

## Dietary carotenoid levels affect carotenoid and retinoid allocation in female Chinook salmon *Oncorhynchus tshawytscha*

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This study examined the effect of dietary carotenoid availability on carotenoid and retinoid concentrations in the flesh, plasma, skin and eggs of female Chinook salmon *Oncorhynchus tshawytscha*. Carotenoid concentrations in all tissues were closely related to dietary availability. Early in the breeding season, carotenoids were stored primarily in the muscle, with a flesh carotenoid concentration of  $9.9 \mu\text{g g}^{-1}$  in fish fed a high carotenoid diet compared with  $1.9 \mu\text{g g}^{-1}$  in fish fed a low carotenoid diet. During the breeding season, carotenoid reserves were mobilized predominantly to the eggs and also to the skin. By the end of the breeding season, carotenoid concentrations in the eggs were  $17.9 \mu\text{g g}^{-1}$  in fish fed a high carotenoid diet and  $3.9 \mu\text{g g}^{-1}$  in fish fed a low carotenoid diet. Conversely, egg retinoid concentrations were only *c.* 20% lower in fish fed a low *v.* high carotenoid diet, which suggests that retinoid concentrations were not limited by the availability of carotenoid precursors. Egg carotenoid concentrations were not correlated with either skin carotenoid concentration or colouration, which suggests that female carotenoid displays are not a reliable signal that males can use to evaluate egg carotenoid resources.

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### INTRODUCTION

Carotenoids are important natural pigments principally produced by photosynthetic organisms and accumulated by many animals through their diet (Goodwin, 1986). These yellow to red pigments are widely distributed in arthropods, birds, reptiles and fishes, and may serve a number of physiological functions (Goodwin, 1984). First, carotenoids may function as antioxidants, which protect against the otherwise damaging oxidizing effects of free radicals (Burton, 1989; Krinsky & Yeum, 2003; but for evidence against a major role of carotenoids as antioxidants see Hartley & Kennedy, 2004; Costantini & Møller, 2008). Second, carotenoids may improve resistance to pathogens by increasing the production of antibodies or the proliferation of immune cells (Bendich, 1989; Jyonouchi *et al.*, 1994; McGraw & Ardia, 2003). Third, carotenoids may serve as a precursor to retinoids (vitamin A)

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(Schiedt *et al.*, 1985), which itself may be necessary for immune functioning, vision and development (Stephensen, 2001; Blomhoff & Blomhoff, 2006; Palace & Werner, 2006). Retinoids may be particularly important in developing eggs because they play a key role in cell signalling and patterning during the development of vertebrate embryos (Kawakami *et al.*, 2005; Vermot & Pourquié, 2005; Bowles *et al.*, 2006). Because of these functions, carotenoids are widely abundant in the eggs of fishes and birds and may be a key component of egg quality (Craik, 1985; Blount *et al.*, 2000). Indeed, maternal provisioning of carotenoids to eggs has been linked to hatching success and juvenile resistance to disease and oxidative stress (Palace *et al.*, 1998; Pettersson & Lignell, 1999; Ahmadi *et al.*, 2006; Tyndale *et al.*, 2008; but for a study in which no link was found see Christiansen & Torrissen, 1997). Given their many physiological functions and the inability of animals to synthesize carotenoids directly, for many species, these pigments are an important resource that is limited by dietary availability (Blount *et al.*, 2000).

In addition to physiological functions, in many species, carotenoids provide external pigmentation that may be used to attract mates. This role in mate choice is particularly well characterized for male colouration in a number of birds and fishes, in which females prefer more intense male carotenoid displays (Milinski & Bakker, 1990; Hill, 1991; Endler & Houde, 1995; Blount *et al.*, 2003). These carotenoid displays have been suggested to function as an indicator of male quality, either because they reflect an individual's ability to forage for rare carotenoid resources or because only high-quality males can handicap themselves by allocating physiologically important carotenoid resources to a display (Lozano, 1994; Olson & Owens, 1998). There are only a few examples that show a role of female carotenoid displays in sexual selection; in two-spotted gobies *Gobiusculus flavescens* (Fabricius), males prefer females that have a more orange abdomen, and this colour is largely determined by the carotenoid resources in that female's eggs, which are visible through the body wall (Amundsen & Forsgren, 2001; Svensson *et al.*, 2006); in sockeye salmon *Oncorhynchus nerka* (Walbaum), males prefer redder females that have high skin carotenoid levels, although the reason for this preference is unknown (Foote *et al.*, 2004).

Given the multiple demands for carotenoids, individuals may need to balance the allocation of carotenoids among various tissues and functions. This allocation is likely to be particularly critical in females, who must allocate carotenoids both to their own needs and to their offspring (*e.g.* eggs). In Pacific salmon *Oncorhynchus* sp., individuals are semelparous and thus largely forgo somatic maintenance as they sexually mature in order to invest all their energy and resources into a single spawning season (Quinn, 2005). During maturation, females direct carotenoid resources away from muscle stores and allocate them among two competing locations: the skin and the developing eggs (Kitahara, 1983; Ando *et al.*, 1994). Only a few studies, however, have examined the relationship between carotenoid levels in the skin and carotenoid levels in the eggs within individual females (Nordeide *et al.*, 2006, 2008), and this trade-off in carotenoid allocation among functions remains poorly characterized in *Oncorhynchus* sp. Pacific salmon. Conceivably, if carotenoid allocation to the skin and eggs is positively correlated, then a male preference for redder females could provide a direct benefit of increased egg quality.

This study used Chinook salmon *Oncorhynchus tshawytscha* (Walbaum) to examine carotenoid allocation among tissues in maturing females, and is one of the first

studies to describe the carotenoid composition of different tissues in this species. Carotenoid levels are compared in females experiencing either high or low dietary carotenoid availability to characterize priorities in carotenoid allocation when this resource is either plentiful or scarce. This difference in dietary carotenoid availability parallels the difference in availability between marine environments (carotenoid rich) and freshwater environments (carotenoid poor) and may provide insight into the carotenoid allocation strategies used by freshwater populations of salmonids. Because carotenoids can serve as retinoid precursors, the relationship between dietary carotenoid availability and egg retinoid concentrations is also examined. Female tissue and egg carotenoid concentrations are predicted to mirror the dietary carotenoid concentrations, but because neither diet was deficient in retinoids, low carotenoid availability is predicted to have only a moderate effect on a female's ability to provide retinoids to her eggs. This study concludes by discussing the possible role of female skin carotenoids in sexual selection as well as alternative functions for these pigment displays.

