FINAL REPORT

Astaxanthin digestibility, tissue concentration and body retention in Atlantic salmon (*Salmo salar*) fed graded diet inclusion levels of Antarctic krill (*Euphausia superba*) meal or Carophyll Pink as pigment sources

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- Antarctic krill meal containing 105 mgkg^{-1} astaxanthin esters (20% mono- and 80% diester) was included in experimental Diets 1 to 5 at 0, 10, 20, 30 and 40 % (wet weights of the diets), exchanging approximately 0, 13, 27, 40 and 53% of the dietary fish meal protein, and 0, 7, 14, 21 and 28% of total dietary lipid from fish meal and oil. Natural esterified astaxanthin from the krillmeal provided the only pigment source in diets 2 to 5, Diet 1 contained no added pigment and Diet 6 was supplemented with Carophyll Pink, providing free astaxanthin (80% All-E (trans); 5% 9Z (cis) and 14% 13Z (cis)-astaxanthin) to a level similar to that presumably provided by the highest inclusion level of the Antarctic krill meal (42 mgkg^{-1}).

	Antarctic krill meal	Carophyll Pink	Total astaxanthin in the diets, mg kg^{-1}	
Diet	g100g^{-1} diet	mg kg^{-1} diet	Mono- and diester	Free astaxanthin
1	0	-	<1	1.4
2	10	-	14	1.3
3	20	-	26	1.1
4	30	-	37	1.1
5	40	-	50	1.0
6	0	42	<1	42

- A 12 week feeding trial with Atlantic salmon (initial weight 732) was performed according to a regression design (6 diets, 2 replicates). During the experimental trial fish more than doubled their body weights and showed high growth rates (thermal growth coefficient; TGC = 3.35 ± 0.19; specific growth rate; SGR = 0.97 ± 0.05). Final body weights (1554 to 1708 g) and weight gain showed no difference ($P > 0.05$) between diet groups, and increased level of krill meal in the diet did not affect SGR or TGC ($P > 0.05$). Daily feed intake (0.82 to 0.88 % of mean body weights), and feed conversion (1.06 – 1.11) did not show significant differences that can be attributed to increased inclusion of Antarctic krill meal or the different pigment sources.

- All-E (trans) astaxanthin was the dominating astaxanthin form in salmon tissues, contributing to 83 – 84% of total astaxanthin in whole body and muscle. The 13Z (Cis) astaxanthin showed lower tissue levels (average ~ 13%), while 9Z (Cis) astaxanthin was below the methods' detection limit (<0.10 mg kg^{-1}) in all measured tissues. The ratio between All-E (trans) and 13Z (Cis) astaxanthin in liver was different from that in muscle and whole body, showing lower All-E astaxanthin (48%) and higher 13Z astaxanthin (48%), respectively. The amount of total astaxanthin and All-E-astaxanthin in liver increased relative to the muscle concentrations with increased inclusion of Antarctic krill meal and with Carophyll Pink, suggesting some metabolic adaptive changes due to higher available astaxanthin from the feed. Whole body astaxanthin concentration (of which the muscle exerts 64%) was approximately 60 % when compared to the concentration in muscle, irrespective of pigment source (krill meal or Carophyll Pink) and krill meal inclusion level.

- Whole body and muscle total level of free astaxanthin and All-E (trans) astaxanthin increased and showed significant differences with respect to increased level of krill meal in the diet ($P < 0.01$) and with different pigment sources ($P < 0.05$). Fish fed Carophyll Pink as the pigment source (Diet 6) showed significantly higher specific concentration of astaxanthin in muscle and liver as compared to fish fed the highest inclusion of Antarctic krill meal (Diet

5), indicating more efficient retention of free astaxanthin from Carophyll Pink than esterified astaxanthin from Antartctic krill meal.

- Apparent digestibility of esterified astaxanthin (sum mono- and di-astaxanthin) was average 90.8 ± 1.9 % and showed no differences with increased inclusion of krill meal in the diets ($P > 0.05$). Results indicate a very efficient hydrolysis of esterified astxanthin from Antarctic krill meal and that astaxanthin is probably absorbed in the free form. This was also supported by the fact that esterified astaxanthin was not present in any of the measured tissues.

- Apparent digestibility of total astaxanthin (sum free and esterified astaxanthin) was average 53.4 ± 5.1 % for all fish fed krill meal or Carophyll Pink as the pigment sources (Diet 2 – 6), showing no differences with respect to inclusion level of Antarctic krill meal or the pigment source being krill or Carophyll Pink ($P < 0.05$). In the pigment free control group, apparent digestibility of astaxanthin was negative (-11.1%), indicating net loss of astaxanthin in fish, confirmed by low tissue astaxanthin concentration and by negative whole body retention level in this group.

- Whole body retention of astaxanthin was negative in the pigment free control group (-12.3 %), indicating net loss of astaxanthin, possible through metabolic loss, oxidation and expected dilution of muscle astaxanthin as the fish grow during the 12 week feeding period. Retention values increased with increased inclusion of Antarctic krill meal, showing 1.93, 2.05, 3.97 and 4.03 % efficient body retention of the total amount of astaxanthin eaten in diets with 10, 20, 30 and 40 % krill meal. These results are equivalent to an available level of astaxanthin of 0.27, 0.52, 1.36 and 1.92 mg astaxanthin kg^{-1} fish. The highest retention value of astaxanthin, 6.45%, equivalent to 2.51 mg astaxanthin kg^{-1} fish, was found for Carophyll Pink. This value was not significantly different from diets with 30 and 40 % Antarctic krill meal. However, muscle astaxanthin concentration was significantly higher in fish fed Carophyll Pink, as compared to fish fed the highest krill meal inclusion level. This indicates more efficient retention of free astaxanthin from Carophyll Pink than esterified astaxanthin from the krill meal, directed towards flesh astaxanthin deposition.

- Muscle levels of vitamin A, E and C, and liver levels of vitamins A and C, were not affected by the different diets, reminding of equal additions of these vitamins in all diets, still dietary vitamin E increased with increasing krill additions, probably due to vitamin E added to the ingredient as an antioxidant. Liver levels of vitamin E increased with increasing krill meal inclusion in diets. Significant ($P < 0.003$) higher levels of vitamin E was found in fish fed Diet 4 and 5 (30 and 40% krill inclusion, respectively) and may reflect the higher levels of vitamin E found in these diets compared to diets with a low inclusion of krill meal, although no correlation was found between vitamin E levels in the diets and in liver ($P > 0.05$).

Stability of free and esterified astaxanthin during feed production
- Astazanthin retention values in the krill meal diets were 99% or higher for Diets 1 to 5, indicating high stability of esterified astaxanthin from krill during feed production, but probably also underestimated level of astaxanthin in the krill meal before processing. Free astaxanthin from Carophyll Pink (Diet 6) also showed high stability, with a total loss < 5 % during feed production. The ratio between di- and monoester of astaxanthin (80:20) and the three asta-isomers all-E (trans), 9Z (cis) and 13Z (cis) (80:5:15) were approximately constant during feed production. Evaluation of astaxanthin stability during mixing of feed ingredients, extrusion, drying and coating confirmed the high stability of both free and esterified astaxanthin during feed production.

Storage stability of free and esterified astaxanthin in the feed at 5, 15 and 25 °C
- Esterified astaxanthin showed high stability in the feeds during storage at 5 and 15°C with retention values of 93 – 100 % (5°C) and 93 – 96 % (15°C) in the feeds following 12 weeks of storage, equivalent to a storage loss less than 3 mg kg^{-1} esterified astaxanthin. Storage stability was lower at 25°C (79 – 89%), accounting for a loss of 3 to 6 mg kg^{-1} esterified astaxanthin. The stability of free astaxanthin from Carophyll Pink (Diet 6) was lower as compared to esterified astaxanthin at all temperatures, with retention values of 79 % (9 mg kg^{-1}), 69 % (13 mg kg^{-1}) and 48 % (22 mg kg^{-1}) in feeds stored at 5, 15 and 25°C, respectively.

1. Introduction

Astaxanthin in salmonid diets may account for as much as 15 - 20% of the total feed cost in salmon production (Torrissen 1995). The efficiency of using natural carotenoids from alternative sources such as the yeast Phaffia (Sanderson and Jolly 1994), the green algae Haematococcus sp. (Choubert et al., 2006; Barbosa et al., 1999) and crustaceans (Torissen et al., 1981/1982; Choubert and Luquet 1983) for complete or partial replacement of Carophyll Pink, has mostly shown to be inferior or cost-ineffective as compared to synthesized free astaxanthin. Natural carotenoids produced at a lower trophic level are widely abundant in different krill species and products thereof, of which Antarctic large krill (*Euphausia superba)* contain high natural levels of astaxanthin as the principal pigment, while *E. pacifica* may contain even higher levels (Nicol et al., 2000). Antarctic krill has a large potential of commercial harvesting, with an estimated standing biomass of 44 mill tonnes, of which less than 120 000 tonnes was exploited in 2003/2004 (CCAMLR 2005). Astaxanthin in Antarctic krill is present almost entirely as mono-ester (20%) and di-ester (80 %) of long-chained fatty acids in the hydroxyl positions of the astaxanthin molecule, and to a smaller extent (1%) in free form (Langmyhr 2005, pers.comm.). Astaxanthin and its esters in krill further consist of three optical isomers, mainly as 3R,3'R-astaxanthin (60-70%) but also as 3R3'S meso-astaxanthin (10-20%) and 3S,3'S-astaxanthin (10-20%) due to its two hydroxyl groups (Takaichi et al., 2003).

Total astaxanthin contents in Antarctic large krill show some seasonal variation, ranging from 93 to 146 mg kg^{-1} of wet weight, equivalent to 70 -150 mg kg^{-1} astaxanthin in the krill meal, most common variation is between 100 to 130 mg kg^{-1} (Langmyhr 2005, pers.comm.). Krill oil may contain much higher levels, approximately 1500 mg kg^{-1} astaxanthin. As commercial salmon feeds contain from 30 to 60 mg astaxanthin kg^{-1}, inclusion of Antarctic krill meal or krill oil in the diet may reduce the need for supplementation of synthetically produced free astaxanthin (e.g. Carophyll Pink or similar products) that is most commonly used as a feed additive for flesh pigmentation in salmonids.

Astaxanthin retention in salmon muscle is reported to be inefficient, and values down to 3 – 18 % of ingested pigment from various sources are considered normal in muscle (referred to in Barbosa et al., 1999). This may partly be explained by poor uptake from the intestinal tract, high conversion rate of astaxanthin to vitamin A in mucosa, and poor retention in muscle of the absorbed astaxanthin (Foss et al., 1987; Wathne et al., 1998, White et al. 2003). Additionally some of the absorbed astaxanthin function as an antioxidant in fish organs, and further some is metabolised and excreted (Torrissen 1995). Few studies have been carried out to investigate the availability of esterified astaxanthin from Antarctic krill meal and -oils. Astaxanthin diester from krill oil was found to be efficiently hydrolysed in the intestine of 270 g Coho salmon (*Oncorhynchus kisutch*) before being absorbed, and apparently no difference was found in absorption and flesh deposition by using krill astaxanthin diester or synthesized free astaxanthin (Mori et al. 1989). Astaxanthin in the intestinal tract and in sera was further composed only of free astaxanthin, indicating that Coho salmon was able to hydrolyze almost all esters in the intestine into free forms. The ability of pink salmon to hydrolyze esters in the intestine with non-specific lipase has previously been reported by Greene and Selivonchick (1987). In Atlantic salmon, astaxanthin from spray-dried krill hydrolysate was found to be 91 % available as compared to free astaxanthin (Forster, I. unpublished data), while the bioavailability of astaxanthin from Antarctic krill meal was much less (Nicol et al., 2000). Other studies have shown that free astaxanthin is superior to

esterified astaxanthin dipalmitate in the pigmentation of salmonids (Schiedt et al., 1985; Storebakken et al., 1987; Foss et al., 1987).

Several dietary factors such as lipid level (Torrissen et al., 1990; Nickell and Bromage 1998), dietary amount of long chain polyunsaturated fatty acid, HUFA (Christiansen et al., 1993; Bjerkeng et al., 1999a) and dietary level of α-tocopheryl acetate (Bjerkeng et al., 1999b) are all known to stimulate astaxanthin deposition in the flesh of salmonids. Lipid contents of Antarctic krill meal may show some variation, ranging from 8.4 to 22.3 g $100g^{-1}$ (2004) and from 21.4 to 26.3 g $100g^{-1}$ (2005), probably due to seasonal variation and different processing conditions in the krill meal production (Langmyhr 2005, pers. comm.). Krill lipids are classified into 60 % neutral lipids (mostly triacylglycerides, TAGs) and 40 % phospholipids (mostly phosphatidylcholin). During processing, most of the TAG is separated into the oil fraction, while the remaining phospholipids accumulate in the Antarctic krill meal (Langmyhr 2005). As Antarctic krill meal is a good source of marine phospholipids and also of HUFA's such as eicosapentaenoate (C20:5n-3) and docosahexaenoate (C22:6n-3), (Phleger et al. 2002), it may contribute to the efficiency of astaxanthin absorption and deposition in the flesh of Atlantic salmon.

