

Why are salmonids pink?

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Abstract: Pigmented flesh, a trait uniquely present in four of the 10 genera of salmonid fishes, is caused by the ubiquitous capacity of these genera for accumulating dietary carotenoids in muscle tissue. Here we combine life history reasoning with current knowledge of carotenoid metabolism to suggest a possible evolutionary scenario accounting for the emergence and maintenance of this phenotype. We claim that a basal carotenoid metabolism and a hormonally controlled targeted degradation of muscle tissue during maturation were established before the anadromous life style and that these proximal features were concomitantly exploited by natural selection in an ancestral anadromous and nest-making salmonid. Three different selection regimes are suggested to account for available empirical data concerning emergence, as well as maintenance, of the flesh pigmentation trait: a primary one driven by the need for enhanced somatic maintenance accompanying nest making under starving conditions, a secondary one driven by sexual selection, and a tertiary one driven by exposure to a meager carotenoid diet in nonanadromous freshwater populations. Finally, an experimental research program thought to be instrumental for fully resolving this evolutionary puzzle is suggested.

Résumé : La chair pigmentée, à cause de leur capacité générale à accumuler dans leurs muscles les caroténoïdes de leur régime alimentaire, est une caractéristique particulière retrouvée chez quatre des 10 genres de poissons salmonidés. Nous combinons ici une argumentation reliée au cycle biologique aux connaissances actuelles sur le métabolisme des caroténoïdes et nous suggérons un scénario d'évolution possible de l'apparition et du maintien de ce phénotype. Nous croyons qu'avant l'établissement du mode de vie anadrome, il s'est développé un métabolisme de base des caroténoïdes et une dégradation ciblée du tissu musculaire sous contrôle hormonal durant la maturation; ces caractéristiques proximales ont alors été exploitées conjointement par la sélection naturelle chez un salmonidé ancestral anadrome et constructeur de nid. Nous présentons trois régimes différents de sélection qui pourraient expliquer les données empiriques disponibles concernant l'apparition et le maintien du caractère de chair pigmentée : un régime primaire stimulé par la nécessité d'un maintien somatique accru reliée à la construction du nid dans des conditions de disette, un secondaire activé par la sélection sexuelle et un tertiaire régi par l'exposition à un régime alimentaire pauvre en caroténoïdes chez les populations d'eau douce non anadromes. Nous suggérons en terminant un programme de recherche expérimental que nous pensons devoir résoudre complètement cette énigme évolutive.

[Traduit par la Rédaction]

Introduction

All species in four out of 10 genera of the fish family Salmonidae are distinguished by the pinkish-red color of their flesh. This color is due to muscle accumulation of carotenoids present mainly in their crustacean prey. In fact, except for all salmon, trout, and char species belonging to the *Oncorhynchus*, *Salvelinus*, *Salmo*, and *Parahucho* genera, no marine or freshwater fish living in the Northern Hemisphere seem to possess this phenotype (Fig. 1).

The uniqueness of this phenomenon is astonishing considering that several marine fish species that have the same diet as these pigmented fish are known to possess very similar carotenoid uptake and utilization patterns and yet have unpigmented flesh. The commercial aspects associated with flesh color in farmed salmonids have given rise to a substantial body of literature dealing with carotenoid uptake and metabolism in fish (Torrissen et al. 1989; Bjerkeng et al. 1992; Aas et al. 2000). The available knowledge on carotenoid metabolism was recently translated into a dynamic model de-

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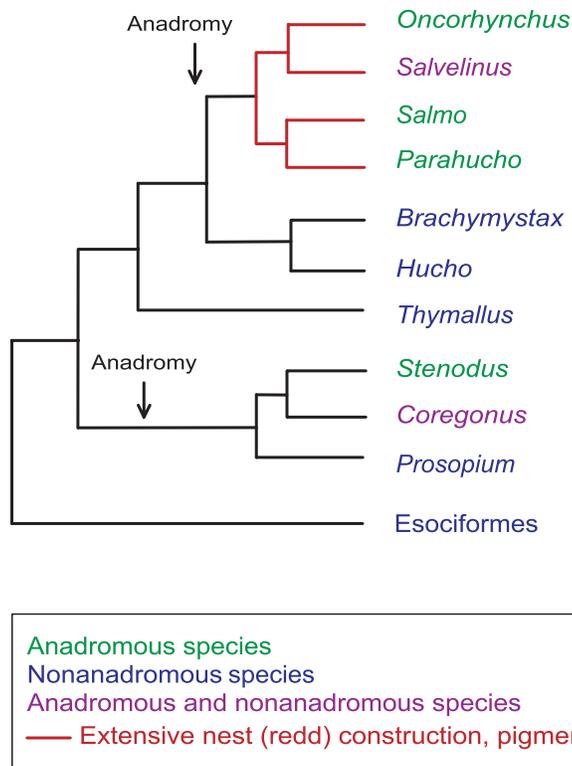
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Fig. 1. Sketch of salmoniform phylogeny, based on Ramsden et al. (2003). Esociformes and Salmoniformes are sister groups, diverging around 95 million years ago (MYA) (Johnson and Patterson 1996). The tetraploid event in salmonid evolution is estimated to have occurred 25–100 MYA (Allendorf and Thorgaard 1984) and took place after the divergence from Esociformes. The divergences among *Salmo*, *Salvelinus*, and *Oncorhynchus* probably occurred on the order of 20 MYA (Crespi and Teo 2002). The branch lengths are arbitrary, and the time scale for evolution of the different salmonid traits is to be interpreted in light of the duplication event.



scribing and analyzing the uptake, utilization, and deposition of carotenoids in Atlantic salmon (*Salmo salar*) (Rajasingh et al. 2006). However, surprisingly little attention has been given to the very interesting evolutionary issues attached to the flesh-pigmentation phenomenon.

The nearly ubiquitous capacity for accumulating carotenoids in all of the species of these four genera suggests that this trait is under positive selection. Here we combine life history reasoning with current knowledge of the proximate mechanisms involved in carotenoid metabolism to suggest a specific evolutionary scenario accounting for observed pigmentation patterns in anadromous and nonanadromous populations, and we also consider why muscle accumulation of carotenoids is specific to these four genera of salmonids. Moreover, we show how the flesh pigmentation can be used to make inferences concerning phylogenetic relationships within the Salmonidae and the monophyletic origin of anadromy within the group. Finally, by identifying the multi-layered selection context and the facilitating life history traits and proximal mechanisms possibly responsible for the evolutionary anatomy of flesh pigmentation, we provide an explanatory scaffold instrumental for identification of further

descriptive, experimental, and theoretical work required for fully resolving this evolutionary puzzle.