## MATERIALS AND METHODS

### SAMPLE COLLECTION

Experiments were conducted using *O. tshawytscha* reared at Yellow Island Aquaculture Ltd (YIAL), Quadra Island, British Columbia, Canada. The YIAL population was founded with gametes from the Robertson Creek hatchery on Vancouver Island and has been maintained since 1986. The Quinsam population was obtained from the Quinsam River hatchery, which has been supplementing *O. tshawytscha* since 1975 and currently releases four million juveniles annually. In the autumn of 2001, eggs were collected from YIAL females and fertilized using milt from Quinsam males. The fertilized eggs were pooled and incubated in Heath trays with a constant water flow of *c.* 15 l min<sup>-1</sup>. During incubation, dead eggs were regularly removed to prevent *Saprolegnia* spp. growth. In March 2002, the fry were transferred to 3000 l tanks in the hatchery building and fed *ad libitum* with food that included no supplemental carotenoids (Micro Crumble Starter Feed, EWOS Canada; www.ewos.com; vitamin A: 10 000 IU kg<sup>-1</sup>). In July 2002, the smolts were divided between two saltwater pens, with one pen fed a high carotenoid diet and the other a low carotenoid diet. Fish were fed *ad libitum* with either a high carotenoid diet augmented with 50 mg kg<sup>-1</sup> astaxanthin from the algae *Haematococcus pluvialis* or a low carotenoid diet that had carotenoid levels <5 mg kg<sup>-1</sup> and included no supplemental astaxanthin (both diets: Chinook grower, Taplow feeds; www.taplow.com; vitamin A: 8000 IU kg<sup>-1</sup>).

Fish were reared for over 3 years on these diets, at which point tissue samples were collected from maturing females at three times: 1 September 2005, 11–12 October 2005 and 10 November 2005. These dates correspond, respectively, to the times when wild fish are in the ocean before starting freshwater migration, when wild fish are starting freshwater migration and when wild fish are nearing the end of the spawning season. Fish on both diets continued to feed throughout the sampling period, with no difference in appetite observed between fish on the high and low carotenoid diets. At each sampling time, five fish from each pen were collected by seine and euthanized. Skin and eggs were examined in an additional three fish from each diet for the October sample. Blood was collected from the caudal vein using an ethylenediaminetetra-acetic acid (EDTA)-treated syringe and centrifuged to isolate plasma. Body length (measured from the front of the fish to the centre of the caudal fin, *i.e.* fork length,  $L_F$ ) and mass were measured, and then fish were photographed under standardized light for digital colour analysis. Colour was determined as in Neff *et al.* (2008) using Photoshop (version 7.0; Adobe; www.adobe.com) to measure the average red, green and blue colours for all pixels within an *c.* 15 cm<sup>2</sup> area on the midline of the fish, anterior to the dorsal fin. These colour values were then corrected using colour standards in each picture (Villafuerte &

Negro, 1998) and combined into two indices with a principal components analysis. The first index captured 94.5% of the variation and was a measure of brightness with positive loadings for all three colours (red = 0.57, green = 0.59 and blue = 0.57). The second index captured an additional 5.4% of the variation and had variable loadings among the colours (red = 0.74, green = -0.05 and blue = -0.67); the second axis largely measured hue with positive values representing a red hue and negative values representing a more blue hue. Eggs, flesh and skin samples were taken from each fish, with flesh and skin sampled below the dorsal fin and immediately above the lateral line. Samples were immediately frozen at  $-20^{\circ}\text{C}$  in the field, and then transferred to the laboratory where they were stored at  $-80^{\circ}\text{C}$ .

## CAROTENOID EXTRACTIONS

Carotenoids and retinoids were extracted from 80 to 320 mg ( $\pm 0.05$  mg) of tissue using the protocol of Li *et al.* (2005) with the following modifications. First, egg mass was determined using one to three eggs per female. To prepare samples for extraction, skin samples were cut into small pieces using a scalpel, flesh samples were homogenized using a glass mortar and pestle, eggs were homogenized using a plastic pestle in a microcentrifuge tube and plasma samples did not require homogenization. After homogenization, 20  $\mu\text{l}$  of retinoic acid in acetone ( $0.11\text{ mg ml}^{-1}$ ) was added to each sample as an internal standard to correct for losses during extraction. Extraction began by adding 400  $\mu\text{l}$  of acetone to each sample and vortexing for 10 s. Samples were centrifuged at 1500  $g$  for 30 s to separate and then collect the acetone phase, after which the precipitate was re-extracted with 400  $\mu\text{l}$  of acetone three more times. The pooled acetone extracts were concentrated in a vacuum centrifuge, after which the carotenoid residue was redissolved in 400  $\mu\text{l}$  of methyl tert-butyl ether (MTBE), with 200  $\mu\text{l}$  of 0.1% HCl in distilled water added to promote phase separation. The MTBE phase was collected, the aqueous phase was then extracted twice more with MTBE and the pooled extracts were concentrated in a vacuum centrifuge. The pigment residue was then extracted three more times with 400  $\mu\text{l}$  MTBE as described above and concentrated with a vacuum centrifuge. Finally, the pigment residue was dissolved in 100  $\mu\text{l}$  of MTBE and sealed in an amber vial for analysis by high performance liquid chromatography (HPLC).

HPLC separation of pigments was based on the protocol of Gilmore & Yamamoto (1991) as described below. The HPLC system was a Beckman Coulter System Gold HPLC with Binary Gradient 126 Solvent Selection Pump, Photodiode Array 168 Detector and 507e Autosampler (Beckman Coulter; [www.beckmancoulter.com](http://www.beckmancoulter.com)). The column was a Beckman Ultrasphere ODS, with 5  $\mu\text{m}$  particle size, 4.5 mm internal diameter and 15 cm length. Pigment separation was achieved at a constant flow of 1  $\text{ml min}^{-1}$ , with two solvents: (A) acetonitrile, methanol, 0.1 M Tris-HCl pH = 8 (7:1:1) and (B) methanol, hexanes (4:1). Separation began by injecting 10  $\mu\text{l}$  of sample, followed by 4 min of 100% solvent A, 2.5 min linear gradient to 100% solvent B, 10 min of 100% solvent B, 2.5 min linear gradient to 100% solvent A and 16 min at 100% solvent A to re-equilibrate the column before loading the next sample. Solvents used for separation were all HPLC grade. The eluent was monitored over the range of 300–600 nm, with quantitation at 325 nm for retinoids, at 480 nm for astaxanthin and at 440 nm for skin carotenoids.

