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Lipid composition of malpigmented and normally pigmented newly settled yellowtail flounder, *Limanda ferruginea* (Storer)

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Abstract

The lipid composition of malpigmented (MP) and normally pigmented (NP), newly settled yellowtail flounder (*Limanda ferruginea*, Storer) was compared in order to elucidate a possible connection between lipids and pigmentation development. Larvae were fed commercially enriched live food for 12 weeks post hatch and then differences in lipid composition and size were analysed. NP fish were found to be significantly larger (standard length 35 mm) than MP fish (32 mm) at 100% settlement. There were higher proportions of triacylglycerols in NP fish ($P = 0.01$), whereas MP fish had an increased percentage of phospholipids ($P = 0.01$). NP fish had a higher percentage of docosahexaenoic acid (DHA) in the polar lipids of their body ($P = 0.03$) and total lipids of their eyes ($P = 0.04$). These data support previously proposed theories for the importance of DHA in pigmentation development. Principal components analysis (PCA) described the majority of the variance (77%) within the data set using just two principal components axes. PCA demonstrated that differences between body zones were greater than those between NP and MP fish within a given zone.

Keywords: pigmentation, DHA, essential fatty acids, larval fish, *Limanda ferruginea*

Introduction

Malpigmentation is common in a number of cultured flatfish species (Seikai, Shimozaki & Watanabe 1987; Rainuzzo, Reitan, Jorgensen & Olsen 1994; Baker, Alves & Bengtson 1998; Gara, Shields & McEvoy 1998; Naess & Lie 1998). Pigmentation abnormalities reduce the market value of fish and consequently represent a significant challenge to the aquaculture industry. In addition, juveniles produced for enhancement of wild stocks are probably more vulnerable to predation as they are unable to blend into their natural benthic environments (Godin 1997). Recently, it has also been suggested that abnormally pigmented Atlantic halibut (*Hippoglossus hippoglossus*) are more susceptible to skin damage from ultraviolet radiation than normally pigmented fish (Bricknell, Bruno, Bowden & Smith 1996).

The exact mechanism behind the development of abnormal pigmentation is not known. However, environmental/husbandry, nutritional and neuroendocrine activity are thought to be important (Kanazawa 1993; Denson & Smith 1997; Venizelos & Benetti 1999; Estevez, Kaneko, Seikai, Tagawa & Tanaka 2001). Numerous studies have shown that early lipid nutrition affects later pigmentation in several flatfish species (Seikai *et al.* 1987; Gara *et al.* 1998). Further, wild copepods have been shown to be more effective in inducing normal pigmentation than rotifers and *Artemia* (Naess & Lie 1998; McEvoy, Naess, Bell & Lie 1998). Rainuzzo *et al.* (1994) found that the docosahexaenoic acid (DHA) to eicosapentaenoic acid (EPA) (DHA/EPA) ratio in the polar lipid of turbot larvae was positively correlated with levels of normal pigmentation. However, other dietary factors, such as the arachidonic acid to EPA (AA/EPA) ratio and levels of vitamin A,

phospholipid and DHA, have also been correlated with pigmentation development (Kanazawa 1993; McEvoy *et al*. 1998; Estevez, McEvoy, Bell & Sargent 1999).

The lipid composition of normally pigmented (NP) and malpigmented (MP) fish has been compared in Atlantic halibut (McEvoy *et al*. 1998) and Japanese flounder, *Paralichthys olivaceus* (Estevez & Kanazawa 1996). Normally pigmented Atlantic halibut had significantly higher amounts of DHA and EPA present in the phosphatidylcholine (PC) fraction of the eye than MP fry, while, in Japanese flounder, MP fish showed better growth and higher neutral lipids in the body. Normal pigmentation was also associated with higher relative amounts of polyunsaturated fatty acids (PUFAs) in the polar lipid of larval brains and eyes.

This study was designed to compare the lipid composition of MP and NP juvenile yellowtail flounder. Lipid classes and fatty acids were examined in different body zones to distinguish between body and neural tissue. The objective of this study was to elucidate a possible connection between tissue lipids and pigmentation development. In addition, we provide baseline data on the lipid composition of different body zones in a new cultured species.