An evolutionary scenario accounting for observed flesh pigmentation in salmonids

In this section we outline what we think is the most probable evolutionary scenario in terms of what constituted the proximal evolutionary basis to be exploited and the three subsequent selection regimes that drove this exploitation. In the following sections we make a detailed validation of our evolutionary synthesis based on available data.

Proximal evolutionary basis

Two widespread physiological features in teleosts defined the proximal backdrop for the initial evolution of carotenoid deposition in the flesh: basic carotenoid metabolism and degradation of white muscle. A well-functioning carotenoid metabolism building upon fatty acid metabolism with regard to uptake over the intestinal wall, transport in the blood plasma by lipoprotein particles and serum albumin, and metabolism by the liver was established in the lineage leading to the Salmonidae long before the appearance of the anadromous life style. Likewise, targeted degradation of white muscle for energy during spawning was also present in this lineage previous to the emergence of anadromy.

Primary selection regime: somatic maintenance

Anadromy and redd (gravel nest for reproduction) making were established before muscle uptake of carotenoids became a fixed evolutionary trait. These factors together with previously established proteolytic machinery for targeted degradation of white muscle provided the life history setting favoring deposition of carotenoids in the flesh. The energy requirements for redd making and associated activities introduced a strengthened selection regime for targeted somatic maintenance of physiological and behavioral traits most vital to reproductive success, while using body tissues as the sole energy and nutrient source. Individuals capable of releasing carotenoids into the bloodstream from degrading muscle tissue were able to preserve vital functions under starvation better than those not possessing this trait.

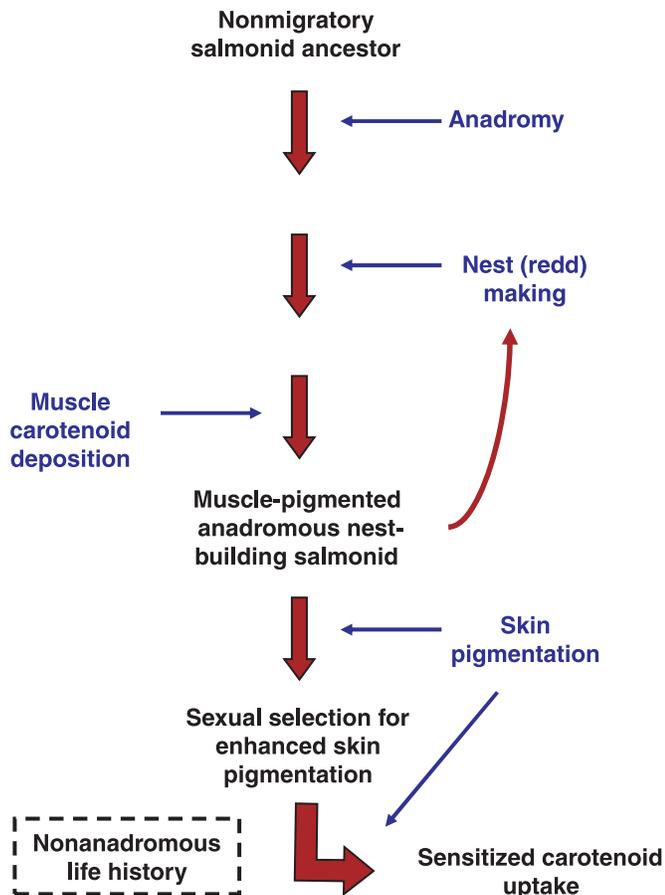
Secondary selection regime: accentuated reproductive traits

The presence of excess amounts of carotenoids in the bloodstream during the freshwater spawning phase provided the foundation for establishment, or substantial accentuation, of reproductive traits: enhanced egg pigmentation, heightened skin pigmentation, and associated mate choice preferences.

Tertiary selection regime: sensitization of uptake in landlocked populations

When becoming secondarily nonanadromous, most salmonid populations experienced much less access to carotenoid-rich prey, but there was still enough to be exploited by further sensitization of their pigment uptake system, thus preserving the above behavioral traits also in the nonanadromous condition. A schematic representation of all of these evolutionary stages is provided (Fig. 2).

Fig. 2. Outline of evolutionary scenario underlying carotenoid pigmentation of salmonid muscle. Selection regimes for four major traits (blue arrows) are suggested to have been responsible for the evolution (red block arrows) of the observed pigmentation characteristics within Salmoniformes. Starting to use the flesh as a sink for carotenoids in the sea and as a source in freshwater might have facilitated further elaboration of the redd-making strategy, as well as enhanced coloration of eggs (red arrow). In those populations already adapted to a strong sexual selection regime associated with skin pigmentation, the change from an anadromous to a nonanadromous life history regime caused further sensitization of the carotenoid uptake system in order to cope with lower levels of carotenoids in the diet.



Empirical rationale for the evolutionary scenario

Proximal evolutionary basis

First, carotenoid metabolism is a well-developed feature in a multitude of teleosts. The uptake in the intestine, transport in the blood, and metabolism in the liver of carotenoids closely follow those of fatty acids (Clevidence and Bieri 1993; Parker 1996). This basic carotenoid metabolic regime is thus most likely an evolutionary pathway far older than the capacity for muscle pigmentation. The oxygen-containing forms of carotenoids are classified as xanthophylls and have been found in varying levels in the skin, eggs, and ovaries of a wide range of marine (Atlantic halibut (*Hippoglossus hippoglossus*), Atlantic cod (*Gadus morhua*,

etc.) and freshwater (northern pike (*Esox lucius*), black goby (*Gobius niger*), goldfish (*Carassius auratus*), etc.) species (Goodwin 1984). The xanthophyll astaxanthin, along with its isomers, is one of the most widespread carotenoids in aquatic animals and the primary pigment in salmonids (Kanemitsu and Aoe 1958; Torrisen et al. 1989; Shahidi et al. 1998). Skin coloration as a result of pigment deposition operates as a nuptial signal and is usually observable during sexual maturation in either or both of the sexes (Goodwin 1984). In eggs, carotenoids are believed to be beneficial to the viability of incubating eggs and the growth and development of the fry (Craik 1985; Shahidi et al. 1998). Unlike in most mammals, xanthophylls have also been shown to act as precursors for vitamin A production in fish, most likely because fish possess a reductive pathway for xanthophyll metabolism (Goodwin 1986; Matsuno 1991), which mammals do not seem to possess. This would further explain the presence of xanthophylls in the eggs and skin of some fish species. However, except for those belonging to the above-mentioned four genera, none of these species has large amounts of pigment in its flesh.