Astaxanthin, vitamin E and vitamin C are all strong antioxidants (Palozza and Krinsky 1992; Hamre *et al*., 1997; Bell *et al*., 2000) and exert important parts of the antioxidant defence system of Atlantic salmon (Hamre et al. 1994 and 1997; Bell et al. 2000). The antioxidant defence system protects the fish against uncontrolled lipid peroxidation, which is initiated by free radicals (Hamre et al. 2004). Fish tissue is rich in HUFAs and hence susceptible to lipid peroxidation (Bell et al. 2000; Hamre et al. 2004). Salmonids are not able to biosynthesize neither carotenoids nor vitamins, and dietary supplies are required to obtain adequate tissue levels of these antioxidants. The availability of astaxanthin from Antarctic krill meal is not known and possible interactions between dietary astaxanthin and antioxidant vitamins that might affect astaxanthin as pigment source has not been investigated by using Antarctic krill meal as pigment source in the diet for Atlantic salmon.

The aim of the present study was to elucidate the efficiency of using natural mono- and diester astaxanthin in Antarctic krill meal for flesh pigmentation in Atlantic salmon and to compare it with the retention of free astaxanthin from Carophyll Pink. Dietary impacts on growth, feed efficiency, digestibility, tissue concentrations and tissue retentions of astaxanthin, were studied in fish fed graded inclusion levels of Antarctic krill meal or Carophyll Pink as pigment sources. Stability of free and esterified astaxanthin during feed processing and during feed storage at three different temperatures is also reported.

2. Material and methods

2.1. Diets

Antarctic krill meal with digestible protein contents of 85.4% (not corrected for indigestible kitin-N), exchanged a 1:1 mixture of two different fish meals with digestible protein contents of 89.8 and 90.6%, as measured biological with mink (Skrede, 1979). Dietary inclusion levels of the Antarctic krill meal was 0, 10, 20, 30 and 40 % (wet weights of the diets), exchanging 0, 13, 27, 40 and 53% of the dietary fish meal protein, and 0, 7, 14, 21 and 28% of total dietary lipid from fish meals and oil in Diets 1, 2, 3, 4 and 5, respectively. Free astaxanthin was not detectable in the krill meal, while high levels of 105 mg kg^{-1} astaxanthin esters was analysed (20% mono- and 80% diester astaxanthin). Natural esterified astaxanthin from the

Antarctic krill meal provided the only pigment source in diets 2 to 5, while Diet 1 (control) contained no added pigment and Diet 6 was supplemented with free astaxanthin (Carophyll Pink) to a level similar to that presumed to be provided by the highest inclusion level of the krill meal (42 mg kg^{-1}). Ingredient composition and chemical contents of the experimental diets are shown in Table 1 and Table 2. The difference in proximate composition of fish meals and krill meal was balanced through a small reduction in crude wheat and fish oil in the diets (Table 1) to keep the dietary protein to lipid ratio constant at 44:30 for all diets. Average protein and lipid levels were 435 ± 2.7 g kg^{-1} and 304 ± 3.3 g kg^{-1}, respectively. The diets were iso-caloric and the energy content ranged from 23.7 MJ kg^{-1} to 24.0 MJ kg^{-1}. All diets were extruded (6.5 mm pellet) at fixed and defined conditions (twin screw) and the physical quality appeared similar for all experimental diets. All diets were kept refrigerated (≤ 5°C) in closed baskets to avoid loss of astaxanthin during the feeding trial.

2.2. Experimental fish and handling

Underyearling Atlantic salmon smolt were transported to Fiskeriforskning's research station in Austevoll, Norway, following smoltification in Aug/Sept 2005, and fed a commercial EWOS Transfer feed, 3 and 5 mm pellet for 27 weeks, prior to a 4 week acclimatization period on a pigment-free experimental diet (~2.4 mg astaxanthin kg^{-1}) prior to start of the experiment 15th March 2006. At the start of the experiment, the salmon (average weight 732 ± 3 g, n = 12, each of 40 fish) was randomly distributed to twelve 2.0x2.0 m glass fibre tanks (3.2 m^3), each of 40 fish. The fish were fed one of the six experimental diets, in duplicate tanks, for a feeding period of 12 weeks. All tanks were equipped for continuous collection of feed refusals. Fish were fed to appetite by automatic feeders, and the daily feed rations were adjusted according to assumed fish biomass and feed intake. The collected feeds were dried in an oven once a week, and the «true» amount of feed eaten was used for determination of feed intake and feed conversion. All tanks were supplied with running seawater taken from 50 m depth. Average water temperature was 9.95 ± 0.6°C during the experiment, controlled by a heat exchanger. Salinity of seawater was 3.2 – 3.3 % throughout the feeding trial and the oxygen content in the outlet water from the tanks was approximately 8.5 mg $liter^{-1}$. The fish were exposed to 24 h light during the experimental period of 83 feeding days.

Individual weight and length of fish were measured at start and at the end of the trial. After a feeding period of 12 weeks, 6 fish from each tank were collected for evaluation of weight, length, gutted weight, liver weight, and for calculation of condition factor, hepatosomatic index (HSI) and dressing out percentage. Collected fish from each tank were anaesthetised, killed with a blow to the head and samples of liver and muscle (n=6 fish/diet) were collected for analysis of lipid, dry matter, astaxanthin, vitamin A, E and C and lipid peroxidation (TBARs). Following a starving period of 5 days to empty dietary remains in the intestine, another 6 fish were collected from each tank according to the same procedure for analysis of lipid, dry matter and astaxanthin in homogenates of whole body, and for calculation of lipid and astaxanthin retention in the body. All samples were thoroughly homogenised and equally distributed to A and B samples at sampling. Samples were kept on dry ice until returning to the laboratories where they were stored at -80°C until further analysis.

2.3. Digestibility

Apparent digestibility of lipid, energy, free and esterified astaxanthin were determined in fish from all diets. Yttrium oxide was added to the feed as an inert marker at a level of 0.1 g kg^{-1}. Pooled samples of about 30 g of faeces (w. w.) were collected following manual stripping of faeces of all fish from each respective tank (n = 30 – 35). In the last week of the feeding trial, some incidences of fish with infected wounds appeared, also resulting in 3 dead fish late in

the trial. In this period feed intake was low in most tanks, and stripping of faeces was repeated following another 8 days of feeding to collect adequate amounts of faeces for the digestibility measurements. Yttrium, lipid and energy, and free and esterified astaxanthin were determined in the six experimental feeds and in pooled samples of faeces from each replicate tank (n = 3 per group). Ethoxyquin (200 mg ethoxyquin kg $^{-1}$ d. m.) was added as a stabilising agent to the pooled samples of faeces, and the mixture immediately frozen and stored at -30 °C. Samples of frozen faeces were lyophilized (final plate temperature 24 °C) and homogenized prior to chemical analyses. Apparent digestibility of nutrients the in experimental diets was calculated from the formula

$$AD = 100 - 100 \; x \; \frac{Y_d \; x \; CX_f}{CX_d \; x \; Y_f}$$

Where d is diet, f is faeces, Y yttrium concentration and CX nutrient concentration.

2.4. Stability of free and esterified astaxanthin during feed production

Stability of free and esterified astaxanthin during initial mixing of ingredients was evaluated by the relative difference between those analysed in the feed blends and those calculated based on inclusion levels and analysed level of esterified and free astaxanthin in the Antarctic krill meal diets and in the Carophyll Pink diet. Stability of free and esterified astaxanthin during heating (88°C, 3 min) and extrusion (120°C, 1 min) of the feed blend; drying (maximum 70°C, 45 min) and coating (45°C, < 2 min) was calculated from the analysed values, only corrected for variable moisture during feed production. Values for astaxanthin in the extruded, dried feeds were however also corrected for variable fat coating to measure impacts of lipid coating during the final production step.

2.5 Stability of free and esterified astaxanthin during storage of feed

Batches of all experimental diets (1 kg) were stored in a cabinet equipped with heating elements to keep the temperature under controlled conditions at 5°C, 15°C and 25°C throughout the feeding period of 12 weeks. Small samples (100 g) of all diets were collected at the start of the trial, and after 4, 8 and 12 weeks for analyses of free and esterified astaxanthin in each respective diet. All samples were collected, homogenized and analysed within one day for accurate measurements of astaxanthin.

2.6. Chemical analysis

All chemical analyses were carried out in duplicate by laboratories accredited by the Norwegian National Accreditation body. In feed ingredients, diets, feces and fish tissue, crude protein (N x 6.25) was determined by the Kjeldahl method (ISO 5983-1997), moisture gravimetrically after drying for 4 h at 105ºC (ISO 6496-1999), and ash after combustion for 16 h at 550ºC (ISO 5984-2002). Lipid content in the fish meals and diets were determined gravimetrically after petroleum ether extraction (Soxhlet technique), (AOCS Ba 3-38) and lipid content in whole body, liver and muscle following acidic extraction (Folch). For digestibility measurements, lipid content in fish meal and faeces were determined according to a modified Bligh & Dyer (1959). Astaxanthin was analysed according to a HPLC method developed by Hoffman La. Roche (1994) following ethanol and dichloride methane extraction of astaxanthin from the experimental diets and fillets. Carophyll Pink in the experimental diets was enzymatically treated in hot water prior to the extraction procedure. Yttrium was determined in feed and faeces by inductively coupled plasma atomic emission spectroscopy (ISO 11885-1996). True protein digestibility was determined in mature male mink as described by Skrede (1979). Levels of vitamin A, E and C were determined in diets, muscle and liver samples. Vitamin A (sum retinol and sum didehydroretinol) was determined by

HPLC and UV detection after saponification and extraction in hexane as described by Moren et al. (2002) modified from Nöll (1996). Vitamin E was determined by HPLC and fluorescence detection after saponification and extraction according to Lie et al. (1994). Vitamin C was extracted in meta-phosphoric acid and measured electrochemically after separation by HPLC as described by Mæland and Waagbø (1998). Certified reference materials, in house control materials and control chart were used for quality assurance. Lipid peroxidation in diets, muscle and liver was measured colorimetrically as thiobarbituric acid reactive substances (TBARs) according to Berntssen et al. (2000) and Hamre et al. (2001).

2.7. Statistical methods and calculations

Biological and analytical data were subjected to regression analyses and one-way analysis of variance (ANOVA) using STATISTICA (Ver 7.1, StstSoft, Tulsa, OK, USA) and differences between means were tested using Tukey HSD test (Sokal and Rohlf, 1981). Effects with a probability $P < 0.05$ were considered significant. Pearson correlation and Spearman's rank correlation analysis was further used to examine possible relationships between feed parameters and dietary responses.

Growth, feed intake, feed conversion were determined according to the following formulas (BW_2 = final body weight, BW_1 = initial body weight):

- Specific growth rate, SGR = $(\ln BW_2 - \ln BW_1)$ / feeding days.
- Thermal growth coefficient, TGC = $(BW_2^{1/3} - BW_1^{1/3}) * 1000 / \sum$ (temp.(°C) * feeding days) according to Cho (1992).
- Daily feed intake per fish = g feed intake / days / number of fish.
- Daily feed intake, % of weight gain = g feed intake / days /$((BW_2 + BW_1)/2 * 100)$ / fish no.
- Feed efficiency = g live weight gain / g dry feed eaten.

