of F-

actin to bind carotenoids such as astaxanthin is higher in these small, juvenile, fish compared with larger, mature, fish but at levels much higher than seen *in vivo* (Table 2).

Competitive binding of carotenoids was explored by delivering a mixture of astaxanthin and canthaxanthin to F-actin isolated from Atlantic salmon at a defined molar ratio. The amount of astaxanthin was constant throughout (at approx. the K_d value determined earlier) and the amount of canthaxanthin was altered so that the total amount of carotenoid delivered to the isolated protein varied between treatments. The data shown in Fig. 3 reveal that in Atlantic salmon astaxanthin is preferentially bound to F-actin compared to canthaxanthin and even the presence of ten-fold higher levels of canthaxanthin does not affect its binding. The amount of total carotenoid (astaxanthin + canthaxanthin) bound to the reconstituted F-actin did not vary significantly between treatments, except when a ratio of 0.1:1.0 astaxanthin : canthaxanthin was used. In this case the shape of the resulting dose curve would strongly suggest non-specific binding of canthaxanthin was present. Such non-specific binding behaviour for canthaxanthin has also been seen when canthaxanthin is used on its own in reconstitution studies with F-actin.

The ability of isolated F-actin to bind a range of carotenoids possessing different structrual features was explored. The data presented in Table 3 shows that F-actin prepared from Atlantic salmon can bind a number of different carotenoids that possess keto-groups at C4 (and/or C4') or hydroxyl-groups at C3 (and/or C3') positions on the molecule. Binding efficiencies are highest for those carotenoids that possess keto-groups such as astaxanthin, canthaxanthin and astacene (3,3'dihydroxy-2,2,2',3'-tetrahydro-β,β-carotene-4,4'-dione). Efficiences were lowest for hydroxy-echinenone (3'-hydroxy-β,β-caroten-4-one) and for the carotenoid diols lutein and zeaxanthin. Carotenoids such as ß-carotene (β,β-carotene; the only hydrocarbon tested), crustaxanthin (β,β-carotene-3,4,3',4'-tetrol) and isozeaxanthin (β,β-carotene-4,4'-diol) did not bind to isolated F-actin. The inability of either crustaxanthin or isozeaxanthin to bind to F-actin suggests that the presence of a hydroxyl group at C4 and/or C4' serves to inhibit binding.

Discussion

Henmi and colleagues (1987) first identified the actomyosin complex as the main site for carotenoid binding in salmonid muscle. When isolated from pigmented

salmonid fillets this caroteno-protein complex does indeed contain carotenoids, as do all fractions that contain filamentous actin (Table 1). The optical characteristics (circular dichrosim) of the reconsituted actomyosin complexes demontrated that carotenoids were only weakly bound to the protein (Hemni *et al*. 1990). However, attempts to reconstitute the complex in the present study with exogenous astaxanthin and canthaxanthin displayed dose behaviour characteristic of a significant level of non-specific binding (data not shown). Such behaviour was not seen with the reconstitution of F-actin purified from Atlantic salmon. Binding of carotenoids was associated only with F-actin but not with myosin or monomeric actin (i.e., globular or G-actin).

The reconstitution of isolated F-actin with selected carotenoids (mainly xanthophylls; see Fig. 1 for structures) was used to explore whether F-actin displayed any preferences in terms of binding. Henmi *et al*. (1989) demonstrated that a range of carotenoids could be bound to isolated actomyosin including acyl esters of astaxanthin, canthaxanthin, echinenone (β,β-caroten-4-one), zeaxanthin and β-carotene. In particular, the binding of acyl esters of astaxanthin to isolated proteins would not be predicted but was not tested with F-actin in the present study. However it is notable that isolated F-actin did not bind the carotenoid hydrocarbon β-carotene in the present study, in contrast to the observations of Hemni *et al*. (1989). Isolated F-actin from Atlantic salmon demonstrates the ability to bind a range of carotenoids, albeit with different efficiencies (Table 3). The lower binding efficiencies seen for hydroxy-carotenoids such as lutein and zeaxanthin would be consistent with the presence of trace amounts of a small number of other oxygenated carotenoids (xanthophylls) in the flesh of farmed and wild Atlantic salmon. Whilst the ability of the protein to bind these xanthophylls may be reduced in salmonids it is also known that the absorption of 3-hydroxy-carotenoids from the diet is less efficient than 4-keto-carotenoids (Schiedt *et al*. 1985). The astaxanthin metabolite idoxanthin is also found in the muscle of salmonids (Schiedt *et al*. 1989) and although not tested here in reconstitution studies, its structure is consistent with the observations above in that it would be predicted to be bound to F-actin. Hemni *et al*. (1990) observed that astaxanthin and canthaxanthin were bound in the all-*E* form to actomyosin. In the current study only all-*E* forms of carotenoids were used and the ability of F-actin to bind *Z*-isomers was not explored. However, in one experiment *Z*-isomers of astaxathin were present in actin-containing fractions isolated from Rainbow Trout. These geometric isomers were not detected in the original fillets and it is possible that they were