Furthermore, binding of astaxanthin to muscle proteins is feasible even in generally nonpigmented fish like haddock (*Melanogrammus aeglefinus*) and Atlantic halibut, indicating that carotenoid-binding capacity is a trait shared by other fish species and thus cannot be the limiting factor in pigment deposition in muscle (Saha et al. 2006). The occurrence of red-fleshed Atlantic cod specimens (Baalsrud 1956; Bligh and Dyer 1959), although rare anomalies, further strengthens the argument that flesh pigmentation does not demand much evolutionary innovation in proximal terms for several species, but that lack of a sufficiently strong positive selection prevents further maintenance of the trait (see below for an elaboration of this).

The second major condition setting the stage for carotenoid deposition is targeted muscle degradation. Because of a negative nutrient and energy budget during sexual maturation, partly caused by reduced or ceased feeding, several fish species seem particularly prone to heavily exploit white muscle tissue to support development of gonads and eggs and for the formation of secondary sexual characteristics (Mommsen 1998, 2004). This phenomenon of targeted muscle tissue degradation and concomitant weight loss is seen in marine (Atlantic herring (*Clupea harengus*), Atlantic cod, Argentine hake (*Merluccius hubbsi*); Iles 1984; Montecchia et al. 1990; Hemre et al. 2002), freshwater (northern pike, roach (*Rutilus rutilus*), southern catfish (*Silurus meridionalis*); Diana and Mackay 1979; Koch and Wieser 1983; Xie et al. 1998), and anadromous (Pacific (*Oncorhynchus* species) and Atlantic (*Salmo salar*) salmon, American shad (*Alosa sapidissima*); Martin et al. 1993; Leonard and McCormick 1999; Mommsen 2004) species. Three different proteolytic systems present in muscle seem to be responsible for this targeted degradation: (i) the ubiquitin–proteasome complex, (ii) the calpains, and (iii) the lysosomal proteases, mainly cathepsin D (Mommsen 2004). These systems break down different parts of the muscle, acting under different physiological and stress conditions, such as starvation, cancer, uremia, etc. (Lecker et al. 2004). In mammals, proteasome is the major proteolytic system responsible for protein turnover in normal as well as pathological states, whereas calpains

are activated during fasting conditions. Although both of these pathways have been identified in fish, they do not seem to play a major role during spawning-associated muscular protein breakdown, at least not in the later stages (Mommsen 2004; Salem et al. 2006). In piscine species, the lysosomal enzymes are likely the key proteolytic system dealing with the mobilization of muscle protein. Controlled activation of these enzymes in the muscle, in particular the cathepsins, has been observed in a variety of spawning fish such as Pacific chum salmon (*Oncorhynchus keta*; Yamashita and Konagaya 1990), Atlantic salmon (von der Decken 1992), rainbow trout (*Oncorhynchus mykiss*; Salem et al. 2006), ayu (*Plecoglossus altivelis*; Toyohara et al. 1991), European pilchard (*Sardina pilchardus*; Gomez-Guillen and Batista 1997), Atlantic herring (Nielsen and Nielsen 2001), etc. Among the cathepsins, cathepsin D seems to have a major role, hydrolyzing the myosin, actin, and tropomyosin filament proteins (Nielsen and Nielsen 2001) of the white muscle. There are indications that the gene for fish cathepsin D might be receptive to estrogen (Carnevali and Maradonna 2003) and corticosteroids, given that its mammalian counterpart has responsive elements for these hormones (Cavaillès et al. 1993; Dardevet et al. 1995). This would imply that the lysosomal enzyme machinery is at least partly under control of hormonal systems responsible for initiating and directing sexual maturation processes in general.

This targeted degradation system connected to sexual maturation is likely to have been a key facilitating system for exploitation of carotenoids stored in the muscle during sexual maturation. In flesh-pigmented salmonids, astaxanthin is bound to the actomyosin filaments of the muscle tissues (Henmi et al. 1987, 1989; Matthews et al. 2006). When the myofibrillar proteins are degraded, carotenoids are released into the bloodstream and are picked up by high-density lipoprotein (HDL) molecules in the blood (Torrissen and Torrissen 1985; Bjerkgeng et al. 1992). The muscle thus acts as a “sink” for carotenoid deposition during the marine growth phase and as a “source” during the freshwater maturation stage. Because of the already established muscle degradation system associated with sexual maturation, no additional evolutionary innovation seems to have been needed to ensure release of muscle-bound astaxanthin at this stage in development. The sexual nature of this phenomenon is strongly supported by the facts that immature Atlantic salmon have to be starved for up to 3 months before muscle-bound astaxanthin starts to be released into the bloodstream owing to breakdown of muscle tissue (Choubert 1985), whereas juvenile chum salmon when treated with methyltestosterone experience rapid muscle degradation and concomitant pigment release (Ando et al. 1986b).

The above considerations suggest that carotenoid deposition in eggs and skin, utilization of the pigments for vitamin A production, and the actual binding of astaxanthin within the muscle cells are characteristics not adaptively linked to muscle pigmentation. However, together with a pre-established hormonally controlled degradation system specifically targeting white muscle during sexual maturation, these traits provided the prerequisites for an ancestral salmonid species to directly exploit the benefits associated with muscle uptake of carotenoids.

Primary selection regime: improved somatic maintenance

During the targeted muscular degradation process outlined above, there is an accompanying increase in free-radical production, mainly in the form of reactive oxygen species (ROS) (Guderley et al. 2003). ROS are highly reactive intermediate compounds, such as superoxide anion, hydroxyl radicals, and peroxides, that are created during normal metabolic activities such as mitochondrial respiration and detoxification and perform a number of functions in processes such as cell signaling, regulation, apoptosis, etc. (Droge 2002). However, when ROS exceed their normal levels, they create oxidative stress resulting in the oxidation of proteins, DNA, and other cellular components and the peroxidation of cell membrane lipids, which ultimately lead to cell damage and death. In biological organisms, ROS are controlled by a well-developed antioxidant system consisting of extracellular molecules (vitamin C, vitamin E, carotenoids, etc.) and several specialized endogenous enzyme systems (the glutathione family of enzymes, superoxide dismutase (SOD), catalase (CAT), etc.) that prevent or reduce free-radical production and tissue damage (Martinez-Alvarez et al. 2005). Food deprivation and breakdown of tissues are known to generate oxidative stress (Guderley et al. 2003; Morales et al. 2004). In cod, the antioxidant enzyme levels either increase or remain constant in the white muscle and liver, even during an extensive period of starvation (Guderley et al. 2003). This suggests that spawning-related targeted muscle degradation does indeed pose an additional oxidative stress load large enough to have caused the evolution of compensatory processes at least in iteroparous fish. Based on the fact that the targeted muscle degradation strategy is shared by a large number of species, one would expect that the basal antioxidant systems in these species have frequently been tuned by natural selection to respond in the same way as observed in cod. This would in any case suggest that upregulation of endogenous antioxidant systems in connection with targeted proteolysis was well established in ancestral salmonids long before muscle deposition of carotenoids arose as a trait.