3. Results

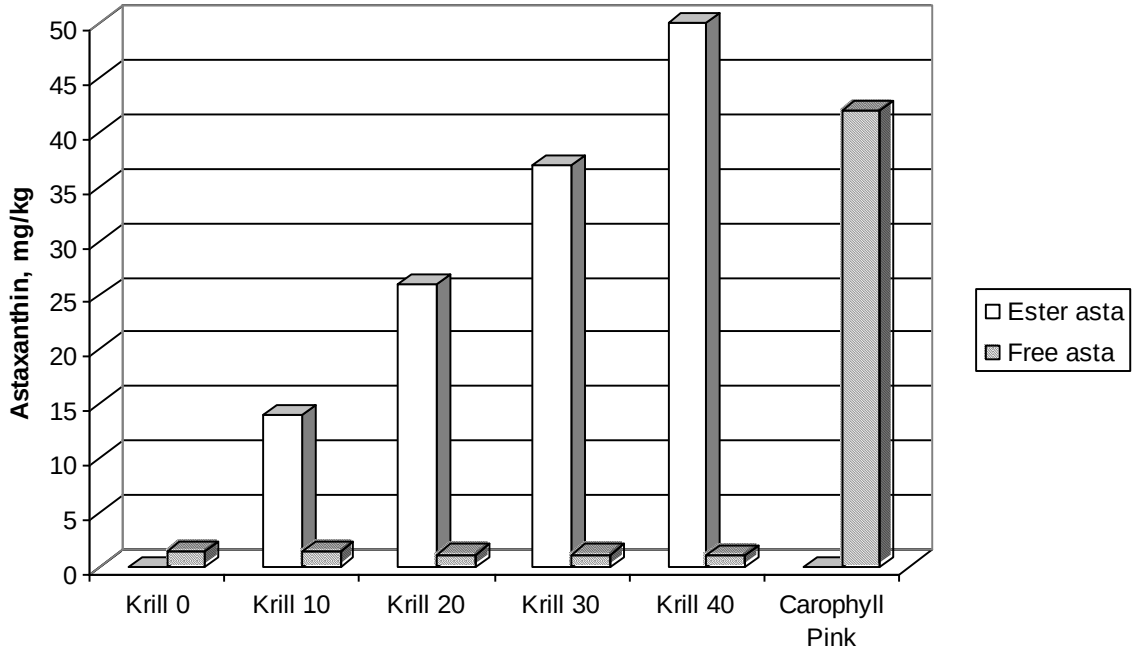
3.1. Dietary composition

Proximate composition of experimental diets showed that the intended levels of nutrients and calculated levels of free and esterified astaxanthin were largely achieved (Table 2). The low basal level of free astaxanthin that was found in Diets 1 to 5 (1.2 ± 0.2 mg kg^{-1}) was expected due to a small contribution of natural astaxanthin from the fish oil. According to the initial analysed value of esterified astaxanthin in the Antarctic krill meal (105 mg kg^{-1}) retention values after diet processing in the krill meal diets were above 100 % for Diets 1 to 5 (Table 2). Analysed levels in the diets imply that at least 125 mg kg^{-1} esterified astaxanthin was present in the krill meal at start of feed production, considering the graded inclusion levels and analysed levels of astaxanthin. Based on the initial analysed value of astaxanthin in the krill meal, only 42 mg kg^{-1} free astaxanthin was included as Carophyll Pink in Diet 6. Dietary levels of free and esterified astaxanthin are shown for all diets in Table 2 and illustrated in Fig. 1.

The levels of vitamin A (sum retinol and sum didehydroretinol) did not differ between the different diets, while some differences in vitamin E and vitamin C levels were seen (Table 2). Vitamin E levels increased with a higher inclusion of Antarctic krill meal, the highest levels of vitamin E were found in diet 4 and 5 (30 and 40% inclusion of Antarctic krill meal, respectively). The vitamin E level in diet 6 (Carophyll Pink) was similar to levels found in the diets with 0 and 10 % inclusion of the Antarctic krill meal. The level of vitamin C varied between the different diets; the lowest level of vitamin C was found in the diet with the

highest inclusion of Antarctic krill meal, and the highest level of vitamin C was seen in diet 2 (10% krill meal). Vitamin C levels in diet 4 and 5 (30 and 40% krill inclusion, respectively) were similar to levels found in diet 6 (Carophyll Pink). TBARs levels varied among the six diets (Table 2). The TBARs levels decreased with increased inclusion of Antarctic krill meal, the lowest levels of TBARs were found in diet 4 and 5 (30 and 40% krill inclusion, respectively). The TBARs level in diet 6 (Carophyll Pink) was higher compared to levels seen in diet 4 and 5, and similar to levels found in the diets with 0 and 10% inclusion of Antarctic krill meal.

Fig. 1 Free and esterified astaxanthin in the diets



3.2. Growth, feed intake and feed conversion

During the experimental trial fish more than doubled their body weights and showed high growth rates (TGC = 3.35 ± 0.19; SGR = 0.97 ± 0.05 %) suggesting that the experimental conditions were good and growth performance close to what can be expected in commercial salmon farming in the southern part of Norway (10°C water temperature). The feeding trial was carried out without any major problems or loss of fish (less than 1.3 % mortality) although some fish with infected wounds appeared late in the trial, but independent of dietary treatment. Final body weights ranged from 1554 to 1708 g, Table 3. Final body weights and weight gain showed no diet dependent differences during the 12 weeks feeding trial ($P > 0.05$) and according to regression analyses, increased level of krill meal in the diet did not affect SGR or TGC ($P > 0.05$). According to ANOVA, TGC and SGR showed significant dietary differences ($P = 0.05$), while the Tukey HSD test failed to confirm this. As fish fed 30 % krill meal in the diet showed high growth performance (ANOVA), it is concluded that the slightly lower growth performance of fish fed 20% krill meal inclusion (Diet 3) could be due to some circumstantial factors interfering with results; e.g. small No. of fish in each tank and variable incidences of fish with infected wounds. In fish fed 40 % krill meal in the diet, a similar small reduction in growth performance and feed intake was found. Overall growth was however high in all groups, with SGR ranging from 0.91 to 1.01 and TGC from 3.10 to 3.54; all within acceptable limits for salmon reared at 10°C.

The daily feed intake ranging from 0.82 to 0.88 % of mean body weights was unaffected by increased inclusion of krill meal in the diet ($P > 0.05$), while a significant difference was found between fish fed 20 % krill meal showing the lowest feed intake, and those fed the Carophyll Pink diet showing the highest feed intake ($P < 0.05$). This can be explained by the slightly lower growth and feed intake in fish fed Diet 3 (20% krill meal). Feed conversion was completely unaffected by the dietary inclusion level of Antarctic krill meal and the different pigment sources ($P > 0.05$).

3.3. Astaxanthin in whole body, muscle and liver

Significant linear regression equations were found for total free astaxanthin ($R^2 \geq 0.90$) and All-E astaxanthin ($R^2 = 0.90$) in whole body and muscle; and for 13Z astaxanthin ($R^2 = 0.87$) in whole body, with increased inclusion of Antarctic krill meal in the diet ($P < 0.001$). Anova one-way analysis further showed significant dietary differences for total level of free astaxanthin and All-E astaxanthin in whole body ($P < 0.001$) and muscle ($P < 0.01$) with respect to increased level of Antarctic krill meal in the diet; as shown in Table 4. Significant differences were also found by using Carophyll Pink as the pigment source relative to diets with high inclusion of the Antarctic krill meal. Total level of free astaxanthin in muscle was significantly higher in fish fed Carophyll Pink, as compared to fish fed 40 % inclusion of Antarctic krill meal in the diet ($P < 0.0001$). Total astaxanthin in whole body showed similar changes, but smaller statistical differences as Diet 5 (40% krill meal) and Diet 6 (Carophyll Pink) was not statistically different ($P > 0.05$).

All-E (trans) astaxanthin was the dominating astaxanthin form, contributing to 83 – 84% of total astaxanthin in whole body and muscle in all dietary groups (Fig. 2 and 3). The 13Z (Cis) astaxanthin showed lower tissue levels (average ~ 13 %) and similar response to the inclusion level of krill meal and Carophyll Pink in whole body and liver ($P < 0.01$), but not in muscle ($P > 0.05$). The 9Z (Cis) astaxanthin was below detection limit in all measured tissues (<0.10 mg kg^{-1}) (Table 4). In the Carophyll Pink diet (Diet 6), the ratio between All-E; 13Z and 9Z-astaxanthin isomers was about 80:14:5. Tissue distribution of the free astaxanthin isomers showed a similar pattern in whole body and muscle for all groups, irrespective of pigment source used. In liver, the isomers followed a different pattern with lower All-E astaxanthin (48%) and higher 13Z astaxanthin (48%), possibly indicating important metabolic functions of 13Z astaxanthin other than those for storage in liver.

Only free astaxanthin (cis/trans) was found in whole body of fish from all dietary groups, indicating no interference with intestinal remains of esterified astaxanthin following the starvation period prior to collection of fish samples. This means that no remains of intestinal contents executed parts of the whole body sample. Esterified astaxanthin was not found in the muscle tissue, while in liver, there appeared to be some small quantities of esterified astaxanthin below detection limit (< 0.10 mg kg^{-1}). This is most likely due to some miscellaneous error or interfering agents, and do not necessarily mean that esterified astaxanthin is absorbed or that re-esterification is taking place following absorption of free astaxanthin.