Pigments were identified by comparison (co-elution, absorbance spectrum) to authentic standards including astaxanthin,  $\beta$ -carotene, lutein and retinyl palmitate, with verification by mass spectrometry (MS) performed on a triple QTOF micro mass spectrometer (water/micromass; [www.waters.com](http://www.waters.com)) equipped with a Z-spray source and run in negative ion and positive ion electrospray mode (Schiedt & Liaaen-Jensen, 1995). Compounds for which standards were not available were identified by absorbance spectroscopy, mass spectrometry and comparison with literature values. Serial dilutions of authentic astaxanthin, lutein, retinoic acid and retinyl palmitate were made fresh on the day they were analysed and used to calibrate the detector. The concentration of skin carotenoids in a sample was calculated using the combined areas of all the carotenoid peaks and was expressed as the equivalent mass of lutein. Concentrations were corrected for losses during extraction using the internal retinoic acid standard. Retinoic acid standards were sealed under nitrogen and stored at  $-20^{\circ}\text{C}$ , with duplicate retinoic acid samples analysed with each batch of tissue samples to calibrate the internal standard.

## STATISTICAL ANALYSIS

Carotenoid and retinoid concentrations in each tissue were analysed using ANOVA with diet (high carotenoid and low carotenoid) and collection date (September, October and November) as fixed factors. *Post hoc* Tukey's HSD tests were used to further explore significant effects. Initial ANOVA included  $L_F$  as a covariate, but it did not have a significant effect on any of the concentrations; hence, this factor was removed from the final models. Egg astaxanthin and retinyl palmitate values were analysed both as the concentration by egg mass and as the mass per egg. The relationships between the mass of astaxanthin and retinyl palmitate per egg and the skin carotenoid concentrations were analysed using ANCOVA, which included diet and collection date as fixed factors. ANCOVA were similarly used to examine the relationship between skin carotenoid concentration and skin brightness and hue. Analyses were performed using JMP (v. 4.0.4, SAS Institute Inc; www.sas.com).

## RESULTS

Astaxanthin was the primary carotenoid present in the flesh samples [Fig. 1(a)]. Flesh astaxanthin concentrations were significantly related to diet, with more astaxanthin in the flesh of fish fed a high carotenoid diet [ANOVA,  $F_{1,24} = 7.32$ ,  $P < 0.05$ , Fig. 2(a)]. Flesh astaxanthin concentrations decreased over time, with a significant drop between September and the two later sampling times [ANOVA,  $F_{2,24} = 13.17$ ,  $P < 0.001$ , Fig. 2(a)]. Flesh astaxanthin was also affected by a significant interaction between time and diet, with the much higher initial flesh astaxanthin concentrations associated with the high carotenoid diet decreasing over time so that there was no significant difference in astaxanthin concentrations between diets at the later dates [ANOVA,  $F_{2,24} = 5.87$ ,  $P < 0.01$ , Fig. 2(a)].

Astaxanthin was also the primary carotenoid found in the plasma samples [Fig. 1(b)]. Plasma concentrations of astaxanthin were higher in fish fed a high carotenoid diet than in fish fed a low carotenoid diet [ANOVA,  $F_{1,24} = 17.8$ ,  $P < 0.001$ , Fig. 2(b)]. Plasma astaxanthin concentrations peaked in October, decreasing significantly in the November samples [ANOVA,  $F_{2,24} = 8.91$ ,  $P < 0.001$ , Fig. 2(b)]. Plasma astaxanthin concentrations showed a significant interaction between time and diet, with concentrations in fish fed the high carotenoid diet increasing from September to October, while concentrations in fish fed the low carotenoid diet were unchanged during this time [ANOVA,  $F_{2,24} = 5.25$ ,  $P < 0.05$ , Fig. 2(b)].

Skin samples contained a group of related carotenoids [Fig. 1(c)]. The properties of the most abundant skin carotenoid were consistent with salmoxanthin, which has previously been described in the skin of chum salmon *Oncorhynchus keta* (Walbaum) (Kitahara, 1983). The positive ion electrospray ionization (ESI)-mass spectrum of this compound revealed two major peaks at 601 and 603  $m/z$  (Fig. 3). These peaks represent  $[M + 1]^+$  ions corresponding to compounds with relative molecular masses ( $M_r$ ) of 600 and 602 and are consistent with salmoxanthin ( $C_{40}H_{56}O_4$ ) and its hydrogenated derivative ( $C_{40}H_{58}O_4$ ). The putative salmoxanthin also had absorption maxima at *c.* 420, 442 and 468 nm, which are similar to the previously reported values for salmoxanthin (Britton *et al.*, 2004). Skin carotenoid concentrations were significantly higher in fish fed the high carotenoid diet than in fish fed the low carotenoid diet [ANOVA,  $F_{1,30} = 7.04$ ,  $P < 0.05$ , Fig. 2(c)]. There was an increase in skin carotenoid concentrations over time but the trend was non-significant [ANOVA,  $F_{2,30} = 2.70$ ,  $P > 0.05$ , Fig. 2(c)], with no interaction between time and diet [ANOVA,  $F_{1,30} = 0.66$ ,  $P > 0.05$ , Fig. 2(c)].