Astaxanthin is a very strong antioxidant, effectively quenching singlet oxygen, scavenging free radicals, and preventing lipid peroxidation (Miki 1991; Shimidzu et al. 1996). It has a molecular structure containing hydroxyl (OH) and keto (C=O) groups at the terminal rings of the carbon chain, and this, together with its lipophilic nature, gives the pigment its high antioxidant properties (Hussein et al. 2006). Astaxanthin has been shown to scavenge peroxy radicals in phospholipid cell membranes and so break the propagation of radical chain reactions (Lim et al. 1992; Palozza and Krinsky 1992; Goto et al. 2001). This prevents further oxidation of the polyunsaturated fatty acids in the membrane, consequently protecting the cell membrane from destruction.

As astaxanthin is such a powerful antioxidant, it is natural to ask whether its release from muscle during sexual maturation in salmonids compensates for the antioxidant up-tuning similar to that observed in cod or whether it adds to the ROS defense machinery, especially in maintaining tissue integrity. Unfortunately, the paucity of empirical data prevents us from settling this issue in a straightforward manner. However, considering that white-fleshed anadromous salmonids, anadromous nonsalmonids, and marine species (Czeczuga 1975;

Goodwin 1984; Czczuga and Czczuga-Semieniuk 2000) all have well-established carotenoid metabolisms without including carotenoid deposition in muscle suggests that this trait was at least not directly selected for as a less costly solution to neutralizing ROS formed during white muscle proteolysis. The fact that the biochemical roles played by dietary antioxidants viz. carotenoids and the endogenous enzymes are fairly different (Cadenas 1995) may also have contributed to the prevention of a compensatory selection regime.

Presuming then that the selection causing fixation of the trait of pigment deposition in muscle was not connected to ROS scavenging, one has to seek explanations based on observed life history characteristics distinguishing the four focal salmonid genera from other genera. Intuitively one may think that the anadromous condition itself involving strenuous river migrations under fasting conditions would qualify to be such a criterion. However, there are a large number of nonpigmented fish species that undertake spawning migrations (either anadromous or catadromous) under equally rigorous starving conditions and, in some cases, even exhibit semelparity (McDowall 1988) (Table 1). For example, the unpigmented American shad has been observed to expend 35%–60% of its energy during spawning migration while still managing to spawn and return (Leonard and McCormick 1999). Furthermore, flesh-pigmented salmonids show considerable inter- and intra-species variability with regard to migratory distances (Quinn 2005), which indicates that the energy expenditure associated with anadromy varies substantially. Based on this we rule out anadromy as the *raison d'être* for muscular pigment uptake in salmonids. Hence, the four genera of pink-fleshed salmonids must have some unique aspect to their life history that differs from other anadromous nonpigmented fish and where the availability of carotenoids would cause a fitness difference.

To the best of our knowledge, the major feature that sets them apart from unpigmented salmonid and nonsalmonid anadromous species is their possession of a more complex mating system centered around extensive redd construction (Quinn 2005), which is associated with considerable energy expenditure for both males and females (Fleming 1998). Females must select or take a suitable site, defend the site, dig nests, assess quality of courting males, spawn, cover the fertilized eggs with gravel, and (in semelparous species) defend the completed redd until they die (Fleming and Reynolds 2004; Quinn 2005). Males must compete or sneak for access to females (Fleming 1998). Also males arrive at the spawning grounds before females and stay there for a longer total period of time (Morbey 2000). Because males rarely run out of gametes, reproductive success is not limited by available gametes but by the ability to successfully court and spawn with females (Quinn 2005). Both sexes may need to avoid terrestrial predators during this entire process. In some populations of sockeye salmon in the Wood River Lakes system in Alaska, more than 50% of adult salmon are killed by brown bears (*Ursus arctos*; Quinn et al. 2003).

Unpigmented salmonid and nonsalmonid anadromous species are in general non-nesting, broadcast spawners (Table 1). An exception is the huchen (*Hucho hucho*), a salmonid closely related to the pigmented genera (Fig. 1), which digs a shallow hole in the gravel, indicating primitive redd-making behavior (Holcik 1995). An adequate supply of

oxygenated water in the nest is of vital importance for the successful incubation of eggs and alevins to emergent fry (Rombough 1988; Groot and Margolis 1991; Quinn 2005). However, the presence of white-fleshed Chinook salmon (*Oncorhynchus tshawytscha*) populations in several rivers in southeastern Alaska and British Columbia (Hard et al. 1989) showing apparently no behavioral differences from pigmented Chinook populations as related to redd making strongly suggests that an increased astaxanthin egg content beyond the basal uptake acquired while in the sea is not necessary for the establishment of redd construction. The fact that extensive redd-making behavior is only observed in salmonids that store carotenoids in their flesh is, of course, no proof that there is a strong evolutionary association between these two traits. However, if there is such an association, we argue that the strategy of pigment deposition in muscle was built upon a (not necessarily fully refined) redd-making strategy, and not the other way around. If there is a connection, it is likely to be based on the extra energy requirements associated with redd making and accompanying territorial behaviors. Unfortunately, we do not have empirical data of the energy expenditure of flesh-pigmented salmonids compared with that of other similar anadromous nonpigmented species in the spawning period. However, considering that the two groups seem to deplete muscle reserves at about the same levels (Leonard and McCormick 1999), this suggests that there is a limit to how much muscle tissue that can be exploited while still leaving the individual capable of reproduction. It is thus reasonable to suggest that the physically demanding reproductive behavior observed in pigmented salmonids has to be fueled by tissues or organs other than muscle. In such a situation, excess carotenoid release into the bloodstream may represent an efficient way to counteract the additional oxidative stress and other negative effects on somatic maintenance functions. In other terms, an individual capable of releasing carotenoids into the bloodstream may be able to maintain the integrity of general, as well as more specific, biological processes in tissues and (or) organs better or for a longer time than individuals that do not. There is some circumstantial evidence for this. Carotenoids are known to aid in shielding a variety of vital body functions such as vision and immunity. In Atlantic salmon, astaxanthin has been shown to protect against cataract formation (Waagbo et al. 2003) and increase resistance to disease pathogens (Christiansen et al. 1995). In rainbow trout, astaxanthin and beta-carotene have been found to increase serum complement and phagocytic activity of kidney leukocytes, as well as upregulate the nonspecific cytotoxicity of lymphocytes (Amar et al. 2001).