Fig. 2 Whole body astaxanthin

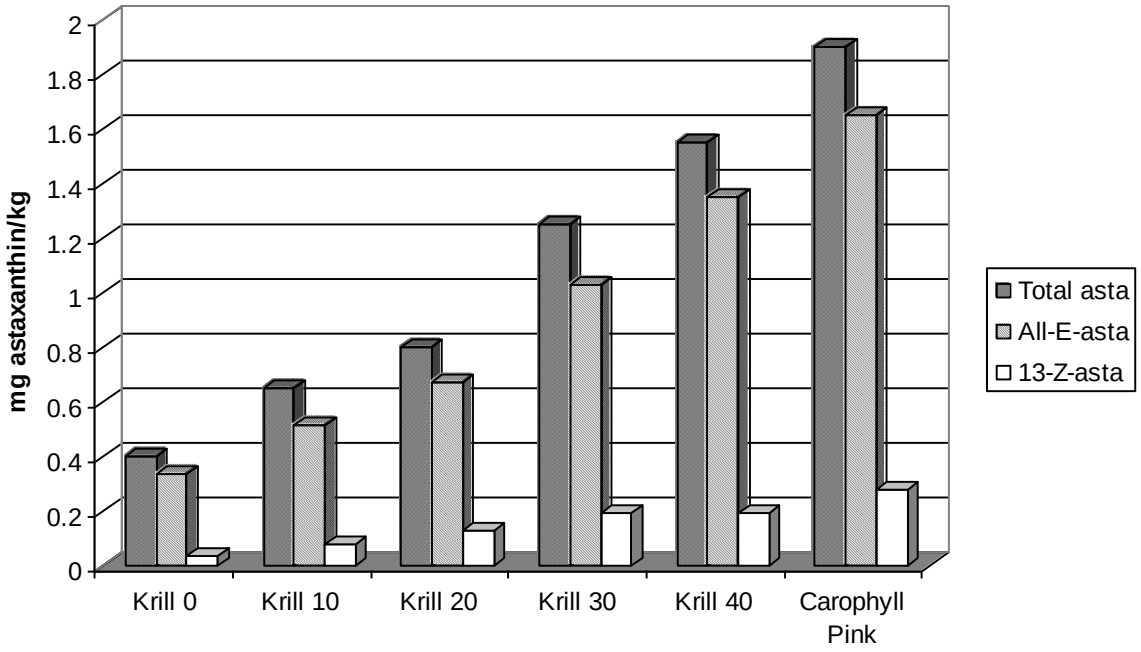


Fig. 3 Muscle astaxanthin

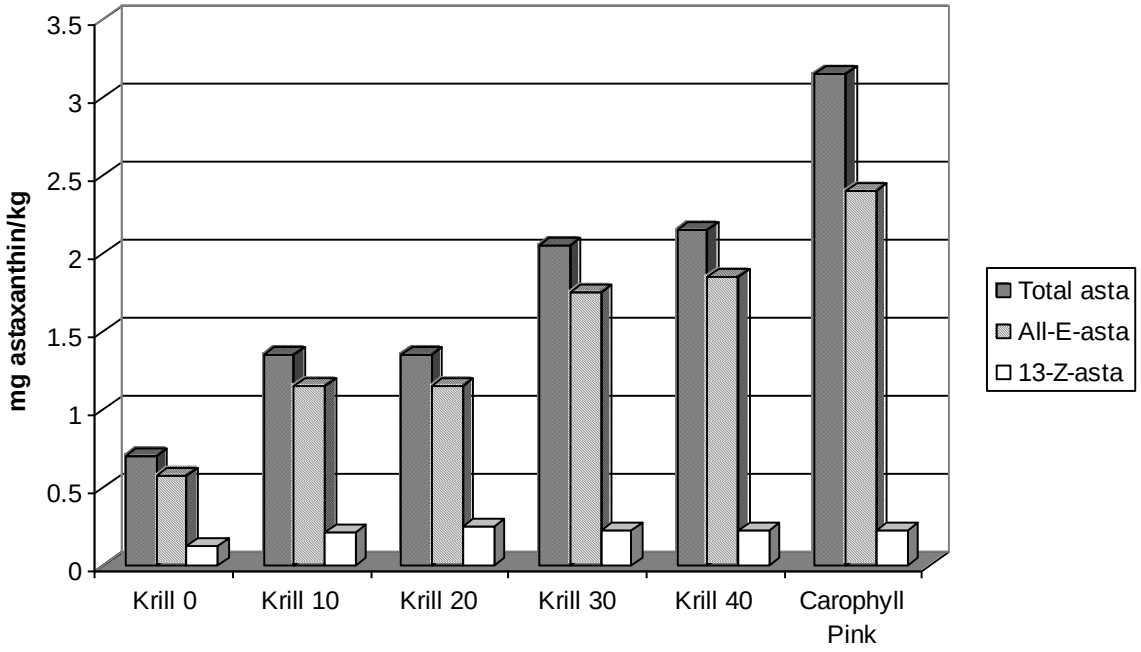
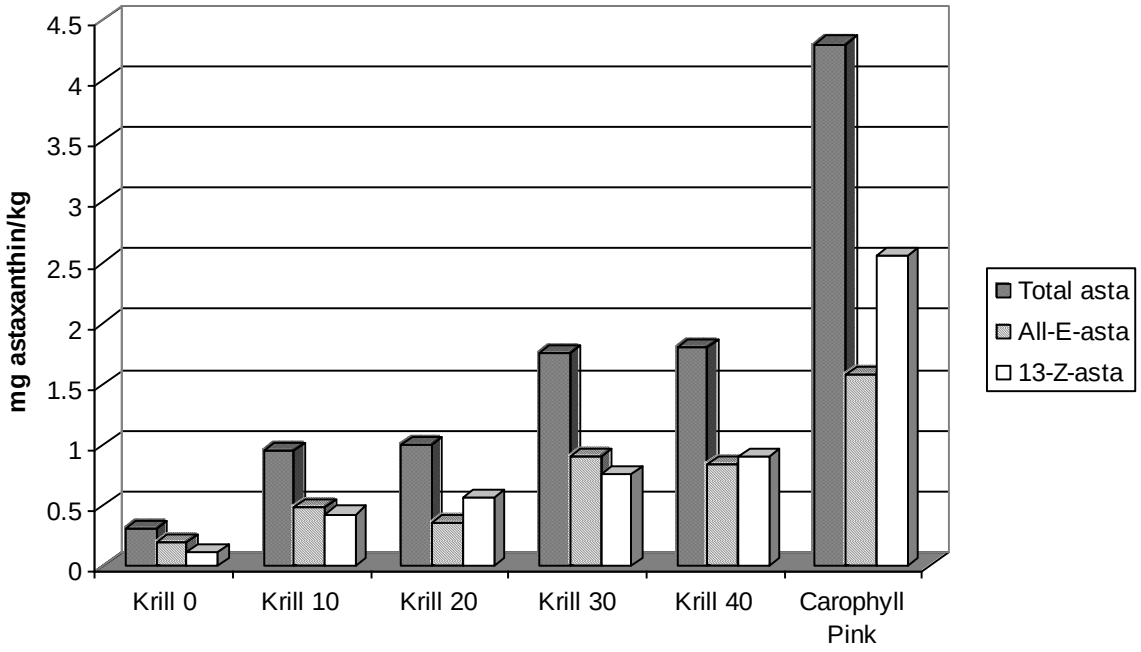


Fig. 4 Liver astaxanthin



Significant linear regression equations were found for total free astaxanthin ($R^2 = 0.85$), All-E astaxanthin ($R^2 = 0.65$); and 13Z astaxanthin ($R^2 = 0.93$) in liver, with increased inclusion of Antarctic krill meal in the diet ($P < 0.01$). Significant dietary differences were however only found for 13Z-astaxanthin ($P < 0.0001$); Diet 5 (40% Antarctic krill meal) producing higher 13Z-astaxanthin in liver as compared to diets with 0, 10 and 20 % Antarctic krill meal (Table 4). Total level of free astaxanthin and all-E-astaxanthin in liver did not show statistically significant differences with respect to increased level of Antarctic krill meal in the diet ($P > 0.05$), although the responses in liver closely resembled and significantly correlated to that in whole body ($r = 0.84/0.73$; $P < 0.05$) and muscle ($r = 0.91/0.80$; $P < 0.05$). The SEM values showed large variations between replicates for diet groups 4, 5 and 6 (Table 4), probably explaining why no significant differences appeared. Dietary differences were found between the two different pigment sources, Carophyll Pink producing higher total astaxanthin levels in the liver relative to any of the Antarctic krill meal diets ($P < 0.01$). The ratio between All-E (trans) and 13Z (Cis) astaxanthin in liver was different from that in muscle and whole body, showing lower trans (All-E) astaxanthin (48%) and higher Cis (13Z) astaxanthin (48%), but also showing changes due to increased inclusion of Antarctic krill meal in the diets as well as large in between replicate variation. The relative amount of total astaxanthin and All-E-astaxanthin in liver increased from 50 to 85 % and from 30 to 50%, respectively, relative to the muscle concentrations, with increased concentration of Antarctic krill meal in the diet. Fish fed the Carophyll Pink diet showed even higher relative astaxanthin values of respectively 138 % (total astaxanthin) and 72 % (All-E-astaxanthin), as compared to the muscle concentrations. This indicates some metabolic adaptive changes due to increased Antarctic krill meal inclusion levels and to the different dietary pigment sources, in liver. Whole body total astaxanthin and All-E-astaxantin was average 60 % of the concentration in muscle, irrespective of diets and pigment sources, reflecting the large relative muscle mass in the body.

3.4. Lipid in whole body and muscle

The pigment free control diet produced close to significant lower whole body lipid levels as compared to Diet 2 (10 % Antarctic krill meal) and Diet 6 (Carophyll Pink), Table 4 ($P = 0.06$). A tendency towards increased body lipid content appeared by inclusion of Antarctic krill meal in the diet, but this was not confirmed by regression analysis ($P > 0.05$). Carophyll Pink in the diet produced high body lipid level, similar to that produced by the Antarctic krill meal diets and nearly significantly higher than the level found in fish fed the pigment free control diet ($P < 0.10$). The lipid content in the muscle did not differ between the dietary groups ($P > 0.05$) (Table 4). Significant correlations were found between whole body lipid level measured on dry weight basis, and total astaxanthin contents in whole body and muscle, All-E (trans) astaxanthin in muscle and 13Z (cis) astaxanthin in liver ($r = 0.6$, $p < 0.05$) by including all diets. These findings warrant further studies, as they indicate a variable retention or β-oxidation of krill-lipid compared to fish-meal lipid. Particularly important if fatty acid profiles in muscle of salmon fed Antarctic krill that may results in changes in the amount and ratio of n-3 HUFAs such as DHA and EPA (see Introduction, Cht.1). DHA and EPA is described to be the most important fatty acids for human health.

3.5. Digestibility of astaxanthin and lipid

High and increasing levels of free astaxanthin were detected together with smaller amounts of esterified astaxanthin in faeces of fish fed increased inclusion of Antarctic krill meal in the diet (Diet 2 – 5), Fig. 5. This indicates efficient intestinal hydrolyses of esterified astaxanthin in the Antarctic krill meal diets. Apparent digestibility of esterified astaxanthin (sum mono- and di-astaxanthin) was average 90.8 % ± 1.9 % and showed no differences with respect to the inclusion level of the krill meal ($P > 0.05$) (Table 5). As astaxanthin is probably only absorbed in free forms, this suggests that approximately 10 % esterified astaxanthin was not hydrolysed in the intestine. Esterified astaxanthin appeared in small quantities in faeces of fish fed the pigment free control diet and in the diet with Carophyll Pink, suggesting that some interference in measuring of the digestibility values probably occurred. However, subtraction of this presumably endogenous produced esterified astaxanthin would increase the digestibility values to 97.5%, indicating an even more efficient hydrolysis of esterified astaxanthin in the intestine.

Apparent digestibility of total astaxanthin (sum free and esterified astaxanthin) was averagely 53.4 % ± 5.1 % for all fish fed Antarctic krill meal or Carophyll Pink as the pigment sources (Diet 2 – 6), showing no differences with respect to the inclusion level of krill meal or the pigment source ($P > 0.05$) (Table 5). The digestibility method reports the intestinal remains subtracted from the total in the diet, claiming the difference to be the absorbed. Possible urinary excretion is however not measured, and hence the actual amount of ingredient absorbed is not measured. Further, the site and rate of hydrolytical cleavage of esterified astaxanthin may also put some limitations to the amount of free astaxanthin available for absorption. In the pigment free control diet (Diet 1), apparent digestibility of astaxanthin was negative (-11.1%), indicating net loss of astaxanthin in the fish. In the same group, low tissue astaxanthin levels and negative whole body retention levels appeared, confirming net loss of astaxanthin during the feeding period (Fig. 6).

A significant linear regression equation was found for lipid digestibility ($R^2 \geq 0.56$) with increased inclusion of Antarctic krill meal in the diet ($P < 0.01$), in accordance with the tendency towards higher body lipid levels by feeding Antarctic krill meal. The relative increase in lipid digestibility was approximately 5 % in Diet 5 (40% krill meal), Table 5. Carophyll Pink in Diet 6 did not affect lipid digestibility and was left out of the regression

model. Lipid digestibility (average 88.1 ± 2.0%) measured for all diets did not show significant dietary differences ($P > 0.05$) between diet groups..

Fig. 5 Free and esterified astaxanthin in feed and faeces

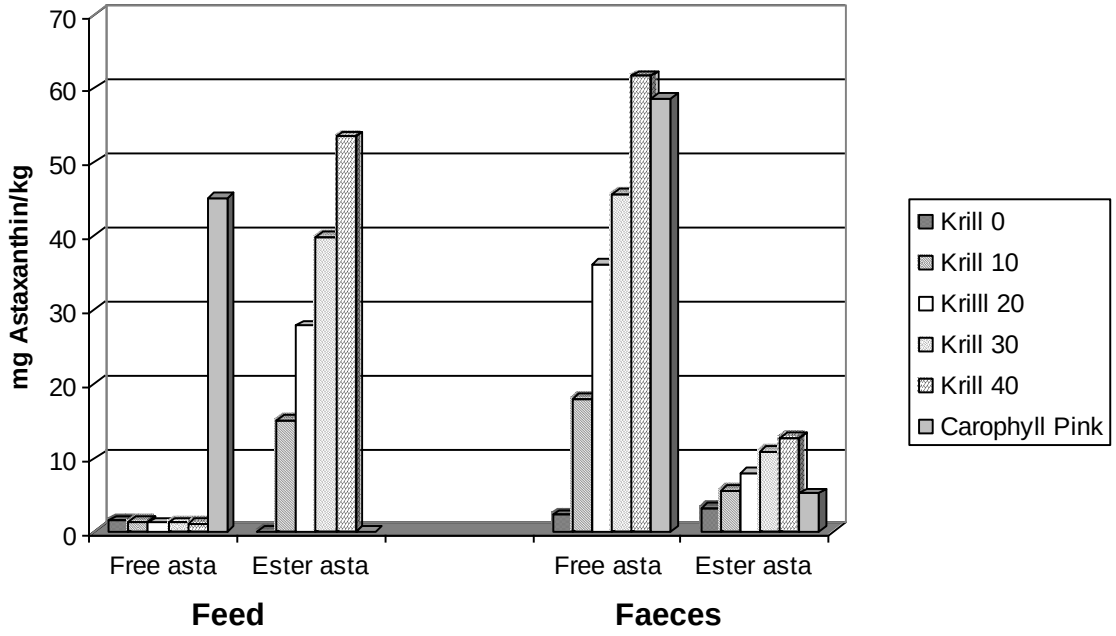
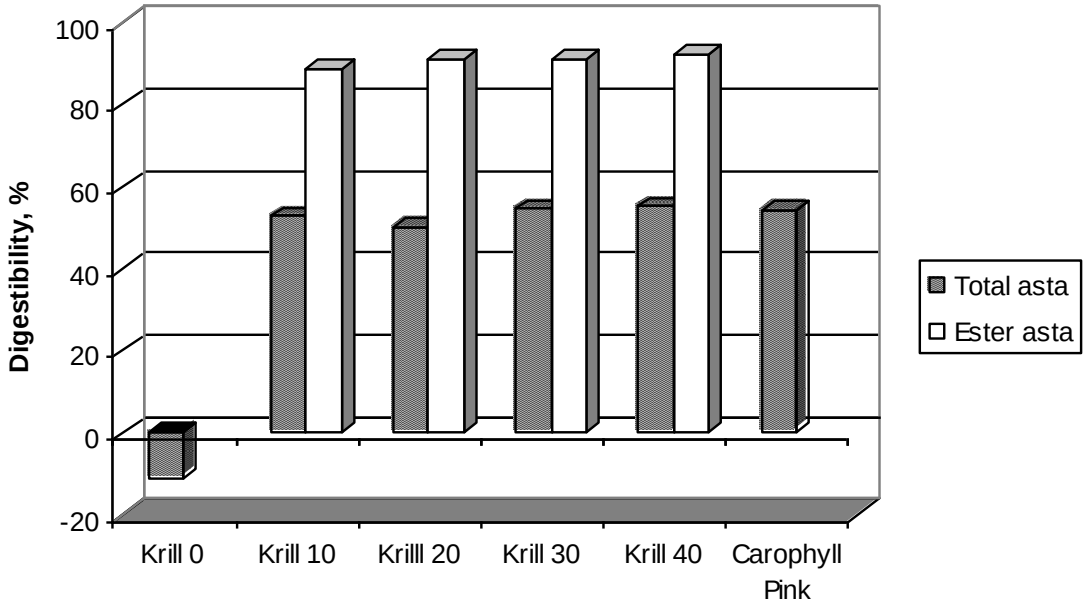