produced as an artefact of the isolation procedure. Racemic astaxanthin was used for reconstitution in the current study. This mixture of optical isomers (the (3*S*,3'*S*), (3*R*,3'*S*) and (3*R*,3'*R*) forms) are known to be utilised equally when fed individually to salmonids (e.g., Storebakken *et al*. 1985).

It has been proposed that only one cyclic end-group of a carotenoid molecule is involved in binding so that the carotenoid was effectively held in a hydrophobic 'binding pocket' (Henmi *et al*. 1989). The structure of F-actin possess a number of hydrophobic 'grooves' located in between the G-actin subunits (von der Ecker *et al*. 2015). α-Actinin is a cross-liking protein that serves to organise the actin filaments into bundles, as present in the F-actin fraction in this study. Factors such as the arrangement of the actin filaments (exposing any putative carotenoid binding sites) may also influence binding and it is likely that α-actinin will play a role in this.

Binding of oxygenated carotenoids (xanthophylls) to proteins occurs in a wide range of organisms. A study of the xanthophyll-binding protein found in the human retina (Yemelyanov *et al*. 2001) showed that a number of different carotenoids could readily bind to this protein. Similarly, *in vitro* reconstitution studies on both invertebrate (e.g. α-crustacyanin; Britton *et al*. 1997) and plant (e.g., the light-harvesting complexes; Phillip *et al*. 1996) pigment-protein complexes reveal extensive plasticity in carotenoid binding. In the light-harvesting complexes from higher plants a strong preference for carotenoids that possess a hydroxyl group at C-3 is observed (Philip *et al*. 1996). The general dependence for binding of carotenoids with functional groups at C-3 and/or C-4 on one or both cyclic end-groups is common across a range of carotenoid binding proteins. In the present study, carotenoids possessing a hydroxy-group at C-3 and/or a keto-group at C-4 are readily bound to F-actin from Atlantic salmon. Indeed the data suggest a preference for carotenoids possessing the latter. An interesting observation was the ability of F-actin to readily bind astacene *in vitro*. This carotenoid results from the oxidation of astaxanthin (particularly under basic conditions) and when used as a pigment in salmonid diets it fails to result in muscle colouration (Bernhard 1990). For this carotenoid, its ability to bind to the muscle protein *per se* has been demonstrated and further suggests that uptake and/or subsequent transport via lipoproteins are the main limiting factors in pigmentation and not protein-binding *per se*.

The data obtained from this examination of the *in vitro* binding behaviour of carotenoids suggests that there is little discrimination between astaxanthin and

canthaxanthin in terms of their ability to bind to actin filaments isolated from Atlantic salmon. There is little difference in the capacity of isolated F-actin to bind either carotenoid, except in small (juvenile) or organically-reared fish (Table 2). It is not clear why actin isolated from organically-reared salmon (originally pigmented with carotenoids from the yeast *Phaffia rhodozyma*) should behave differently. The overall binding capacity of F-actin for carotenoids is also highest in smaller fish, although the overall efficiencies of binding are lower.