The above explanation applies to iteroparous as well as semelparous species as both groups will benefit from being able to better maintain somatic integrity. The ancestral species of the oncorhynchid genus, containing the present-day semelparous species, is assumed to have been a repeat spawner (Finch 1994), and the flesh-pigmentation trait was presumably already present. It is easy to envisage a gradual change to semelparity while still making active use of deposited carotenoids.

The fact that these four genera of salmonids possess this trait signifies that deposition of carotenoids in the muscle has been under positive selection for a very long time. There

Table 1. Migration- and spawning-related life history traits of certain examples of anadromous species taken from different fish families

Name	Migration	Feeding during migration	Flesh pigmentation	Extensive redd construction	Eggs brightly pigmented	Accentuated skin pigmentation	Type of anadromy
Family Salmonidae							
Atlantic salmon (<i>Salmo salar</i>)	Ocean–freshwater	No	Yes	Yes	Yes	Yes	Iteroparous
Pacific salmon (<i>Oncorhynchus</i> species)	Ocean–freshwater	No	Yes (except for white-fleshed Chinook)	Yes	Yes	Yes	Semelparous
Atlantic whitefish (<i>Coregonus huntsmani</i>), Arctic cisco (<i>Coregonus autumnalis</i>)	Coastal water – freshwater	No	No	No	Yes	No	Iteroparous
Inconnu (<i>Stenodus leucichthys</i>)	Coastal water – freshwater	No	No	No	No	No	Iteroparous
Japanese huchen (<i>Parahucho perryi</i>)	Ocean–freshwater	No	Yes	Yes	Yes	Yes	Iteroparous
Family Osmeridae							
Rainbow smelt (<i>Osmerus mordax</i>)	Coastal water – freshwater	No	No	No	No	No	Iteroparous
Eulachon (<i>Thaleichthys pacificus</i>)	Coastal water – freshwater	No	No	No	No	No	Semelparous
Family Acipenseridae							
Atlantic sturgeon (<i>Acipenser oxyrinchus</i>)	Ocean–freshwater	No	No	No	No	No	Iteroparous
Family Salangidae							
Japanese icefish (<i>Salangichthys microdon</i>)	Coastal water – freshwater	n/a	No	No	No	No	Semelparous
Family Retropinnidae							
Cucumberfish (<i>Retropinna retropinna</i>)	Ocean–freshwater	No	No	No	No	No	Semelparous
Family Aplocheilichthyidae							
Tasmanian whitebait (<i>Lovetia sealii</i>)	Ocean–freshwater	No	No	No	No	No	Semelparous
Family Clupeidae							
Alewife (<i>Alosa pseudoharengus</i>)	Ocean–freshwater	No	No	No	No	No	Iteroparous
Atlantic or American shad (<i>Alosa sapidissima</i>)	Ocean–freshwater	No	No	No	No	Yes	Iteroparous
Family Percichthyidae							
Stripped bass (<i>Morone saxatilis</i>)	Coastal water – freshwater	Yes	No	No	No	No	Iteroparous
Family Gadidae							
Atlantic tomcod (<i>Microgadus tomcod</i>)	Coastal water – freshwater	n/a	No	No	No	No	Iteroparous

Note: Information obtained from FishBase (<http://www.fishbase.org/>), McDowall (1988), Morrow (1980), and Scott and Crossman (1975). n/a, not available.

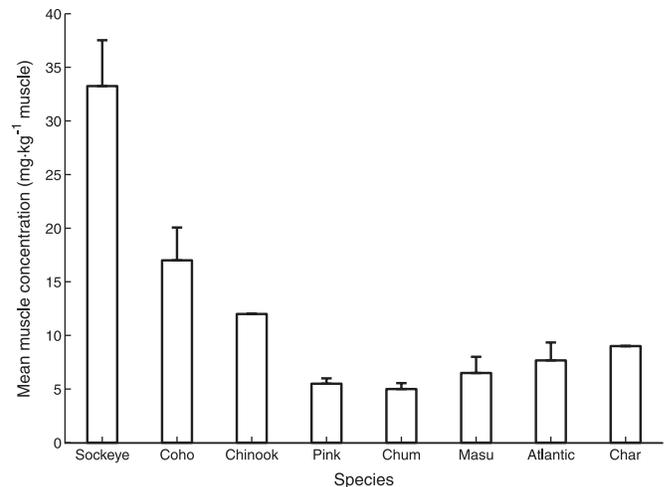
are large inter- and intra-species variations in levels of pigment deposition in muscle (Kitahara 1984; Torrissen et al. 1989) (Fig. 3), but the trait as such is nevertheless ubiquitously present in these genera. This variation probably reflects adaptations to a variety of biotic and abiotic conditions, but in addition to the somatic maintenance aspect, we think that accentuated evolution of the reproductive traits dealt with in the next section have been particularly important.

Secondary selection regime: accentuated reproductive traits

The intensity of egg pigmentation seems to follow the intensity of flesh pigmentation (Craik 1985) and is most probably caused by a greater supply of carotenoids and not deliberately evolved mechanisms. Even though we argue that somatic maintenance issues provided the primary selection setting for fixation of carotenoid deposition in the flesh, it could also be that an increased uptake of astaxanthin into eggs owing to the release from degrading flesh also provided an important early selective advantage (and thus qualifying to be part of the primary selection regime). A widely held hypothesis is that oxygen consumption increases proportionally with egg volume and that smaller eggs are selected for under conditions with limited oxygen availability, i.e., in finer gravel (Quinn et al. 1995; Hendry et al. 2001). However, large salmonid eggs have indeed been shown to have higher survival rates in oxygen-poor conditions than smaller ones (Einum et al. 2002). If these experimental results are confirmed, they suggest that larger egg size was selected for in the redd-making phase previous to the appearance of flesh pigmentation. An increased carotenoid content in the enlarged egg could then have increased survival probability due to various beneficial effects on somatic processes at various developmental stages. In this connection it is worthwhile to observe that the European grayling (*Thymallus thymallus*), a nonanadromous nonpigmented salmonid exhibits primitive nest building, whereas its sister species, the Arctic grayling (*Thymallus arcticus*), spawns over the gravel substrate without digging a redd (Breder and Rosen 1966). If the European grayling turns out to have higher amounts of carotenoids in its eggs than the Arctic grayling, this would strongly imply that the nest-making strategy causes selection for increased carotenoid uptake in the egg, and it would emphasize the importance of the egg in connection with fixation of the strategy of pigment deposition in muscle.