Fig. 6 Digestibility of astaxanthin



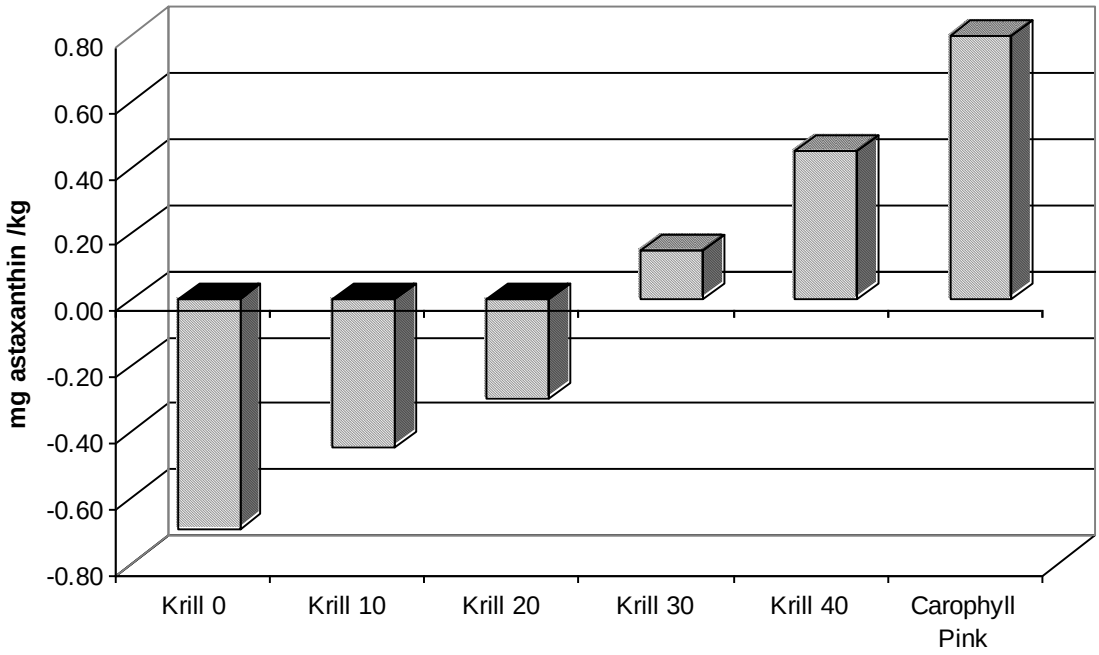
3.6. Whole body retention of astaxanthin and lipid

A significant regression equation was also found for whole body astaxanthin retention ($R^2 \geq 0.62$) with increased inclusion of Antarctic krill meal in the diet ($P < 0.01$). Retention value of

astaxanthin was negative in the pigment free control diet (-12.3%), Table 5, and significantly different from all other diets ($P < 0.0001$). This indicates net loss of astaxanthin during the 12 week feeding period. This was also shown by negative digestibility values (Table 5) and low tissue astaxanthin levels (Table 4). Whole body astaxanthin was lower than the initial level at start of the feeding trial (1.1 mg kg^{-1}) in diets with 0, 10 and 20 % krill meal as illustrated in Fig. 7 showing the difference between final and initial levels. The increased astaxanthin retention values to approximately 2 % of the total amount of astaxanthin eaten in Diets 2 and 3 (10 and 20% krill meal inclusion, respectively), Table 5, hence did not produce adequate amounts of available astaxanthin to maintain the initial tissue levels during this 12 week feeding period (Table 5). Fish fed diets with 30 and 40 % krill meal showed higher astaxanthin retention, about 4 % (Table 5) and increased body concentrations as compared to the initial level (Fig. 7). Retention of astaxanthin from Carophyll Pink was higher (6.5 %) but not statistically significant different from the retention values produced by the Antarctic krill meal included at 30 and 40 % of the diet ($P < 0.05$). Only diets with 30 and 40 % Antarctic krill meal, and the Carophyll Pink diet, produced muscle astaxanthin levels higher than the initial level (1.7 mg astaxanthin kg^{-1}).

The increased astaxanthin retention values produced by Diet 1 to 6 were further shown by increased levels of available astaxanthin measured as mg astaxanthin kg^{-1} weight gain, equivalent to -0.16, 0.27, 0.52, 1.36, 1.92 and 2.51 in Diets 1 to 6, respectively (Table 5). Linear increases in specific astaxanthin retention by increased inclusion of Antarctic krill meal was found according to regression analyses ($R^2 = 0.89$; $P < 0.0001$).

Fig. 7 Whole body astaxanthin deposition (final - initial level)



According to regression analysis, whole body lipid retention did not show significant changes due to increased inclusion of Antarctic krill meal in the diet ($P > 0.05$), although tendencies towards higher lipid digestibility and body lipid levels by feeding Antarctic krill meal was found. Whole body retention (average 85.2 ± 3.4%) of the dietary lipid were 79 % in the

pigment free control diet (Diet 1), 84 to 88 % for the Antarctic krill meal diets (Diets 2 to 5) and 87 % for the Carophyll Pink diet (Diet 6), indicating a very high retention of digested lipid from all diets and no significant dietary differences ($P > 0.05$), Table 5. Whole body lipid content and lipid retention in the body were however lowest in the pigment free control diet (Diet 1) accompanied by the lowest flesh astaxanthin concentration and retention values (Table 4 and 5).

3.7 Vitamin A, E, C and TBARs in muscle and liver

All vitamin levels in muscle were lower compared to levels found in liver (Table 6). The different diets did not influence vitamin levels in muscle, as no significant differences ($P > 0.05$) in the levels of vitamin A, E or C between dietary treatments were found (Table 6). In liver the levels of vitamin A and C were not affected by dietary treatments, while the level of vitamin E increased with increased inclusion of Antarctic krill meal. Significant ($P < 0.003$) higher levels of vitamin E were found in liver from fish fed diet 4 and 5 (30 and 40% inclusion of Antarctic krill meal, respectively) as compared to the non-pigmented control diet. The liver vitamin E levels correlated well, although not significantly ($P > 0.05$), with dietary vitamin E levels, which varied slightly due to high inclusion of Antarctic krill meal (Table 2). TBARs values were higher in liver compared to muscle (Table 6). In neither muscle nor liver TBARs varied between diet groups ($P > 0.05$). The low TBARSs levels found in muscle and liver suggest low lipid peroxidation in these tissues.

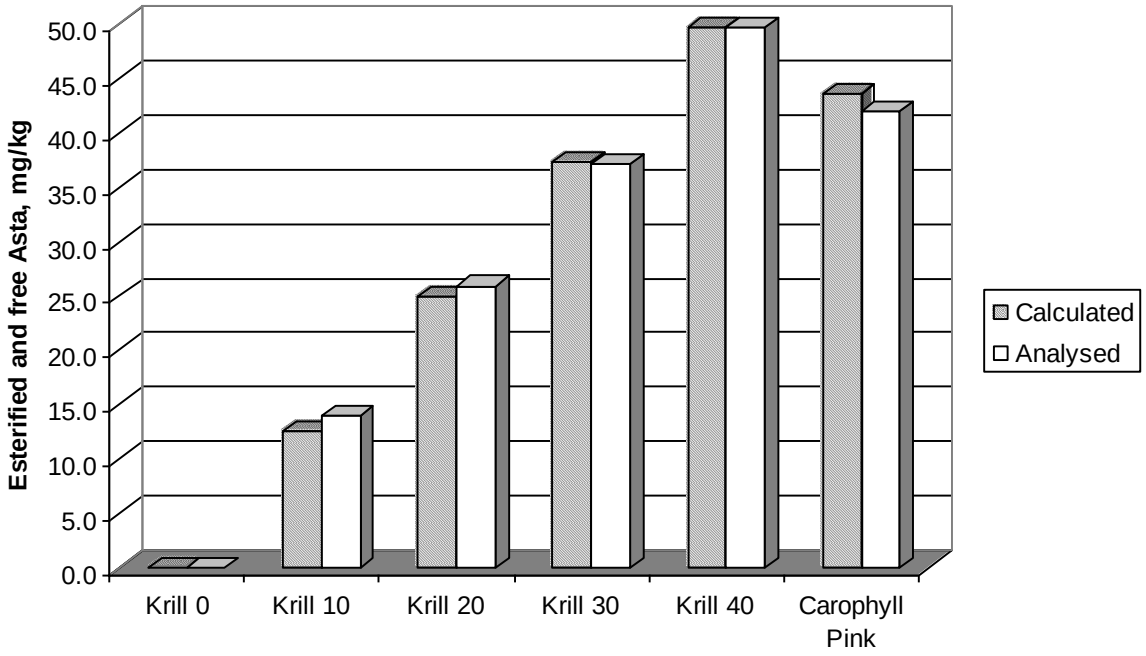
3.8 Condition-factor, HSI and dressing out percentage

According to regression analyses, weights of sampled fish in each tank correlated to final body weights ($r = 0.79$, $p < 0.05$), with the exceptions of results in tank 19 (Diet 4) and tank 18 (Diet 5) showing respectively lower and higher weights. Mean weights of sampled fish were approximately similar, except for Diet 2 and 3, showing respectively slightly elevated and reduced weights, as compared to the other diets. No significant differences were found between dietary groups for body weight, length, gutted weight or liver weight of sampled fish ($P > 0.05$), or the calculated indices condition-factor, dressing out percentage and HSI ($P > 0.05$) (Table 6).

3.9. Stability of free and esterified astaxanthin during feed production

According to the analysed value of esterified astaxanthin (adjusted) in the Antarctic krill meal (111 mg kg^{-1}) retention values in the krill meal diets were 99% or higher for Diets 1 to 5 (Table 2, Fig. 8). Analysed levels in the diets imply that at least 125 mg kg^{-1} esterified astaxanthin was present in the krill meal at start of feed production. Based on the first analysed value in the krill meal (105 mg kg^{-1}), 42 mg kg^{-1} free astaxanthin was included as Carophyll Pink in Diet 6 (Table 1). By calculating the sum of free astaxanthin from Carophyll Pink (42 mg kg^{-1}) and from the other feed ingredients (1.2 mg kg^{-1} diet), a total loss less than 4 % free astaxanthin was found during the feed production (Fig. 8). The ratio between di- and monoester of astaxanthin (80:20) and the three asta-isomers all-E, 9Z and 13Z (80:5:14) were kept approximately constant during feed production (data not shown).

Fig. 8 Astaxanthin in the experimental diets



The corrected level of esterified astaxanthin (125 mg kg^{-1}) was used for calculation of the stability of esterified astaxanthin during mixing of feed ingredients, extrusion, drying and coating of the experimental feeds, to measure loss of astaxanthin during feed production. The calculated and analysed values are shown in Table 7, and the range of relative retention values listed below, all values calculated in dry matter to correct for variable moisture during feed production. Due to small dietary loss, only sum of esterified (mono- and diester) and sum free astaxanthin (all-E, 9Z and 13Z) are presented.

	Mixing	Extrusion	Drying	Coating	Feed production
Esterified astaxanthin, %	97 – 98	99 – 104	97 – 103	97 – 105	99 – 113
Free astaxanthin, %	122	96	102	93	97

3.10. Storage stability of free and esterified astaxanthin in the feed at 5, 15 and 25 °C
Esterified astaxanthin showed high stability in the feeds during controlled storage at 5 and 15°C (Fig. 9 and Fig. 10), with retention values of 93 – 100 % (5°C) and 93 – 96 % (15°C), equivalent to a storage loss less than 3 mg kg^{-1} esterified astaxanthin. Storage stability was lower at 25°C (79 – 89% retention), accounting for a total loss of 3 to 6 mg kg^{-1} esterified astaxanthin (Fig. 11).