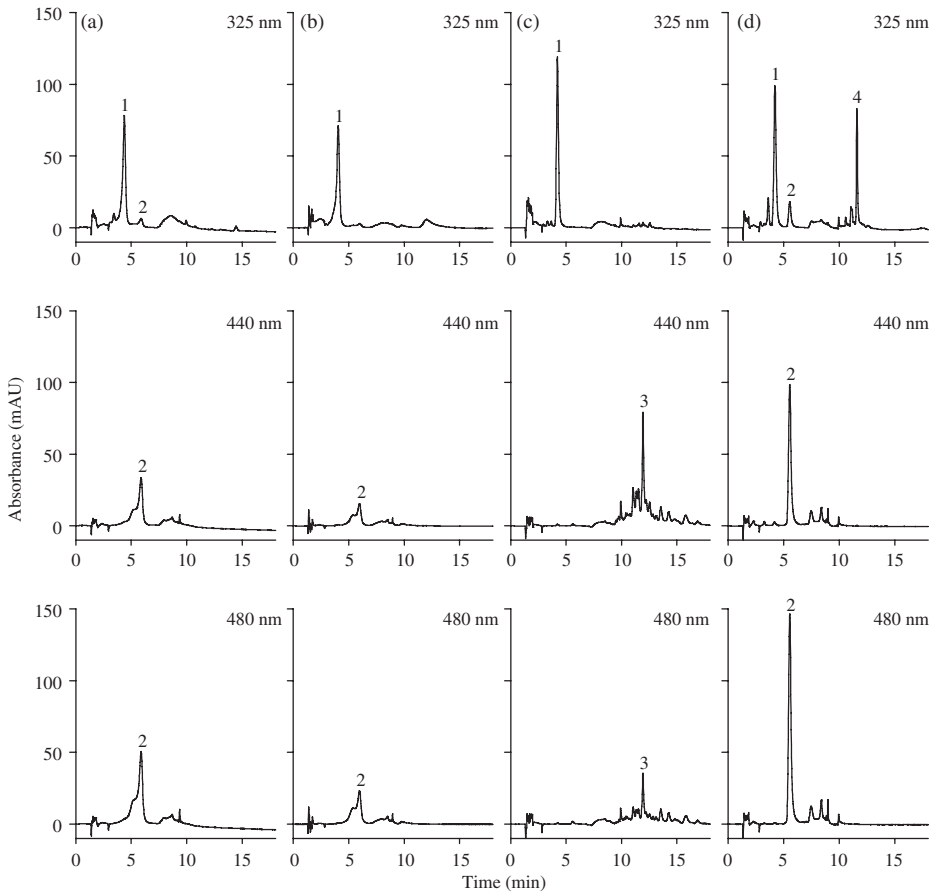


FIG. 1. Representative chromatograms following high performance liquid chromatography separation of tissue samples from *Oncorhynchus tshawytscha*. Plots are presented at three wavelengths (325, 440 and 480 nm) for each of the four tissues that were sampled (a) flesh, (b) plasma, (c) skin and (d) egg. Major peaks are labelled: 1, retinoic acid, an internal standard; 2, astaxanthin; 3, salmoxanthin; 4, putative retinyl palmitate.

Astaxanthin was the primary carotenoid (>85% total) in eggs, which also contained a major retinoid [Fig. 1(d)]. This compound co-eluted with retinyl palmitate and had a typical retinyl palmitate absorbance spectrum ( $\lambda_{\max} = 325$ ). Mass spectral analysis of the retinoid region of the HPLC trace revealed evidence for the carotenoids  $\beta$ -carotene and lutein but was inconclusive for retinyl palmitate. The mass of astaxanthin per egg was higher in the fish fed the high carotenoid diet [ANOVA,  $F_{2,30} = 177$ ,  $P < 0.001$ , Fig. 4(a)]. Astaxanthin mass per egg increased consistently over time for both diets [ANOVA,  $F_{2,30} = 54.6$ ,  $P < 0.001$ , Fig. 4(a)]; however, the mass of astaxanthin per egg increased more over time in the fish fed the high carotenoid diet [ANOVA,  $F_{2,30} = 31.0$ ,  $P < 0.001$ , Fig. 4(a)]. The retinoid mass per egg was likewise higher in fish fed the high carotenoid diet [ANOVA,  $F_{1,30} = 10.3$ ,  $P < 0.01$ , Fig. 4(b)]. The retinoid mass per egg increased significantly from September to November in both groups [ANOVA,  $F_{2,30} = 4.73$ ,  $P < 0.05$ ,



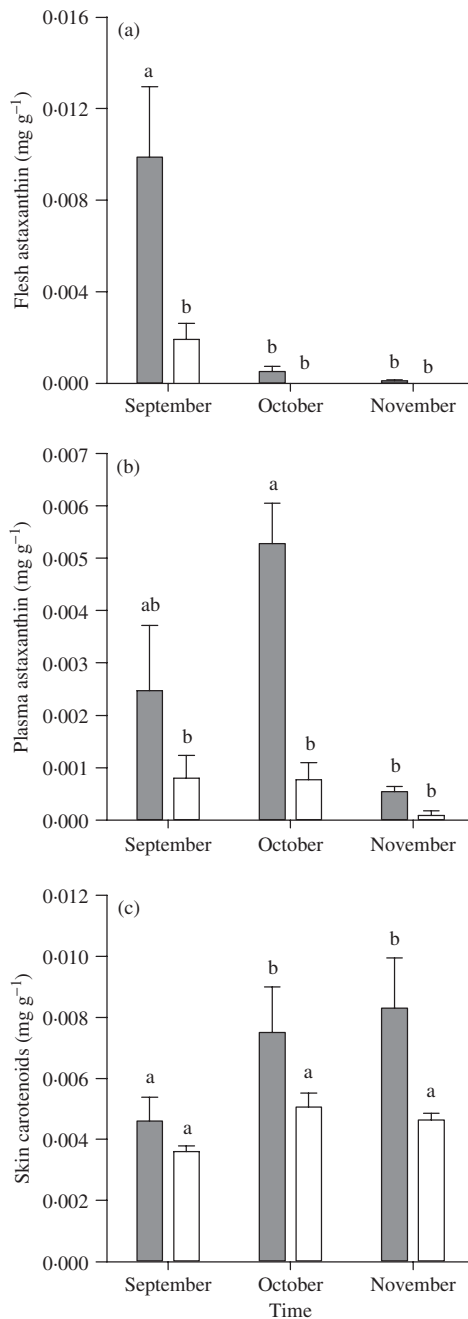


FIG. 2. Mean + s.e. carotenoid concentrations in three tissues from *Oncorhynchus tshawytscha*. The tissues comprise (a) flesh, (b) plasma and (c) skin. Fish were raised for 3 years on either a low carotenoid diet ( $\square$ ) or a high carotenoid diet that was supplemented with  $50 \text{ mg kg}^{-1}$  astaxanthin ( $\blacksquare$ ). Eggs were sampled at three times, which correspond to the ocean phase before the start of freshwater migration, the start of freshwater migration and late in the spawning season. Different lowercase letters denote significant differences ( $P < 0.05$ ) between groups.