However, it should be noted that there is still no consensus concerning the exact benefits of having a large carotenoid content in the egg with respect to fertilization rates and egg and alevin survival and fitness (Christiansen and Torrissen 1997; Pettersson and Lignell 1999; Ahmadi et al. 2006). Astaxanthin released from the muscle is transported to the ovaries by vitellogenin (Ando et al. 1986a) and is found to bind to the yolk protein lipovitellin in the eggs (Ando and Hatano 1986), with both vitellogenin and lipovitellin being essential egg constituents. It can be argued that because the chemical characteristics of vitellogenin and lipovitellin are under positive selection for other reasons, astaxanthin present in the bloodstream will become imported into the egg out of biological necessity. In this situation, egg pigmentation would be an epiphenomenal trait of neither positive nor negative selective value. Even given that there

Fig. 3. Mean astaxanthin levels in the muscle of prespawning wild Pacific salmon species (genus *Oncorhynchus*), Atlantic salmon (*Salmo salar*), and Arctic char (*Salvelinus alpinus*) (Crozier 1970; Kitahara 1983, 1984, 1985; Skrede and Storebakken 1986; Scalia et al. 1989; Ando et al. 1992; Turujman et al. 1997). The standard error was calculated in those cases where measurements of several individuals were available. As the bar graph is made from several independent studies, it should be interpreted with some caution. Species: sockeye, *Oncorhynchus nerka*; coho, *Oncorhynchus kisutch*; Chinook, *Oncorhynchus tshawytscha*; pink, *Oncorhynchus gorbuscha*; chum, *Oncorhynchus keta*; masu, *Oncorhynchus masou*.



are direct benefits to having carotenoids in the eggs, threshold levels could exist for the amount required for improved egg survival and fitness, with the additional pigment content being of no biological value. All or parts of the observed egg pigmentation patterns in flesh-pigmented salmonids, as well as in other fish, could thus essentially represent exaptations (Gould and Vrba 1982). It has to be acknowledged that the jury is still out on this issue.

We claim that the release of large amounts of carotenoids into the bloodstream during sexual maturation made possible the activation of a sexual selection regime connected to skin coloration, perhaps through pre-existing sensory bias for the color red (Basolo 1990; Rodd et al. 2002). The skin contains a much larger variety of carotenoid pigments than the flesh (Kitahara 1983). Astaxanthin in the skin is predominantly found in the esterified form, along with some of its metabolites, e.g., lutein, zeaxanthin, idoxanthin, etc. (Schiedt et al. 1986; Matsuno 1991; Bjerkeng et al. 2000). The esterification process is believed to take place once the pigments enter the skin tissues, as the astaxanthin in the plasma and the muscle is in the free form (Hata and Hata 1975; Torrissen et al. 1989). The carotenoid is deposited in the chromatophores, specifically in the erythrophores (Steven 1948, 1949). It is also thought to bind to the melanin found in the dorsal cutis (Torrissen and Ingebrigtsen 1992). At present we have very little knowledge about the underlying proximal changes in skin uptake and utilization of carotenoids (as well as other changes) that are likely to have accompanied the unfolding of the anticipated sexual selection regime. However, there is a clear connection between astaxanthin concentration in the flesh and the degree of skin coloration during spawning, as the pigment from the muscle

is transferred to the skin (Kitahara 1983; Bjerkeng et al. 1992; Craig and Foote 2001). This indicates that selection for increased skin coloration has been responsible for sensitizing the astaxanthin-uptake system. Skin pigmentation is believed to signal higher disease resistance and antioxidant functions, as well as higher sperm quality (Milinski and Bakker 1990; von Schantz et al. 1999; Skarstein et al. 2001). In sockeye salmon (*Oncorhynchus nerka*), it has been shown that fish exhibiting a breeding color are more successful in intersexual selection than unpigmented individuals (Craig and Foote 2001; Foote et al. 2004). Another aspect concerning selection for skin color as a secondary sexual characteristic is the part that it plays in male–male competition. The color red is known to be an agonistic signal in male birds with regard to territorial defense (Pryke et al. 2001), and carotenoid-based coloration is viewed as a good indicator of fighting ability in fish and birds (Evans and Norris 1996; Godin and Dugatkin 1996; Pryke et al. 2001).

Although carotenoid-based secondary sexual colors are seen in many fish species (Goodwin 1984), the use of the muscle tissue as a sink and later source for astaxanthin has made it possible for anadromous salmonids to develop astonishing skin pigmentations even under food-deprived conditions. In conclusion, we suggest that carotenoid deposition in the muscle tissues made it possible to further exploit adaptational options associated with redd making, as well as providing the proximal basis for elaborate sexual selection regimes based on extensive skin coloration.

Tertiary selection regime: sensitization of uptake in nonanadromous populations

A third selection regime arose when certain salmonid species became nonanadromous, by either becoming landlocked or remaining in the freshwater lakes instead of migrating to saltwater. One common example of such morphs is sockeye salmon, which exists in both the anadromous (sockeye) and nonanadromous (kokanee) forms, often spawning in the same river and lake systems (Groot and Margolis 1991). They are assumed to have diverged genetically in sympatry, with the sockeye being the ancestral form (Wood and Foote 1996), and this has happened independently in many locations. As they inhabit freshwater lakes, kokanee have a carotenoid-poor diet (Gross et al. 1988). Both sockeye and kokanee change their skin color when they mature and migrate to their spawning sites (Groot and Margolis 1991). Kokanee are able to exhibit the same bright red breeding color by being threefold more efficient at taking up and storing the obtainable carotenoids compared with sockeye living in a similar carotenoid-poor environment (Craig and Foote 2001). Consequently, progeny of sockeye that remain in freshwater (“residuals”) turn olive green at maturity, not red. Kokanee are assumed to have evolved from sockeye via these residuals after the last ice age (Ricker 1940). This suggests that kokanee and sockeye have converged on the same red phenotype through genetic divergence concerning carotenoid utilization efficiency (Craig and Foote 2001; Foote et al. 2004; Craig et al. 2005) and that there was, in proximal terms, still room for a dramatic sensitization of the astaxanthin-uptake and -utilization systems in sockeye, even though it was already superior among pigmented salmonids. However, there are nonanadromous oncorhynchid morphs in

which sensitization of the pigment uptake does not seem to have occurred. Rainbow trout can be found in both the anadromous and nonanadromous forms (called steelhead), with the nonanadromous trout often having lower pigment levels in the flesh compared with their anadromous counterparts. As the anadromous rainbow trout does not exhibit the bright red nuptial coloration that sockeye does, this would suggest that in this case nonanadromy did not invoke a selection regime for increased sensitization because the skin color associated selection was less predominant.