The binding characteristics of astaxanthin and canthaxanthin to F-actin are almost identical within a 'physiologically-relevant' range of pigmentation (i.e., the levels seen in F-actin isolated from pigmented whole fish) for both Atlantic salmon and Rainbow trout (data not shown). However, at much higher carotenoid concentrations in Rainbow trout saturation binding of either astaxanthin or canthaxanthin was not acheived, suggesting that substantial non-specific binding of carotenoid occurred (data not shown). In reconstitution studies, saturation binding capacities for astaxanthin to actin isolated from Atlantic salmon are in the range 1.3-3.4 μg carotenoid mg^{-1} protein (Table 2). These values are much larger than those seen in F-actin isolated from pigmented farmed Atlantic salmon (typically up to 0.25 μg carotenoid mg^{-1} protein). This indicates that the inherent capacity of isolated actin filaments to bind carotenoids is not, in itself, a limiting factor in the desposition of dietary carotenoids to the muscle of Atlantic salmon. Based on the properties of muscle proteins alone, it should theoretically be possible to significantly increase the amounts of bound carotenoid and overall pigmentation in farmed salmonids. However, factors such as the absorption, metabolism and transportation of dietary carotenoids are clearly much more important in influencing the deposition of carotenoids in the muscle of salmonids. Whilst the lipid content of the feed is an important factor in determining pigment deposition in farmed fish (e.g., Bjerkeng *et al*. 1997) , the high levels currently adopted in fish farming are not regarded as limiting. Similarly the age or size of the fish may influence deposition of pigments into the muscle of farmed fish (see Schiedt 1998). In this study we have shown that F-actin purified from juvenile Atlantic salmon can, *in vitro*, bind higher levels of carotenoid than older fish (Table 2). A similar observation was also made by Saha *et al*. (2005, 2006). In addition, actin prepared from juvenile fish displayed a higher saturation binding level for canthaxanthin than astaxanthin – a reverse of the situation seen in actin isolated from older Altantic salmon (Table 2). These differences between carotenoid binding behaviour in actin from juvenile and

older fish are small and not understood. However, it is worth noting that the ratios of carotenoid:protein achieved in these *in vitro* studies are considerably higher than those seen *in vivo* in either farmed or wild fish and outside a physiologically meaningful range.

In fresh fillets of Coho salmon the ratio of carotenoid : F-actin is much higher than that seen in either Atlantic salmon or Rainbow trout (Table 1) – which matches the *in vivo* state as the pigmenting efficiencies in Coho salmon are higher. However, the levels are well below the saturation binding levels seen in F-actin isolated from any salmonids and as such does not in itself explain why farmed Coho salmon have the ability to deposit far higher levels of carotenoid than other farmed salmonids. Unfortunately the F-actin preparations from Rainbow trout did not display saturation binding for either astaxanthin or canthaxanthin and the resulting pattern of binding was characteristic of significant non-specific binding of pigment to the protein. This is similar to the observations of Saha *et al.* (2006).

The behaviour of F-actin isolated from Atlantic salmon in binding exogenous carotenoids is consistent with the presence of a single binding site for carotenoids in the protein. Treatment of reconstituted F-actin with Triton X-100 had little overall effect on the carotenoid content of the complex (i.e., no carotenoid was released from the reconstituted pigment-protein complex) although total lipids were reduced by >50% (data not shown). The observation that no carotenoid was released from the binding site suggests that lipids either do not play a role in carotenoid binding *per se* or that only a fraction of the lipids are essential for such binding. Lipids are however known to be important in carotenoid uptake in salmonids (see Schiedt 1998).

Overall, the data derived from the reconstitution studies conducted here demonstrate that the intrinsic properties of F-actin (and its associated proteins such as α-actinin) are not a major factor in regulating the deposit of carotenoids in the muscle of farmed or wild fish. For example, canthaxanthin may be more effectively deposited than astaxanthin in Atlantic salmon (up to 2.7Kg weight; Kiessling *et al*. 2003) yet the isolated protein does not discriminate between these two carotenoids when each is delivered individually and indeed shows a preference for astaxathin when the two pigments are used together *in vitro*. In addition, the saturation binding levels observed for carotenoid binding to F-actin *in vitro* are far higher than those observed in pigmented fish. We conclude that the carotenoid binding site is not a major limiting factor in regulating pigmentation of the white muscle of in salmonids.

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Figure Legends

Figure 1 Structures of the carotenoids used in this study for reconstitution of F-actin.

Figure 2 Saturation binding curves for the binding of astaxanthin (•) and canthaxanthin (o) to F-actin purified from (**A**) large (~2.5 Kg FW) Atlantic salmon; (**B**) large (~2.5 Kg FW) organic Atlantic salmon; and, (**C**) small (~0.5 Kg FW) Atlantic salmon. Curves were fitted by non-linear regression using SigmaPlot (SPSS UK. Ltd.); mean ± S.E, n = 3-6. The Y-axis is shifted in order to improve clarity of the binding curve at low doses.