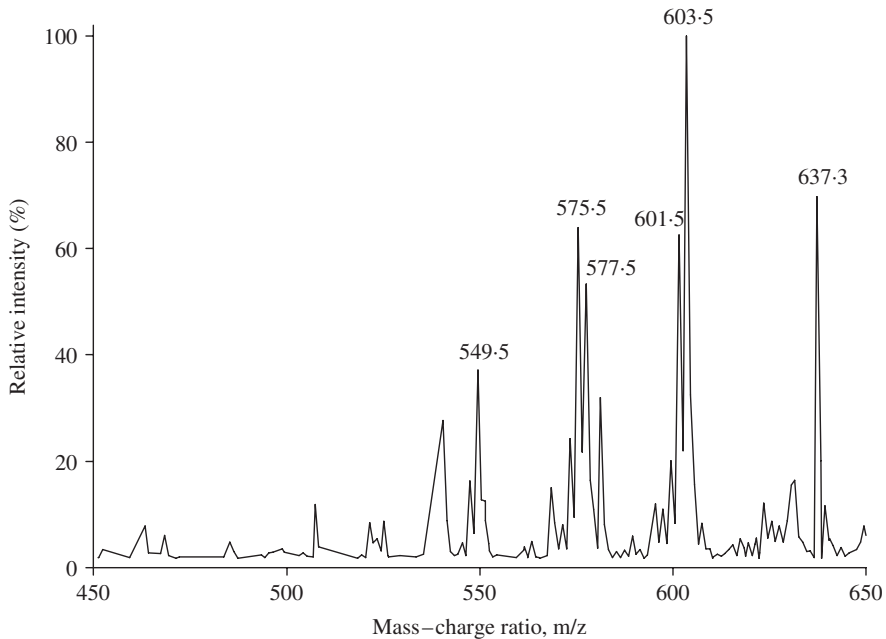


Fig. 3. Positive ion electrospray ionization (ESI)-mass spectrum of the major unidentified carotenoids that were present in the skin of *Oncorhynchus tshawytscha*. The unidentified carotenoids were isolated from other compounds in the skin by high performance liquid chromatography separation before analysis. The  $m/z$  values for the major peaks are shown.

Fig. 4(b)], although there was no interaction between sampling date and diet on the retinoid mass per egg [ANOVA,  $F_{1,30} = 0.61$ ,  $P > 0.05$ , Fig. 4(b)].

Egg mass increased significantly after September (mean  $\pm$  s.e. =  $0.089 \pm 0.010$  g) but was similar in October (mean  $\pm$  s.e. =  $0.231 \pm 0.008$  g) and November (mean  $\pm$  s.e. =  $0.247 \pm 0.010$  g; ANOVA,  $F_{2,30} = 80.8$ ,  $P < 0.001$ ). Egg mass did not differ based on diet or the interaction between sampling date and diet (ANOVA, both  $P > 0.05$ ). The changes in egg mass were associated with minor differences in the patterns of carotenoid and retinoid concentrations compared with the content per egg. Although the amount of astaxanthin per egg increased consistently from September to November, astaxanthin concentrations were flat or lower from September to October before increasing in November [ANOVA,  $F_{2,30} = 5.33$ ,  $P < 0.01$ , Fig. 4(c)]. There was also a significant interaction between sampling date and diet, with astaxanthin concentrations increasing between September and November in fish fed the high carotenoid diet while decreasing in fish fed the low carotenoid diet [ANOVA,  $F_{2,30} = 4.80$ ,  $P < 0.05$ , Fig. 4(c)]. Although the retinoid mass per egg increased over time, there was a highly significant decrease in retinoid concentrations between September and October that corresponded to the increase in egg mass [ANOVA,  $F_{2,30} = 57.9$ ,  $P < 0.001$ , Fig. 4(d)]. The retinoid concentration did not show an interaction between sampling date and diet [ANOVA,  $F_{2,30} = 1.15$ ,  $P > 0.05$ , Fig. 4(d)].

There was no significant correlation between skin carotenoid concentration and the mass of astaxanthin per egg, independent of the differences in these measures