The stability of free astaxanthin from Carophyll Pink (Diet 6) was lower than esterified astaxanthin from the Antarctic krill meal at all temperatures, with relative retention values of 79, 69 and 48 % in feeds stored at 5, 15 and 25°C, respectively, equivalent to storage loss of 9, 13 and 22 mg kg^{-1} (Fig. 12).

Fig. 9 Stability of esterified astaxanthin, 5°C

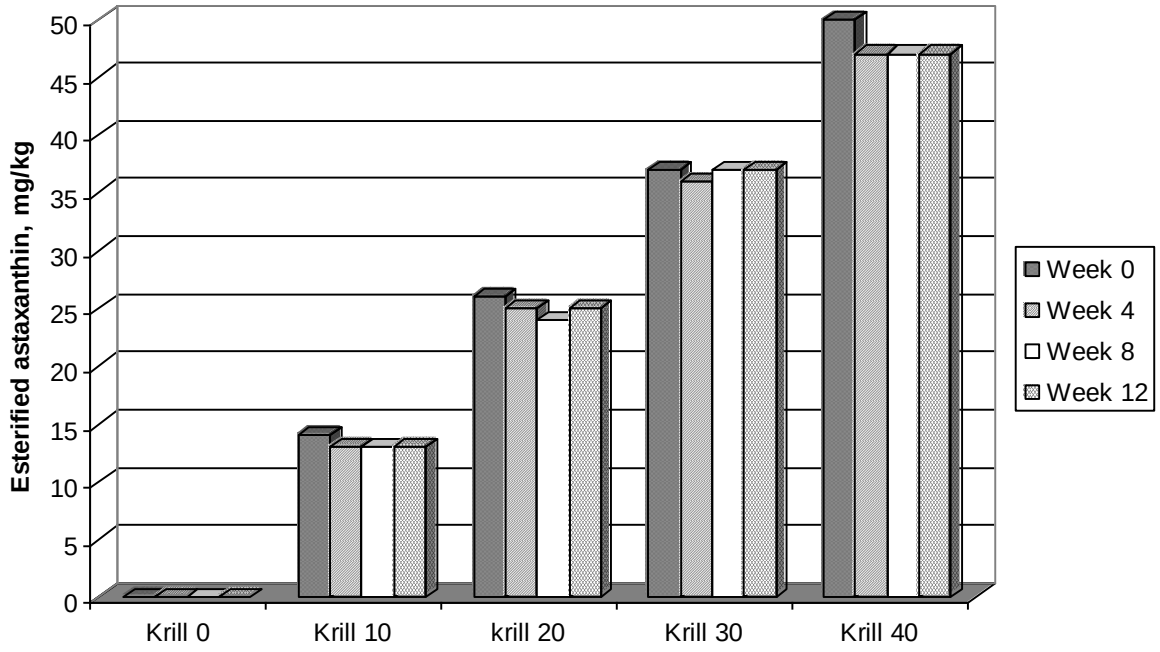


Fig. 10 Stability of esterified astaxanthin,15°C

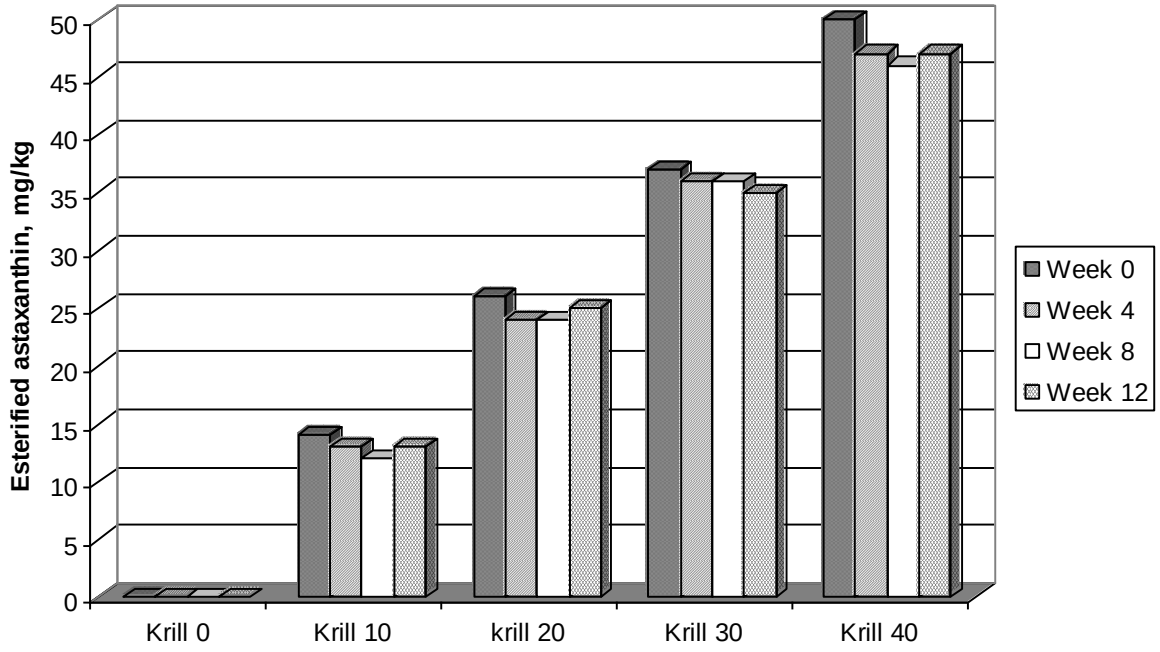


Fig. 11 Stability of esterified astaxanthin, 25°C

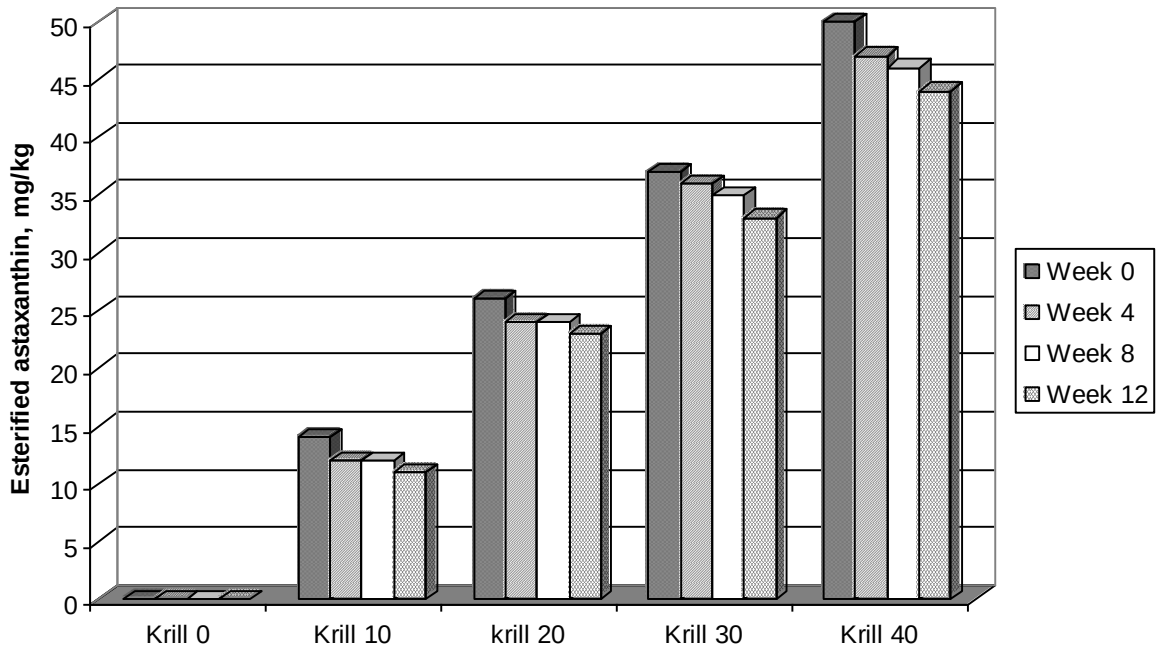
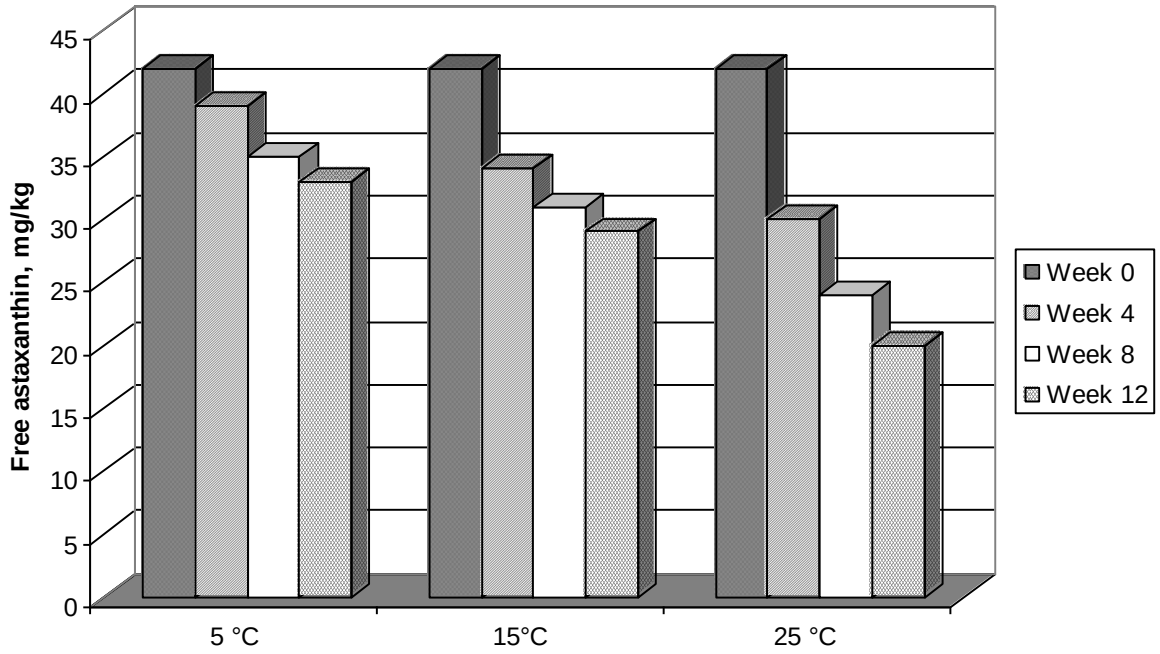


Fig. 12 Stability of free astaxanthin, 5, 15 and 25°C



4. Discussion

The high growth and feed efficiency data in the present 12 week feeding trial, with no differences dependent on krill inclusion level, show that all ingredients and mixtures thereof, supported growth and feed utilization equally well, in agreement with earlier findings where various krill species were tested as protein replacers in salmonid diets (Julshamn et al. 2004, Moren et al. 2006). The slightly lower growth performance and feed intake of fish fed 20 % Antarctic krill meal may be explained by some higher incidence of fish with infected wounds in both replicates, the same fish also showed somewhat reduced flesh coloration. In the present trial, krill was included mainly to evaluate the potential as a pigment source, however, due to krill meal consisting of mainly protein and lipid, and lipid being known to affect pigment metabolism (Torrissen et al. 1990), both lipid digestibility and retention were evaluated. Regression analyses revealed increased lipid digestibility as krill inclusion increased. As growth did not vary between diet groups, this indicates that the krill-lipid was a great energy source, stimulating β-oxidation (Nordgarden et al. 2003a, Oxley et al. 2005, Stubbhaug et al. 2005), resulting in efficient protein sparing (Nordgarden et al. 2003b). The trial clearly demonstrate that esterified astaxanthin from Antarctic krill meal is utilized for flesh pigmentation in Atlantic salmon, but also that the efficiency of utilization is lower as compared to free astaxanthin from Carophyll Pink. Apparent digestibility of astaxanthin were similar for all diets, irrespective of krill meal inclusion levels and pigment sources used, and the small remains of esterified astaxanthin in the intestine also indicated a very efficient hydrolysis of esterified astaxanthin, possibly due to intestinal activity of unspecific lipases. The measured apparent digestibility do not correct for possible differences in excretion rates of astaxanthin by using different pigment sources, that may influence “true” astaxanthin digestibility. According to results from the present study it is difficult to know if the poorer utilization of astaxanthin from Antarctic krill meal is due to slow hydrolysis of esterified astaxanthin into free forms available for absorption, or to reduced solvatization and incorporation of astaxanthin into mixed micelles and lipoproteins during intestinal absorption and blood transport into the liver, as pointed out as some of the limiting factors by Bjerkeng et.al. (1999a,b). The blood clearance rate of carotenoids into the tissues is found to be slow and limited by the low rate of elimination from the different blood lipoprotein fractions (in Wathne et al. 1998). On condition that similar levels of blood astaxanthin were obtained, similar muscle concentrations would be expected. Unfortunately, blood astaxanthin was not analysed in the present study. In liver, the amount of total astaxanthin and All-E-astaxanthin increased relative to the muscle concentrations with increased inclusion of Antarctic krill meal and with Carophyll Pink in the diet. Liver is an important metabolic organ, and the increased relative astaxanthin concentrations found may indicate increased amount of free astaxanthin available for incorporation into the lipoproteins blood transport system and for deposition of astaxanthin into the tissues. This was confirmed by increased muscle astaxanthin concentrations with increased inclusion of Antarctic krill meal in the diet and also by using Carophyll Pink as dietary pigment source.