Specific challenges and unresolved issues

A significant biological exception that does not seem to fit with our explanation is the existence of certain subpopulations of Chinook salmon that exhibit no or very little flesh pigmentation and are known as white-fleshed Chinook, in contrast to the more abundant red-fleshed variety (Milne 1964). The color dichotomy is highly heritable and the inheritance follows to a considerable degree a simple Mendelian two-locus model (Withler 1986). During maturation, the eggs and skin contain pigments, though in much smaller levels than in the red-fleshed variety (Hard et al. 1989; Ando et al. 1992). The flesh also contains small levels of pigmentation in a large proportion of cases (Ando et al. 1992). This suggests that the “white” subpopulations metabolize and utilize carotenoids in much the same way as the “red” ones, but that the ability to accumulate astaxanthin in muscle is substantially reduced. White-fleshed Chinook stocks are not randomly distributed across the species range but spawn mainly in the transboundary rivers of southeastern Alaska and northern British Columbia (Hard et al. 1989). They are seemingly restricted only to coastal areas and spawn in coastal or lower river tributaries, whereas the red Chinook spawn in the upper parts of rivers (Milne 1964). Furthermore, on maturation, the white-fleshed variety enters freshwater in the fall, whereas the red-fleshed variety migrates in summer (Milne 1964). This appears to suggest that they are “ocean-type” Chinook populations and not “stream-type”, which are the two behavioral forms of the species. In contrast to the stream-type populations, ocean-type Chinook are restricted to certain geographical regions and spend most of their life in coastal waters (Healey 1983, 1991). In coastal waters, these Chinook feed mainly on fish such as Pacific sand lance (*Ammodytes hexapterus*), Pacific herring (*Clupea pallasii*), and northern anchovy (*Engraulis mordax*), with crustaceans such as euphausiids forming only a small part of their diet (Healey 1991). Unlike the stream-type, which return in the spring or summer several months before actually spawning, they return to freshwater in the fall, only a few days or weeks before spawning. It has also been suggested that in regions where stream- and ocean-type populations are sympatric, they spawn at different times (Healey 1991). There are no studies determining whether white Chinook populations only exhibit the ocean-type life history. If they do, this may explain why they seem to survive so well relative to the red-fleshed individuals, i.e., because of a greatly reduced freshwater spawning migration and less interpopulation mingling and competition as a result of spatial and (or) temporal separation of spawning. Hence, though flesh pigmentation as a trait was more important in

the past, because of evolved behavioral compensation, the positive selection may have become somewhat relaxed in the white Chinook. However, whether the evolution of this compensatory behavior has been driven by the reduced flesh pigmentation is an open issue. In further support of this, all Chinook salmon prefer spawning microhabitats with high levels of subgravel flow (Healey 1991), e.g., before crests of rapids and below log jams (Vronskiy 1972; Russell et al. 1983). Owing to this preference, it is possible that selection for high carotenoid content eggs is relaxed to a certain degree because available dissolved oxygen in the incubating substrate is high.

White Chinook have been found to be larger in size than the red-fleshed fish (Milne 1964; Withler 1986), indicating greater levels of fecundity and survival fitness. This has led to the suggestion that red flesh occurs at a cost and that there might be a trade-off between fish size at maturity and flesh pigmentation, with the pigmentation trait being selected for in most environments (Hard et al. 1989). However, the white Chinook enter freshwater just before spawning, whereas the red variety spends a longer period in freshwater prior to spawning. A more parsimonious explanation for the difference in sizes would thus be that given their ocean-type life history, white Chinook spend more time feeding in the ocean than the red variety.

The cost issue can be approached in more general terms by considering some of the proximal processes involved. Dietary carotenoid uptake, fatty acid associated transport in the plasma, the ability to bind to muscle tissues, targeted degradation of the muscle during maturation, and uptake in the skin and eggs are all pre-established traits where the costs have already been paid. Moreover, astaxanthin metabolism is based on fatty acid metabolism, and the amount of astaxanthin taken up and processed during the lifetime of an individual is completely dwarfed by the amount of other fatty acids (Sheridan 1988) processed by the same machinery. The only exception to this, as far as we understand the system today, might be the existence of a specific receptor complex in the muscle membrane that only takes up astaxanthin. Compared with the number of receptor complexes needed to handle the total fatty acid traffic over the muscle membrane, the presence of this specific complex can hardly be visible in the cost budget associated with fatty acid metabolism. If it turns out that flesh pigmentation is associated with a high cost, it has thus to involve energetically demanding biochemical processes in the muscle membrane or cytoplasm specific to astaxanthin. As there is absolutely no hint of the presence of such mechanisms in the literature, we claim that there is no exclusive cost of evolutionary importance associated with the flesh-pigmentation trait in salmonids. However, this may not be true for other fish species not making redds. The possibility of a negative cost being associated with enhanced pigmentation uptake in the eggs cannot be ruled out. In marine- and freshwater-spawning species that are open water or substratum spawners, brightly pigmented eggs caused by the mechanism outlined above could cause an additional cost in terms of egg predation if the egg color is already an important visual signal for the predator. This is, of course, highly speculative but could at least reconcile our claims that the muscle uptake mechanism did not demand much evolutionary inno-

vation or involve much cost in redd-making salmonids and that red-fleshed individuals are not more commonly observed in, for example, cod (Baalsrud 1956; Bligh and Dyer 1959) or other fish species with a well-developed carotenoid metabolism.

According to our explanation, the relative fitness of white Chinook should be lower than that of red chinook as long as they maintain redd making, which they seemingly do. It has indeed been observed in some cases that red-fleshed Chinook have higher levels of fecundity (Godfrey 1968), but compensatory behavior (see above) may have made the difference less pronounced over time. Irrespective of this, the fact that the fitness differences do not seem to be dramatic strongly suggests that when not preceded by a long freshwater migration, redd making could very well have been established with only moderate carotenoid levels in the eggs. In fact, nature's own "Chinook experiment" provides the only solid empirical support for this hypothesis.

There are also other reported occurrences of non-pigmented populations within the four pigmented genera. In lake trout (*Salvelinus namaycush*), the flesh might vary from white to orange in color (Morrow 1980), and Atlantic salmon residing in the Baltic Sea are also known to possess white or very palely tinged flesh in certain cases. The latter is known to base its diet mainly on fish (Pettersson and Lignell 1999), and it is likely that the variation observed in lake trout is also due to variation in carotenoid availability. Hence, except for the white Chinook, fish that are living under conditions resulting in the white-fleshed phenotype have seemingly not been reported to lose their ability to accumulate the pigment in the flesh when becoming exposed to a more carotenoid-rich diet. This may indicate that the uptake mechanism in the muscle membrane also serves other positively selected functions and that the capacity for pigment uptake started initially as just an associated artifact. We have sound reasons to assume though that the uptake system has been molded by natural selection in connection both with sexual selection for skin pigmentation (see above) and with sensitizing the kokanee to lower astaxanthin availability in the diet. The latter phenomenon probably characterizes many non-anadromous populations. Thus, once established, it can confidently be claimed that the astaxanthin-uptake mechanism has been under positive selection. However, it cannot be excluded that the initial fixation of the uptake mechanism might have been based on selection for a process that caused muscle uptake of pigment as a side effect. Flesh pigmentation would, in this case, have started as an exaptation (Gould and Vrba 1982) before becoming an adaptation. But such alternative processes are not easy to identify.