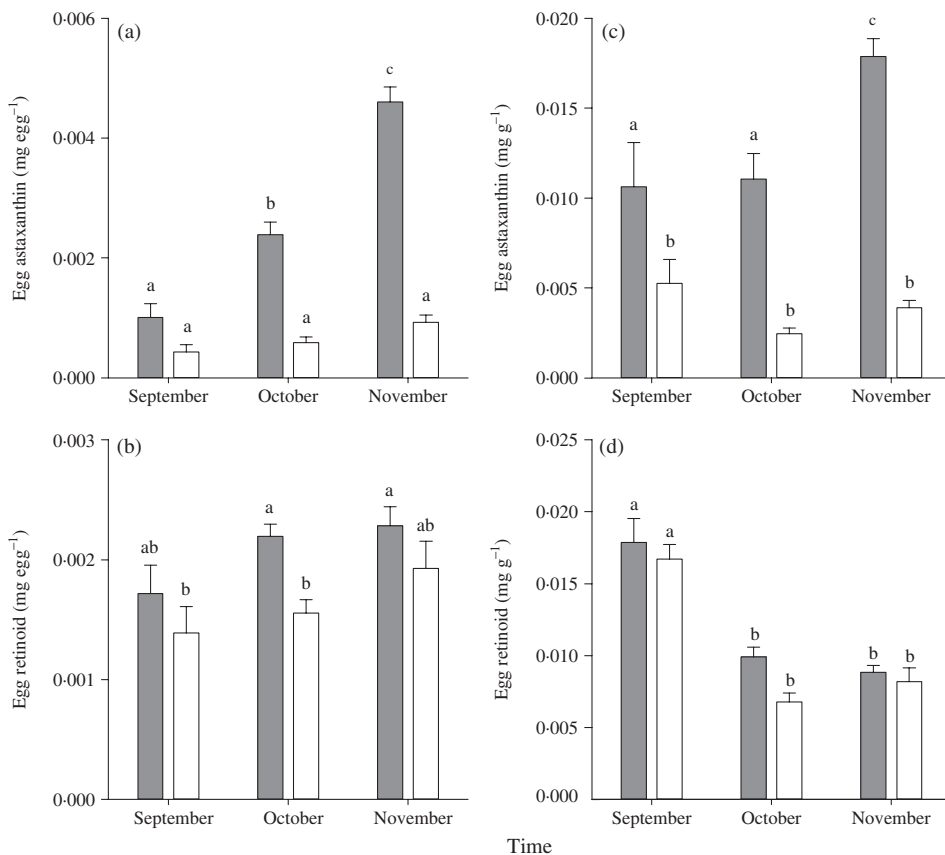


FIG. 4. Mean + s.e. (a), (c) astaxanthin and (b), (d) retinoid concentrations in eggs from *Oncorhynchus tshawytscha* (a), (b) per egg and (c), (d) by egg mass. Fish were raised for 3 years on either a low carotenoid diet (□) or a high carotenoid diet that was supplemented with 50 mg kg<sup>-1</sup> astaxanthin (■). Eggs were sampled at three times, which correspond to the ocean phase before the start of freshwater migration, the start of freshwater migration and late in the spawning season. Different lowercase letters denote significant differences ( $P < 0.05$ ) between groups.

based on diet and sampling date [ANCOVA,  $F_{1,29} = 0.004$ ,  $P > 0.05$ , Fig. 5(a)]. Similarly, there was no significant correlation between skin carotenoid concentration and the retinoid mass per egg, independent of the differences in these measurements based on diet and sampling date [ANCOVA,  $F_{1,29} = 0.98$ ,  $P > 0.05$ , Fig. 5(b)]. No relationship was observed between skin brightness and skin carotenoid concentrations [ANCOVA,  $F_{1,32} = 0.89$ ,  $P > 0.05$ , Fig. 6(a)] or between skin hue and skin carotenoid concentrations [ANCOVA,  $F_{1,32} = 1.55$ ,  $P > 0.05$ , Fig. 6(b)].

## DISCUSSION

This study tracked changes in carotenoid and retinoid concentrations in female *O. tshawytscha* during the spawning season. Before maturation, most carotenoids were stored as astaxanthin in the muscle, with muscle astaxanthin concentrations

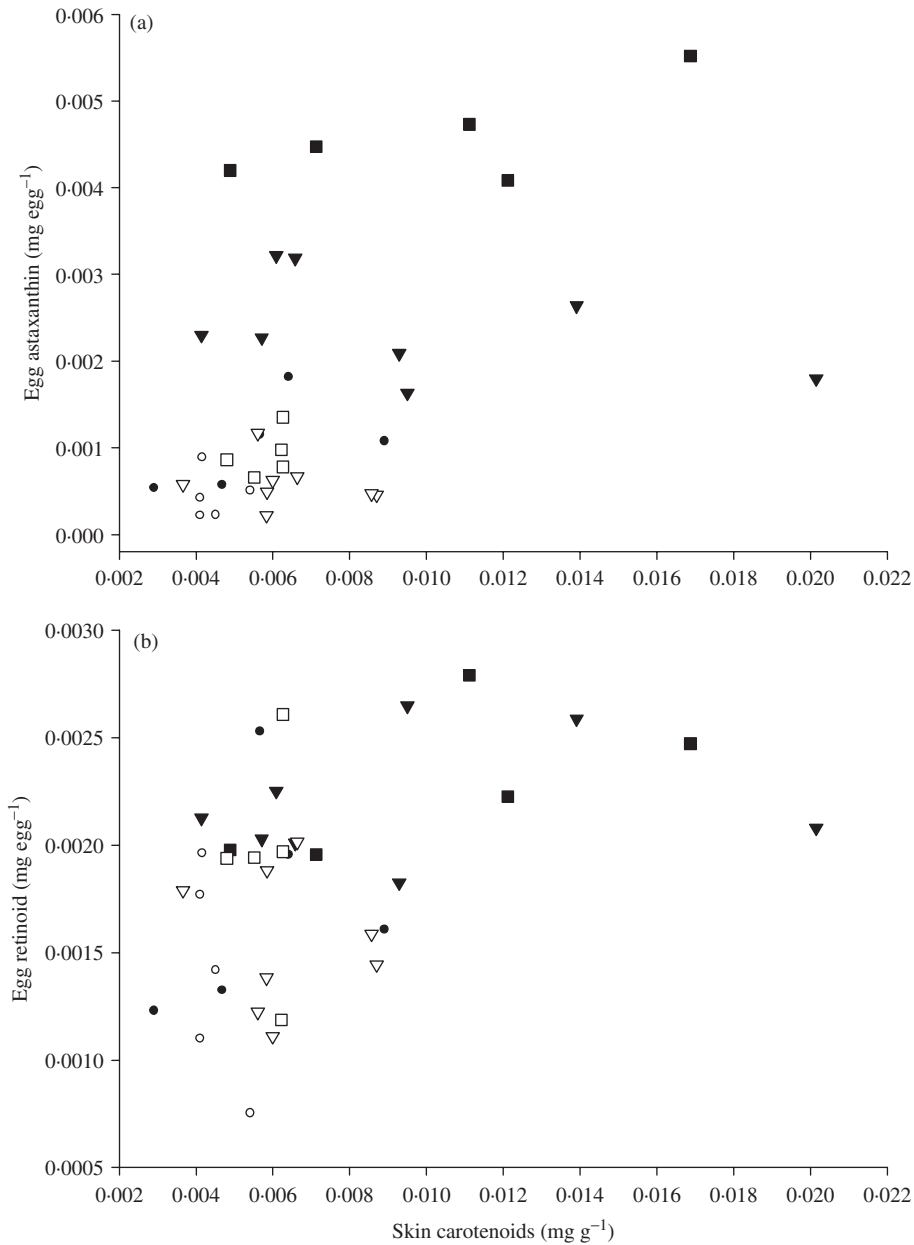


FIG. 5. Relationships between skin and egg carotenoid concentrations in *Oncorhynchus tshawytscha*. Plots present correlations between total skin carotenoid and the amount of (a) astaxanthin or (b) retinoid per egg. Fish were raised for 3 years on either a low carotenoid diet or a high carotenoid diet that was supplemented with 50 mg kg<sup>-1</sup> astaxanthin. Eggs were sampled at three times, which correspond to the ocean phase before the start of freshwater migration (○, low carotenoid diet; ●, high carotenoid diet), at the start of freshwater migration (◐, low carotenoid diet; ◑, high carotenoid diet) and late in the spawning season (□, low carotenoid diet; ■, high carotenoid diet). The relationships between skin carotenoids and astaxanthin or retinoid concentrations were not significant ( $P > 0.05$ ), independent of the differences in these measurements among diets and sampling dates.