Figure 3 Competitive binding of astaxanthin and canthaxanthin in F-actin purified from large (~2.5 Kg FW) Atlantic salmon (mean ± SE, n=3). Ratio of astaxanthin : canthaxanthin bound to F-actin compared to the ratio of the two carotenoids delivered to the isolated protein.

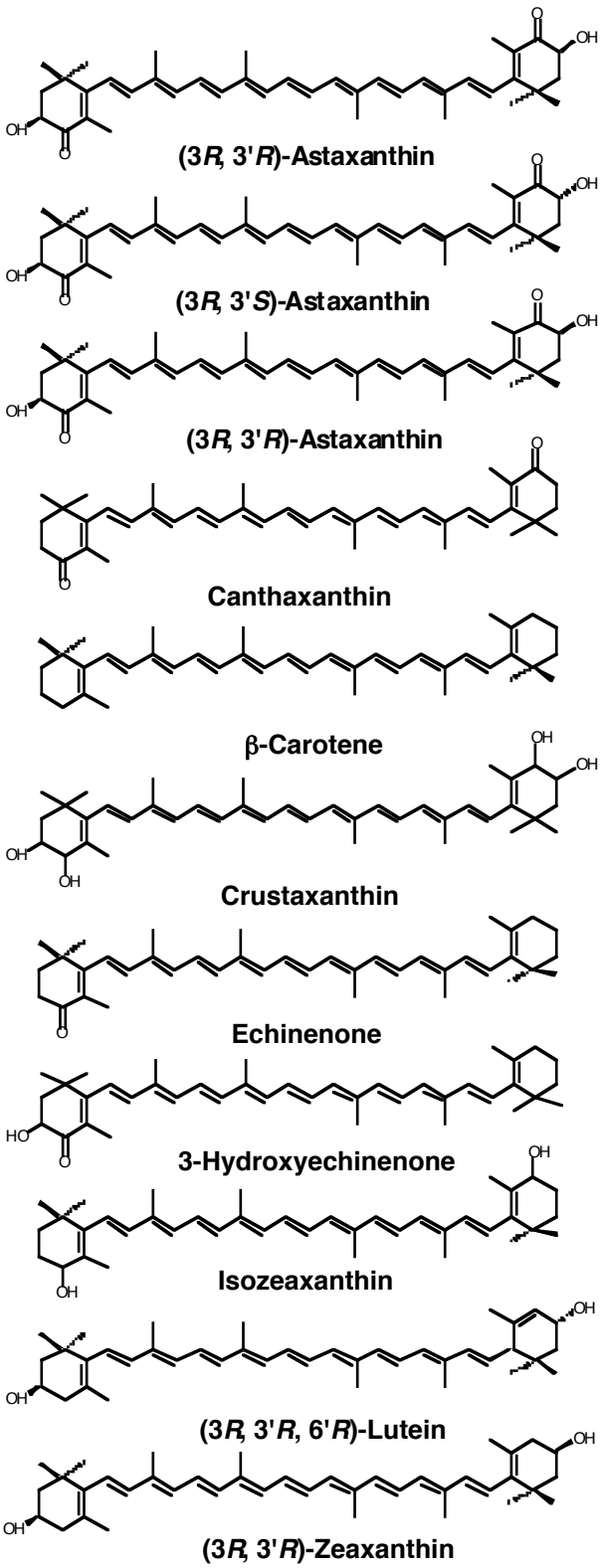
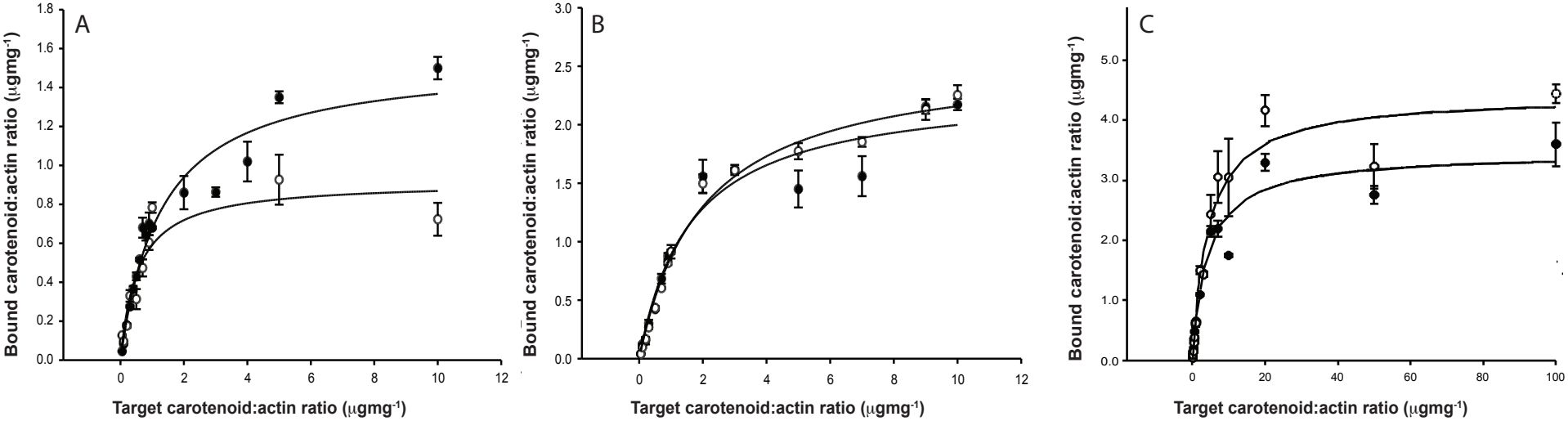