Retention value of astaxanthin from Carophyll Pink (6.5%) are well in line with other studies showing 6.0 to 7.4% retention in Atlantic salmon fed diets with different fish oils (Bjerkeng et al., 1999a; Bjerkeng et al., 1999b) and provided with either 40 or 50 mg astaxanthin kg^{-1} diet. In one of these studies, it was shown that retention value of astaxanthin was significantly increased by feeding lower amounts of astaxanthin in the diet (30 mg kg^{-1}) while the retention leveled off at dietary concentrations of approximately 60 mg astaxanthin kg^{-1} dry feed (referred to in Wathne et al. 1998). In the present study, available astaxanthin from esterified astaxanthin steadily increased with increased amounts of Antarctic krill meal in the diet,

indicating that the lipoprotein transport system is not yet saturated and thus limiting for astaxanthin utilization. According to this, the rate of hydrolytical cleavage of the esterified astaxanthin in the intestine, and/or the solvatization and incorporation of astaxanthin into mixed micelles during intestinal absorption might be limiting for efficient utilization of esterified astaxanthin in Antarctic krill meal.

The stable levels of vitamins E and C in muscle and levels of vitamin C in liver indicate that increases in dietary krill did not lead to increased demand for antioxidant vitamins. All diet ingredients, including the krill meal, were stabilized before processing, and the diets held low and acceptable TBARs values, the results were therefore as expected. The low muscle and liver TBARs values further confirm good quality conditions as regards rancidity in the final product. Vitamins E and C are earlier found to be highly affected by lowered diet quality (Hemre et al. 1997), and also to highly interact if one of these vitamins are limited e.g. does vitamin C regenerate vitamin E at the surface of the cell membrane, sparing vitamin E to protect the HUFAs against peroxidation (Hamre et al. 2004). All present diets held high HUFA-levels, evaluated based on the oils used (Langmyhr 2005), readily to be peroxidized if the balance of antioxidants were not acceptable. The variable vitamin E concentrations registered in liver were correlated, although not significantly, to dietary vitamin E levels, and also coincided with the higher inclusion of krill meal in those diet groups (30 and 40%), reminding of equal additions of vitamin E in all diet groups. It might be that the antioxidant properties of the pigment in the krill interacted in the defense system against rancidity both in diets and in fish tissue, adding to the explanation of the very good results at the 30 and 40% inclusion. Unfortunately our study did not include data on muscle storage stability of the fish muscle; it has however been found that mackerel with high tissue vitamin E was more stable against rancidity compared to fish with lower tissue vitamin E levels, when evaluated during six months of freeze storage (Hemre et al. 1997). The stable vitamin A concentrations found both in muscle and liver, indicate that the increase in available astaxanthin from the krill, did not act as a provitamin A precursor in these organs. Astaxanthin has been found to be converted efficiently to vitamin A especially in salmonid intestinal mucosa (White et al. 2003). The provitamin A function of astaxanthin might explain at least parts of the low retention of this pigment, even if digestibility of astaxanthin were quite high. Strong correlations between vitamin and TBARs levels in diet and vitamin levels in tissues were found, indicating that all three vitamins participates in the antioxidant defense system of the salmon.

5. Conclusions

The present trial clearly demonstrates that esterified astaxanthin from Antarctic krill meal is efficiently hydrolysed into free astaxanthin in the intestine and further absorbed and deposited in the tissues of Atlantic salmon. Despite similar digestibility of astaxanthin from Antarctic krill meal and from Carophyll Pink, free astaxanthin from Carophyll Pink is more efficiently utilized for body retention and flesh pigmentation, showing higher tissue levels and higher body retention values as compared to esterified astaxanthin from Antarctic krill meal at any dietary inclusion level.

- By using mean value of astaxanthin in fish fed Diet 4 (30% krill meal) and Diet 5 (40% krill meal), producing a dietary level of average 43.5 mg kg^{-1} esterified astaxanthin, the relative astaxanthin concentration in whole body, muscle and liver were 74, 67 and 41 %, respectively, as compared to the astaxanthin concentrations in fish fed Carophyll Pink as dietary pigment source (42 mg kg^{-1} free astaxanthin). Retention values in the body reflected

the specific astaxanthin concentrations in muscle, showing a relative value of 62 % as compared to Carophyll Pink fed fish. A dietary inclusion of 30 % krill meal was required to support adequate amounts of available astaxanthin for maintenance and/or increased flesh astaxanthin deposition in fast growing fish during this 12 week feeding trial.

- Storage stability of free and esterified astaxanthin during feed production was very high, as more than 99 % astaxanthin was recovered in the feed by using the Antarctic krill meal and more than 95 % by using Carophyll Pink in the diet. Storage stability of esterified astaxanthin exceeded that of free astaxanthin during storage of the experimental feeds at different temperatures. More than 93% astaxanthin was recovered in the krill meal diets at 5 and 15 °C, equivalent to a storage loss less than 3 mg kg^{-1} esterified astaxanthin, while the storage stability was lower at 25°C (79 – 89%), accounting for a loss of 3 to 6 mg kg^{-1} esterified astaxanthin. The corresponding stability of free astaxanthin from Carophyll Pink was lower at all temperatures, with retention values of 79 % (9 mg kg^{-1}), 69 % (13 mg kg^{-1}) and 48 % (22 mg kg^{-1}) in feeds stored at 5, 15 and 25°C, respectively.

6. Suggestions for further trials

Commercial aspects related to improved quality of the edible part of the salmon (muscle) by increased amount of EPA and DHA are indicated in fish fed 40 % Antarctic krill meal (not reported). Complete analyses of fatty acids and phospholipids in all experimental feeds, muscle (edible part) and liver (metabolic important organ) would strengthen the possibility to explain interrelationships between lipid and astaxanthin metabolism by using Antarctic krill meal as pigment and lipid source in salmon diets. The total budget is calculated to 140.000 kr.

Further trials are required to identify whether the lower efficiency of astaxanthin utilization from esterified astaxanthin in the Antarctic krill meal is related to the rate or site of hydrolytical cleavage of the esterified astaxanthin in the intestine, and/or to reduced solvatization and incorporation of astaxanthin into mixed micelles and lipoproteins during intestinal absorption and blood transport into the liver by using Antarctic krill meal as the dietary pigment source.

It would also be of particular interest to study the efficiency of astaxanthin utilization from Antarctic krill meal in larger size fish during longer term trials. A longer feeding period might possibly reduce the relative difference in flesh deposition as compared to Carophyll Pink fed fish, as the efficiency of flesh deposition levels out when a certain muscle astaxanthin level is reached. In market size fish, dietary pigments are mainly used to maintain a certain level of astaxanthin in the muscle, unless too low colour is produced by the diet during the production time.

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Table 1. Diet composition

	Antarctic krill meal in the diet, g100g^{-1}					Carophyll Pink
	0	10	20	30	40	42 mgkg^{-1}
Ingredient content, g kg^{-1}						
Fish meal[a]	297	257	216	175	135	297
Fish meal[b]	298	256	215	175	134	298
Antarctic krill meal[c]	0	100	200	300	400	0
NorSalmOil[d]	240	227	214	200	187	240
Wheat flour	151	146	141	136	130	151
Carophyll Pink (10%)	-	-	-	-	-	0.42
Vitamin mixture[f]	10	10	10	10	10	10
Mineral mixture[g]	4	4	4	4	4	4
Yttriumoxide	0.1	0.1	0.1	0.1	0.1	0.1

[a] Fish meal , SILFAS, N-5892, Bergen, Norway. Protein: 732 gkg^{-1}, Lipid: 92 gkg^{-1}, ash: 115 gkg^{-1}, moisture: 62 gkg^{-1}.

[b]Fish meal , SILFAS, N-5892, Bergen, Norway. Protein: 677 gkg^{-1}, Lipid: 104 gkg^{-1}, ash: 135 gkg^{-1}, moisture: 96 gkg^{-1}

[c]Antarctic krill meal, Protein: 582 gkg^{-1}, Lipid: 212 gkg^{-1}, ash: 116 gkg^{-1}, moisture: 70 gkg^{-1}

[e] Norsalmoil, Norsildmel AL, N-5141 Fyllingsdalen, Norway

[f] Provided per kg of feed: vitamin $D_{3,}$ 3000 I.E. ,160 mg; vitamin E, 136 mg; thiamin, 20 mg; riboflavin, 30 mg; pyrodoxine-HCl, 25 mg; vitamin C, 200 mg;; calcium pantothenate, 60 mg; biotin. 1 mg; folic acid, 10 mg; niacin, 200 mg; vitamin $B_{12,}$ 0,05 mg; menadion bisulphite, 20 mg

[g]Provided per kg of feed: magnesium 500 mg; potassium, 400 mg; zinc, 80 mg; iron, 50 mg; manganese, 10 mg; copper, 5 mg.

Table 2. Proximate composition of experimental diets, values for vitamin A, E and C, and TBARS are given as mean ± SD (n =2)

	Antarctic krill meal in the diet, g100g^{-1}					
	0	10	20	30	40	Carophyll Pink 42 mgkg^{-1}
Ingredient content, g kg^{-1}						
Protein	433	434	437	433	440	435
Lipid	309	304	304	302	299	305
Moisture	71	72	67	72	65	70
Ash	82	84	85	87	89	83
Gross energy MJ kg^{-1}	24.0	23.9	23.9	23.7	23.9	23.9
Free astaxanthin, mgkg^{-1}	*1.4*	*1.3*	*1.1*	*1.1*	*1.0*	*42*
all-E-Astaxanthin	0.8	0.8	0.8	0.6	0.6	33
9Z-Astaxanthin	0.4	0.4	0.3	0.4	0.3	2.1
13Z-Astaxanthin	0.2	<0.1	<0.1	<0.1	<0.1	6.0
Esterified astaxanthin, mgkg-^{1}	*<1*	*14*	*26*	*37*	*50*	*<1*
Diester	<1	10	21	30	40	<1
Monoester	<1	3.3	5.8	7.7	9.9	<1
Vitamin A, sum retinol, mg kg^{-1}	36.0 ± 2.3	43.1 ± 2.6	38.3 ± 0.8	40.8 ± 3.2	39.4 ± 1.6	43.1 ± 2.6
Vitamin A, sum didehydroretinol, mg kg^{-1}	1.19 ± 0.08	1.54 ± 0.21	1.27 ± 0.05	1.46 ± 0.01	1.28 ± 0.01	1.54 ± 0.21
Vitamin E, mg kg^{-1}	167 ± 4	170 ± 2	177 ±	185 ± 6	193 ± 4	170 ± 2
Vitamin C, mg kg^{-1}	111 ± 4	89 ± 4	103 ± 3	96 ± 2	73 ± 6	89 ± 4
TBARS, nmol g^{-1}	209 ± 2	190 ± 11	159 ± 10	116 ± 2	126 ± 7	190 ± 11

Table 3. Growth, feed intake and feed conversion of A.salmon fed diets with increasing substitution of fish meal with Antarctic krill meal and with Carophyll Pink as pigment source for 12 weeks, all values given as means ± SEM (n=2).