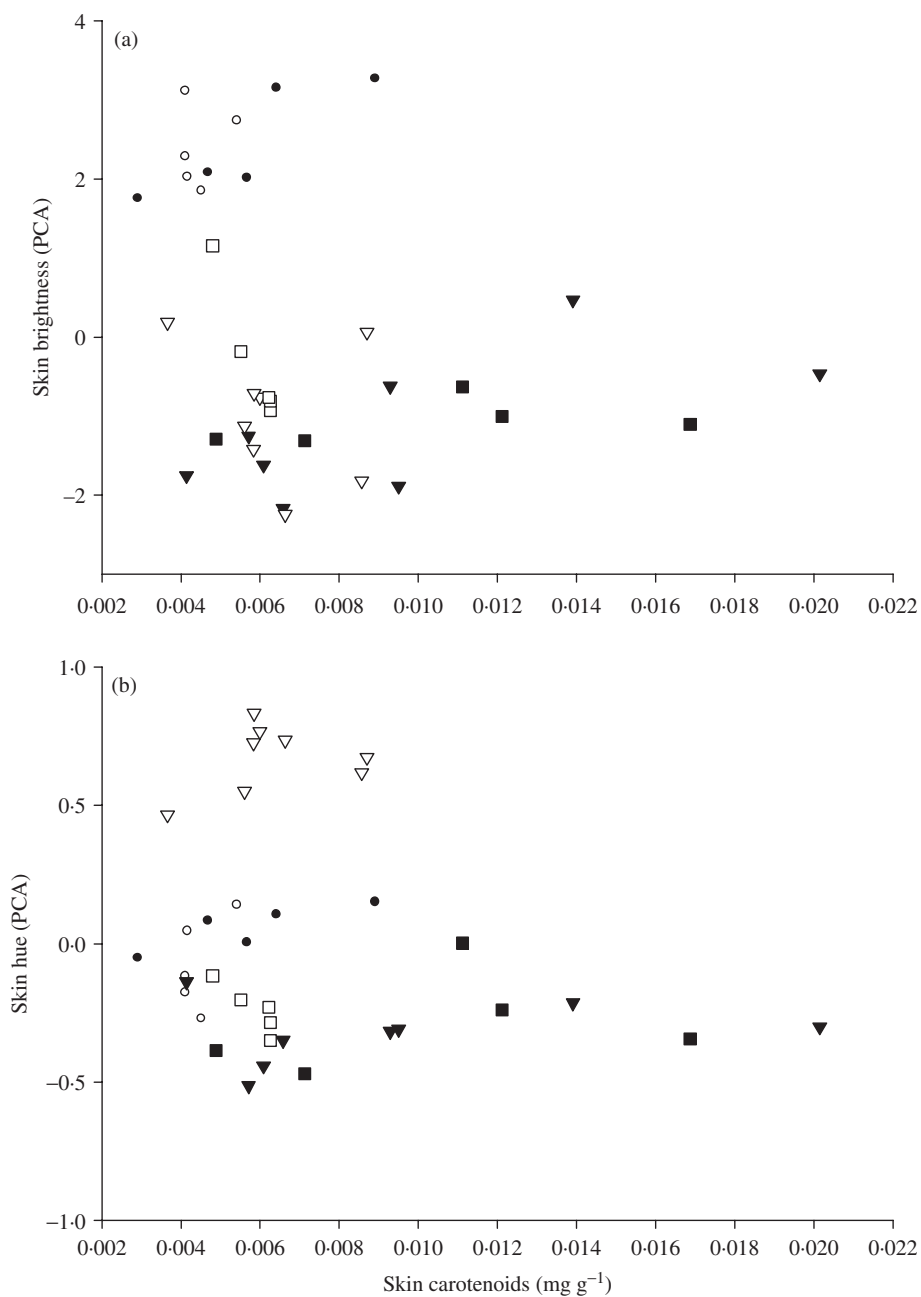


FIG. 6. Relationships between skin carotenoid concentration and skin colour (a) skin brightness and (b) skin hue in *Oncorhynchus tshawytscha*. Fish were raised for 3 years on either a low carotenoid diet or a high carotenoid diet that was supplemented with 50 mg kg<sup>-1</sup> astaxanthin. Eggs were sampled at three times, which correspond to the ocean phase before the start of freshwater migration (O, low carotenoid diet; ●, high carotenoid diet), at the start of freshwater migration (▽, low carotenoid diet; ▼, high carotenoid diet) and late in the spawning season (□, low carotenoid diet; ■, high carotenoid diet). The relationships between skin carotenoids and skin brightness or skin hue were not significant ( $P > 0.05$ ).

decreasing 100-fold during maturation as carotenoids appeared to be mobilized to the skin and eggs. This mobilization was associated with changes in plasma astaxanthin concentrations, which peaked in October before declining in the final November sample. Both skin and plasma carotenoid concentrations reflected dietary carotenoid content, with higher concentrations in fish fed the high carotenoid diet. Skin carotenoid concentrations increased during maturation by *c.* 80% in fish fed the high carotenoid diet and 30% in fish fed the low carotenoid diet. Before maturation, egg astaxanthin concentrations were more than twice as high in fish fed the high carotenoid diet, and this difference was magnified during maturation, with astaxanthin concentrations rising 360% in fish fed the high carotenoid diet compared with 120% in fish fed the low carotenoid diet. In contrast, egg retinoid concentrations increased by only 35% during the spawning season and were *c.* 30% higher in fish fed the high carotenoid diet on each sampling date.