(3R, 3'R)-Astaxanthin
(3R, 3'S)-Astaxanthin
(3R, 3'R)-Astaxanthin
Canthaxanthin
β-Carotene
Crustaxanthin
Echinenone
3-Hydroxyechinenone
Isozeaxanthin
(3R, 3'R, 6'R)-Lutein
(3R, 3'R)-Zeaxanthin

Figure 1


A
B
C
Bound carotenoid:actin ratio (μgmg-1)
Target carotenoid:actin ratio (μgmg-1)

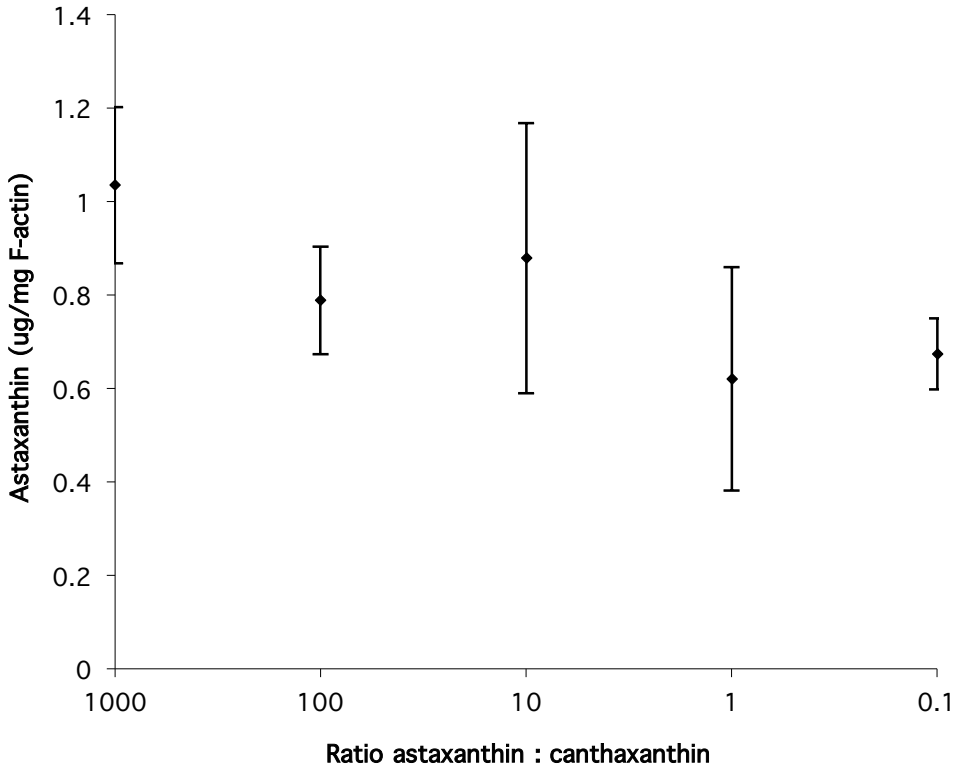

1.4
1.2
1
0.8
0.6
0.4
0.2
0
Astaxanthin (ug/mg F-actin)
1000
100
10
1
0.1
Ratio astaxanthin : canthaxanthin

Figure 3.