	Antarctic krill meal in the diet, $g100g^{-1}$					Carophyll Pink	
	0	10	20	30	40	42 $mgkg^{-1}$	ANOVA $P^{1}<$
Total feeding period, week 1-12							
Body weight at start, g	732 ± 2	732 ± 2	732 ± 3	735 ± 3	733 ± 4	731 ± 1	ns
Body weight at end, g	1669 ± 39	1665 ± 32	1554 ± 19	1708 ± 30	1562 ± 38	1678 ± 40	ns
Weight gain, g/fish	937 ± 37	933 ± 30	822 ± 17	974 ± 26	829 ± 34	947 ± 41	ns
SGR	0.99 ± 0.03	0.99 ± 0.02	0.91 ± 0.01	1.02 ± 0.02	0.91 ± 0.02	1.00 ± 0.03	0.05[2]
TGC	3.44 ± 0.10	3.43 ± 0.08	3.10 ± 0.04	3.54 ± 0.07	3.12 ± 0.09	3.47 ± 0.12	0.05[2]
Daily feed intake; per fish [3]	10.4 ± 0.06[a1]	10.4 ± 0.10[a]	9.33 ± 0.17[b]	10.56 ± 0.03[a]	9.44 ± 0.08 [b]	10.54 ± 0.01[a]	<0.001
Daily feed intake, % [4]	0.87 ± 0.01[ab]	0.87 ± 0.01[ab]	0.82 ± 0.01[b]	0.86 ± 0.01[ab]	0.82 ± 0.01[ab]	0.88 ± 0.01[a]	<0.05
Feed efficiency[5]	1.08 ± 0.04	1.09 ± 0.01	1.06 ± 0.01	1.11 ± 0.03	1.06 ± 0.03	1.08 ± 0.05	ns
Mortality, number of fish[6]	1	2	1	0	1	1	

[1] Statistical significant differences within rows are shown with different superscript letters($P < 0.05$), ns = not significant
[2] Tukey HSD post hoc comparison test did not show dietary differences
[3] Daily feed intake per fish = g feed intake / days / number of fish.
[4] Daily feed intake, % of weight gain = g feed intake / days /((BW_2 + BW_1)/2 * 100) / fish no.
[5] Feed efficiency = g live weight gain / (g feed intake).
[3] 5 individual fish from different diets died following handling at start of the experiment

Table 4. Lipid and astaxanthin in whole body, muscle and liver of A. salmon fed diets with increasing substitution of fish meal with Antarctic krill meal and with Carophyll Pink as pigment source for 12 weeks, all values given as means ± SEM (n=2).

	Antarctic krill meal in the diet, g100g^{-1}					Carophyll Pink	
	0	10	20	30	40	42 mgkg^{-1}	ANOVA P<
Whole body, g kg^{-1}							
Lipid	18.7 ± 0.1	20.1 ± 0.5	19.3 ± 0.15	19.2 ± <0.01	19.5 ± 0.1	20.0 ± 0.4	0.06[1]
Free astaxanthin, mg kg^{-1}	0.4 ± <0.01 [a]	0.65 ± 0.05 [a]	0.8 ± 0.2 [ab]	1.25 ± 0.05 [bc]	1.55 ± 0.15 [cd]	1.9 ± 0.1 [d]	0.0001
all-E-Astaxanthin	0.34 ± 0.03 [a]	0.52 ± 0.08 [ab]	0.67 ± 0.2 [ab]	1.03 ± 0.07 [bc]	1.35 ± 0.15 [cd]	1.65 ± 0.05 [d]	0.001
9Z-Astaxanthin	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	-
13Z-Astaxanthin	0.04 ± 0.01 [a]	0.08 ± 0.03 [a]	0.13 ± 0.01 [ab]	0.20 ± 0.01 [bc]	0.19 ± 0.02 [bc]	0.28 ± 0.03 [c]	0.01
Muscle, g kg^{-1}							
Lipid (g/100g)	12.8 ± 0.4	13.3 ± 0.8	12.1 ± 0.3	12.9 ± 0.9	12.9 ± 0.8	12.9 ± 0.2	ns
Free astaxanthin, mgkg^{-1}	0.7 ± 0.1 [a]	1.35 ± 0.05 [b]	1.35 ± 0.05 [b]	2.05 ± 0.05 [c]	2.15 ± 0.05 [c]	3.15 ± 0.15 [d]	0.00001
all-E-Astaxanthin	0.57 ± 0.08 [a]	1.15 ± 0.05 [ab]	1.15 ± 0.05 [ab]	1.75 ± 0.05 [bc]	1.85 ± 0.05 [bc]	2.4 ± 0.4 [c]	0.01
9Z-Astaxanthin	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	-
13Z-Astaxanthin	0.13 ± 0.02	0.21 ± 0.01	0.25 ± 0.05	0.22 ± 0.02	0.23 ± 0.10	0.23 ± 0.07	n.s.
Liver, g kg^{-1}							
Lipid (not analysed)							
Free astaxanthin, mg kg^{-1}	0.3 ± 0.1 [a]	0.95 ± 0.15 [a]	1.00 ± <0.01 [a]	1.75 ± 0.25 [a]	1.8 ± 0.2 [a]	4.3 ± 0.7 [b]	0.001
all-E-Astaxanthin	0.19 ± 0.02	0.49 ± 0.06	0.35 ± 0.01	0.90 ± 0.21	0.84 ± 0.17	1.57 ± 0.63	0.10
9Z-Astaxanthin	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	-
13Z-Astaxanthin	0.11 ± 0.08 [a]	0.43 ± 0.09 [ab1]	0.56 ± 0.06 [b]	0.75 ± 0.01 [bc]	0.91 ± 0.05 [c]	2.55 ± 0.05 [d]	0.00001

[1] Pigment free control diet (Diet 1) nearly significantly different from Diet 2 (p< 0.06) and Diet 6 (p<0.10)

Table 5. Apparent digestibility of lipid, total astaxanthin (free and esterified) and esterified (sum mono- and diester) astaxanthin, and whole body retention of lipid and total astaxanthin in A.salmon fed diets with increasing substitution of fish meal with Antarctic krill meal and with Carophyll Pink as pigment sources for 12 weeks, all values given as means ± SEM (n=2).

	Antarctic krill meal in the diet, g100g^{-1}					Carophyll Pink	
	0	10	20	30	40	42 mgkg^{-1}	ANOVA P<
Apparent digestibility, %							
Lipid	86.2 ± 1.2	87.3 ± 0.6	87.1 ± 2.1	89.9 ± 0.9	90.4 ± 0.8	87.3 ± 0.6	ns
Total astaxanthin[1]	-11.1 ± 15.3 [a]	52.8 ± 2.2 [b]	50.3 ± 4.6 [b]	54.6 ± 3.3 [b]	55.2 ± 6.8 [b]	54.1 ± 4.2 [b]	0.01
Esterified astaxanthin	-	88.7 ± 0.5	91.1 ± <0.1	91.3 ± 0.8	92.3 ± 2.1	-	ns
Whole body retention, %[2]							
Lipid	78.9 ± 2.4	88.2 ± 5.6	84.0 ± 1.6	85.9 ± 2.2	86.6 ± 2.0	87.4 ± 0.3	ns
Astaxanthin	-12.3 ± 0.35 [a]	1.93 ± 0.35 [b]	2.05 ± 1.51[3] [b]	3.97 ± 0.14 [bc]	4.03 ± 0.7 [bc]	6.45 ± 0.28 [c]	0.0001
Astaxanthin, mg kg^{-1} weight gain	-0.16 ± 0.01 [a]	0.27 ± 0.05 [ab]	0.52 ± 0.39 [ab]	1.36 ± 0.09 [bc]	1.92 ± 0.29 [c]	2.51 ± 0.22 [c]	0.001

[1] Calculated by the sum of free and esterified astaxanthin in feed and faeces
[2] Net nutrient (N) utilization: Total N retained in the body (g) / total N eaten (g)

Table 6. Vitamin A, E, C and TBARS in muscle and liver of A. salmon fed diets with increasing substitution of fish meal with Antarctic krill meal and with Carophyll Pink as pigment sources for 12 weeks, all values given as means ± SEM (n=2). Body weight and slaughter quality of fish sampled for analyses are also given as means ± SEM (n=2, each of 6 individual fish).

	Antarctic krill meal in the diet, g100g^{-1}					Carophyll Pink	
	0	10	20	30	40	42 mg kg^{-1}	ANOVA P<
Muscle, mg kg^{-1}							
Vitamin A, sum retinol	0.20 ± 0.01	0.24 ± 0.03	0.221 ± 0.003	0.21 ± 0.02	0.233 ± 0.02	0.23 ± 0.004	ns
Vitamin A, sum didehydroretinol	0.25 ± 0.01	0.25 ± 0.04	0.27 ± 0.03	0.28 ± 0.01	0.31 ± 0.02	0.26 ± 0.01	ns
Vitamin E (α-tocopherol)	13.3 ± 0.3	13.8 ± 2.2	13.5 ± 0.2	16.0 ± 1.4	16.0 ± 0.6	14.1 ±1.2	ns
Vitamin C	30.5 ± 0.0	30.5 ± 3.0	28.6 ± 4.0	27.0 ± 1.1	30.0 ± 1.3	27.0 ± 0.8	ns
TBARs (nmol g^{-1})	2.40 ± 0.30	2.03 ± 0.48	2.40 ± 0.10	2.09 ± 0.63	2.23 ± 0.01	2.70 ± 0.22	ns
Liver, mg kg^{-1}							
Vitamin A, sum retinol	168.0 ± 20.3	164.4 ± 21.1	165.3 ± 4.1	175.8 ± 18.5	169.0 ± 7.3	176.4 ± 20.2	ns
Vitamin A, sum didehydroretinol	199.6 ± 32.6	184.6 ± 20.3	197.2 ± 16.2	209.9 ± 15.7	195.8 ± 10.5	199.4 ± 25.5	ns
Vitamin E (α-tocopherol)	94.5 ± 7.8 ^{a}	171.8 ± 16.7 ^{b}	161.3 ± 4.0 ^{ab}	244.2 ± 7.0 ^{c}	203.8 ± 18.8 ^{bc}	161.5 ± 38.4^{ab}	0.003
Vitamin C	232.7 ± 8.3	270.6 ± 28.1	241.5 ± 4.5	244.5 ± 14.7	270.7 ± 21.5	234.6 ± 7.3	ns
TBARs (nmol g^{-1})	9.0 ± 0.4	8.7 ± 1.4	7.3 ± 0.8	7.4 ± 0.6	7.6 ± 0.9	8.4 ±0.3	ns
Slaugther quality							
Weight, g	1647 ± 131	1787 ± 39	1551 ± 1	1643 ± 77	1671 ± 23	1680 ± 84	ns
Condition factor[1]	1.49 ± 0.03	1.51 ± 0.05	1.45 ± 0.01	1.44 ± 0.02	1.43 ± 0.02	1.49± 0.05	ns
HSI[2]	1.21 ± 0.14	1.16 ± 0.08	1.09 ± 0.08	1.06 ± 0.04	1.10 ± 0.01	1.14 ± 0.05	ns
Dressing out percentage[3]	12.6 ± 0.8	11.6 ± 0.9	12.3 ± 0.6	11.4 ± 0.6	11.6 ± 0.5	11.9 ± 1.1	ns

[1]Condition factor: (fish weight * 100)/ (body lenght)^{3}

[2]Hepatosomatic index: liver weight (g) * 100 / fish weight (g)

[3]Dressing out percentage: (fish weight – gutted fish weight) / fish weight x 100

Table 7. Sum of esterified astaxanthin (mono- and diester) in Diets 2 to 5 (Krill meal 10, 20, 30 and 40%) and free astaxanthin in Diet 6 (Carophyll Pink) during blending, extrusion, drying and coating in the feed production, all values given in dry matter

	Antarctic krill meal in the diet, g100g^{-1}					Carophyll Pink
	0	10	20	30	40	42 mgkg^{-1}
Esterified asta, mg kg^{-1}dry matter						
Feed blend (calculated)[1]	-	16.7	33.6	49.9	65.8	-
Feed blend	-	16.3	32.7	48.9	64.0	-
Extruded feed	-	16.9	33.9	49.7	63.1	-
Dried extruded feed	-	17.4	33.7	48.1	62.4	-
Oil coated feed (calculated)[2]	-	14.4	28.4	41.2	54.3	-
Oil coated feed	-	15.1	27.9	39.9	53.5	-
Free astaxanthin, mg kg^{-1}dry matter						
Feed blend (calculated)[1]	-	-	-	-	-	60.9
Feed blend	-	-	-	-	-	74.2[3]
Extruded feed [2]	-	-	-	-	-	58.7
Dried extruded feed	-	-	-	-	-	59.8
Oil coated feed (calculated)[2]	-	-	-	-	-	48.7
Oil coated feed	1.51	1.4	1.2	1.2	1.1	45.2

[1] Dietary astaxanthin loss during initial mixing of ingredients is based on calculated values from the dietary feed ingredients.
[2] Astaxanthin in the feed following coating is corrected for oil inclusion level in each diet. [3] High value, needs to be verified.