The patterns of carotenoid allocation among tissues observed during the spawning season in this study were generally consistent with those previously reported in *Oncorhynchus* sp.. Studies of *O. tshawytscha* have similarly identified astaxanthin as the primary carotenoid in flesh and eggs (Ando *et al.*, 1992, 1994; Li *et al.*, 2005). The rise in plasma astaxanthin during maturation was consistent with the transport of carotenoid resources from the muscle to the eggs and skin, which has been shown to occur through binding carotenoids to lipoprotein transporters (Ando & Hatano, 1988). Salmoxanthin has previously been identified as a major skin carotenoid component in *O. keta* (Kitahara, 1983), but this study is the first to note its presence in *O. tshawytscha*. The presence of salmoxanthin and small quantities of unidentified carotenoids indicated that carotenoid resources, which were predominantly stored in flesh as astaxanthin, were heavily modified in the skin, as was noted in *O. keta* (Kitahara, 1983). The identity of the most abundant retinoid in the eggs was not confirmed, but it co-eluted with and had an absorbance spectrum identical to retinyl palmitate. While mass spectral data were inconclusive, this egg retinoid is probably retinyl palmitate. If true, this would be the first suggestion of a retinyl ester as a major retinoid allocated to the eggs of *O. tshawytscha*, although studies of other salmonids and fishes have similarly identified retinyl esters as major vitamin A stores in eggs (Plack & Kon, 1961; Irie & Seki, 2002) and have shown that vitamin A reserves are critical for egg production and development (Furuita *et al.*, 2001, 2003; Palace & Werner, 2006).

Dietary carotenoid availability may be a key determinant of a female's carotenoid and retinoid reserves and ultimately her ability to provision these resources to her eggs. Indeed, tissue carotenoid concentrations in salmonids have been widely reported to correlate with dietary carotenoid concentrations (Storebakken *et al.*, 1987; Smith *et al.*, 1992; March & MacMillan, 1996). In this study, low dietary carotenoid availability was associated with greatly reduced astaxanthin storage in the muscle, which subsequently limited the ability of maturing females to mobilize carotenoids to the eggs, skin and plasma. Thus, although female *Oncorhynchus* sp. may continue to feed throughout the breeding season (Garner *et al.*, 2009), it is the carotenoid reserves stored in the muscle that appear to be the primary source of carotenoids allocated to the eggs (Kitahara, 1983; Ando & Hatano, 1988). In contrast to the clear effect on carotenoid reserves, the effect of dietary carotenoid concentrations on retinoid reserves and transfer to eggs is less clear. In birds, a high carotenoid diet was associated with elevated egg retinoid concentrations in quail *Coturnix coturnix japonica*

(Temminck & Schlegel) (Karadas *et al.*, 2005), but diet had no effect on egg retinoid concentrations in blue tits *Parus caeruleus* (Biard *et al.*, 2005). In the present study, retinoid concentrations were lower in fish fed a low carotenoid diet than in fish fed a high carotenoid diet, which is consistent with carotenoids functioning as retinoid precursors (Schiedt *et al.*, 1985). Low dietary carotenoid concentrations, however, resulted in only *c.* 20% reduction in egg retinoid concentrations compared with a 75% reduction in egg astaxanthin concentrations. Thus, *O. tshawytscha* faced with low dietary carotenoid availability were able to maintain close to normal egg retinoid concentrations, despite considerably lower carotenoid concentrations, which suggests that most retinoids found in the eggs were not derived from dietary carotenoids but were instead obtained directly from the diet. Direct allocation of dietary retinoids may thus be able to provide eggs with sufficient retinoids to meet the demands of development and immune function (Stephensen, 2001; Blomhoff & Blomhoff, 2006; Palace & Werner, 2006) and may help ease the demand for carotenoids as retinoid precursors when carotenoid availability is low, as for example when a population of salmon transitions from a high carotenoid marine diet to a low carotenoid freshwater diet.

Female carotenoid displays may be a cue that males use to select mates. For example, male preference for female carotenoid displays has previously been shown in *G. flavescens* and *O. nerka* (Amundsen & Forsgren, 2001; Foote *et al.*, 2004). Theory predicts that males will prefer intense female carotenoid displays when these cues signal some underlying aspect of mate quality or maternal investment (Olson & Owens, 1998; Svensson *et al.*, 2006). Thus, males may prefer intense female carotenoid displays because they indicate maternal provisioning of carotenoids to eggs, which has been linked to hatching success and juvenile resistance to disease (Ahmadi *et al.*, 2006; Tyndale *et al.*, 2008). The present study of *O. tshawytscha* provides only limited evidence that female skin carotenoid concentrations convey information about egg quality, at least with respect to egg carotenoid content. Females fed a high carotenoid diet had significantly higher concentrations of carotenoids in both the skin and the eggs than females fed the low carotenoid diet. Thus, female skin carotenoid concentrations could allow males to identify females with high egg carotenoid concentrations if a population experienced large differences in dietary carotenoid availability. Within diets, however, there was no relationship between the concentrations of carotenoids in the skin and eggs of individual females, so female skin carotenoid concentrations may often provide no signal of egg quality to males. Moreover, skin carotenoid concentrations were not related to any of the measures of skin colour, so it is not clear that skin carotenoids were associated with a phenotypic cue that males could use to discriminate among females, even when egg quality differed. Indeed, a study of mating behaviour in *O. tshawytscha* showed no effect of female colouration on reproductive success, which suggests that skin pigments were not involved in mate choice (Neff *et al.*, 2008). An alternative function for these skin carotenoids is suggested by a study of brown trout *Salmo trutta* L., which showed that low carotenoid levels were associated with abnormally pale chromatophores in the skin (Steven, 1949). Thus, rather than functioning in mate choice, the yellow pigments in *O. tshawytscha* skin may contribute to the natural brown colour of these fish and make them less conspicuous against the riverbed. If so, this would represent an interesting divergence in the use of skin pigments in *Oncorhynchus* sp., as bright red carotenoid displays in *O. nerka* are used to attract mates (Foote *et al.*, 2004),

while the yellow carotenoid displays in *O. tshawytscha* may be used to provide crypsis.

Overall, dietary carotenoid availability appeared to be the primary determinant of tissue carotenoid concentrations in *O. tshawytscha*. Egg retinoid levels were somewhat lower when dietary carotenoid availability was low, but the ability to continue allocating retinoids to the eggs directly may help females to compensate for a carotenoid poor diet. Further investigation of the combined role of carotenoids and retinoids in determining egg fitness would clarify the degree to which the importance of carotenoids arises from their ability to function as retinoid precursors. Subsequent investigations could likewise address the more traditional role of carotenoid displays by male *O. tshawytscha* in mate choice, as male colour has been shown to correlate with reproductive success in this species (Neff *et al.*, 2008).

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