Considering the evolution of muscular accumulation of carotenoids as a trait in certain salmonid genera makes it also possible to further elucidate the evolutionary history within the Salmonidae family. It has been suggested that anadromy arose independently in the genera *Salmo* and *Oncorhynchus* (Oakley and Phillips 1999). Others have suggested that though some form of anadromy may be ancestral, the elaboration of anadromy involving long migrations, returning to natal streams to reproduce, and semelparity or a very low degree of iteroparity evolved in parallel (Crespi and Fulton 2004). If the former scenario is true, then redd making and the muscle uptake and subsequent exploitation

of carotenoids must also have evolved in parallel in the two genera (Fig. 1). For reasons of parsimony, we find this scenario rather improbable compared with the latter. Based on the above life history reasoning associated with the flesh-pigmentation trait, a more likely explanation is thus that (a probably less polished version of) anadromy, redd making, and the capacity for muscle pigmentation were established in their common ancestor. This is also in agreement with the proposition of Ramsden et al. (2003).

The question of whether the salmonid ancestor had a marine or freshwater origin before taking up the anadromous lifestyle is under much debate due to lack of conclusive empirical data (McDowall 2001). Our suggested evolutionary scenario is in principle insensitive to the type of origin and thus cannot bring any new insight to the table.

Issues for further research

There are several unresolved issues of proximal, as well as ultimate, character that need to be settled before we can pretend to have reasonably solid empirical support for our evolutionary scenario. This opens the door for experimentation that combines proximal and ultimate perspectives to test the hypotheses that we presented above. Some possible avenues of study are suggested below.

1. Which molecular machinery is responsible for the transport of carotenoids over the muscle membrane and what was the key molecular innovation compared with fatty acid uptake mechanisms present in other fishes?
2. What are the molecular changes that account for the sensitization of the carotenoid uptake between Atlantic salmon and sockeye and between sockeye and kokanee?
3. Are there differences between the antioxidant machineries active in spawning flesh-pigmented salmonids and other fish species?
4. What are the mechanisms involved in maintenance of somatic functions by astaxanthin during migration and spawning? (a) Are there physiologically or morphologically measurable differences between severely physically stressed individuals with and without flesh pigmentation (possibly hormonally induced sexual maturation to initiate targeted reduction of white muscle)? (b) Which reproductive behavioral traits are influenced by these differences? (c) What are the underlying molecular mechanisms involved?
5. Is egg coloration adaptive? (a) What is the relation between egg coloration and egg size within and among species? (b) How does astaxanthin in the egg influence egg survival for various egg sizes under various oxygenation regimes? (c) What is the relationship between egg and early-development vitamin A demand and initial astaxanthin content in the egg? (d) How does the antioxidant capacity of astaxanthin influence egg survival and are there also other prominent beneficial properties of the pigment? (e) Is egg color as a predation signal bringing with it a negative cost for species not constructing nests, i.e., are pigmented eggs more vulnerable to egg predators?
6. Does egg coloration directly reflect the amount of carotenoids bound to vitellogenin in the blood or is the coloration under specific control?

7. What molecular machinery underlies the skin pigmentation trait, to which degree is the skin pigmentation under direct hormonal control, and how has it been molded by sexual selection in different species?

Several of these questions can be addressed by deliberately focusing on the *Oncorhynchus* genus. *Oncorhynchus*, containing eight species, is the most diverse genus of the four flesh-pigmented genera. These species vary in flesh carotenoid content (Fig. 3) and life history patterns, including distribution, spawning habitat, rearing habitat, fresh- and salt-water residency duration, spawning migration difficulty, run timing, diet, size at maturity, and egg size and number (Hendry and Stearns 2004). There is a striking variability in skin coloration at sexual maturity among them (Quinn 2005). Because of the extensive management and study of these commercial species, there exists a large amount of baseline data, including distribution, historical changes, and heritability of phenotypic traits (Gjerde and Schaeffer 1989; Hebert et al. 1998; Kinnison et al. 2001). Their growing importance as model organisms for evolution and their recently resolved phylogeny pave the way for phylogenetically controlled comparisons among species (Crespi and Fulton 2004). In particular, sockeye salmon is a good model organism for research programs addressing the issues we present here. Sockeye exhibit the greatest variability of life history patterns among *Oncorhynchus* species (Burgner 1991). Owing to their precise natal homing, they are locally adapted at a fine spatial scale. In a single lake, there may be many populations adapted to different local spawning habitats (Wood and Bain 1995). There are many nonanadromous populations that may be landlocked or in sympatry with anadromous populations (Wood and Foote 1996). The genetic relationships of populations are described from micro- to macro-scales (Kinnison and Hendry 2004). Sockeye also have the highest recorded amounts of muscle carotenoids (Fig. 3), and this may make room for a high degree of variability in populations living under differing environmental conditions.

Concluding remarks

We have presented a consistent evolutionary scenario accounting for why capacity for carotenoid pigmentation of the flesh is ubiquitously present in the *Salmo*, *Oncorhynchus*, *Salvelinus*, and *Parahucho* genera of Salmonidae in the anadromous, as well as the nonanadromous, condition. Our explanation is based on three major elements. First, astaxanthin deposition in muscle fostered improved somatic maintenance in connection with nonspecific degradation of tissues. Second, it made possible further elaboration of the redd-making strategy. Third, it provided the biological backdrop for the buildup of a complex sensory and behavioral repertoire associated with skin pigmentation and sexual selection. Considering the large variation in availability of carotenoids in the diet, the diversity of life history regimes within these four genera, the large variation in astaxanthin flesh content among and within species, the large variation in egg and skin coloration, and the fact that past and present interaction of these elements may manifest in unique and complex ways in different lineages, it may seem somewhat bold to claim that these three explanatory elements can account for all empirical observations. We certainly acknowledge the need for further research but find it

very encouraging that the various empirical pieces of information available can be put together into a well-defined explanatory structure. Moreover, by compiling available empirical data into an evolutionary synthesis, we hope to have facilitated the identification of a focused research program that will lead to a clear understanding of the evolutionary history of flesh pigmentation in proximal, as well as ultimate, terms.

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