Materials and methods

Live food

Rotifers were cultured on baker's yeast and culture Selco (INVE, Dendermond, Belgium) for 5 days prior to enrichment. They were then taken from stock cultures and placed into 300-L enrichment vessels at a density of 3×10^5 rotifers L^{-1}. Rotifer batches were enriched for approximately 18 h using 0.3 g of Algamac-3010 per 10^6 rotifers (Bio-Marine, Hawthorne, CA, USA). Algamac was added to rotifer enrichment vessels at time zero and after 9 h of enrichment. Rotifers were sampled in triplicate for lipid analysis twice during this experiment.

Second instar stage *Artemia* were stocked in 300-L tanks at a density of 2×10^5 animals L^{-1}. During enrichment, the temperature was maintained at 26 °C and vigorous bottom aeration was applied. Algamac 3010 was added at a concentration of 2 g per 10^6 animals. After 12 h of enrichment, *Artemia* were transferred to a new enrichment vessel to receive a second 12-h enrichment. *Artemia* were sampled for lipid analysis in triplicate twice during the experimental period.

Larviculture

Eggs for this experiment were obtained from yellowtail flounder broodstock between 4 July and 9 July 1999 and were pooled to obtain the required quantity. They were then incubated in a 300-L cylindroconical upwelling tank and hatched at approximately 65 degree-days. At 100% hatch, larvae were transferred into two 300-L cylindroconical upwelling tanks at a density of 30 larvae L^{-1}. Water flow was set at 2 L min^{-1} and one air stone was placed in the centre of each tank to provide aeration. Larvae were reared under ambient temperatures (12–18 °C) using a 24-h photoperiod with the light intensity of ~800 lux.

Enriched rotifers were added to tanks twice per day at a density of 7000 prey L^{-1} for the first 4 weeks post hatch (Puvanendran & Brown 1995; Rabe & Brown 2000). Tanks were 'greened' daily with 5 L of *Isochrysis galbana*. After 4 weeks, larvae were transferred into a 3000-L tank at a density of 0.3 larvae L^{-1} and fed *Artemia* twice per day at a density of 2000 prey L^{-1}. The water level was reduced to ~1000 L from week 8 until the end of week 12 to aid settling behaviour.

Laval sampling

At the end of 12 weeks ~100% of the fish demonstrated settling behaviour and pigmentation and eye migration could be defined. At this time, juveniles were randomly sampled with a dip net for both morphometric measurements and lipid analysis. The possible pigmentation and eye migration patterns observed in yellowtail flounder are shown in Table 1. Five normally pigmented (pigmentation 5, eye migration 3) and five malpigmented (pigmentation 1, eye migration 3) juveniles were sampled for standard length and body depth. Standard length was defined as the length in mm from the tip of the snout to the end of the notochord. Body depth was defined as the width of the larvae just posterior to the anus not including the fin fold. Their eyes, heads and bodies were then dissected and measurements of wet weight and dry weight were taken on these three body zones. For dry weight measurements, fish tissues were placed on preweighed foils and dried at 60 °C for 48 h. Foils were then stored in a desiccator and reweighed.

Ten normally pigmented (NP) and 10 malpigmented (MP) fish were sampled for lipid analysis.

Table 1 Categories of pigmentation and eye migration used in evaluation of yellowtail flounder (after Gara *et al.* 1998)

Categories	Definition
Pigmentation	
1	No pigmentation visible
2	Pigmentation visible only on the head
3	Pigmentation visible on the head and tail
4	Pigmentation visible on the head and abdomen
5	Completely pigmented on the ocular side
6	Completely pigmented on both the ocular and blind side
Eye migration	
0	Blind side eye not yet visible
1	Blind side eye only partially visible but not the full diameter
2	Blind side eye diameter fully visible but not past the dorsal margin
3	Blind side eye visible and fully past the dorsal margin

Juveniles were sacrificed using an overdose of MS-222 and their standard length, body depth and wet weights were recorded. The eyes, heads and bodies were dissected on ice and the parts from two fish were pooled into each lipid sample. This resulted in five samples of NP and five samples of MP fish for eye, head, and body zones. Lipid samples were placed directly in chloroform and stored under nitrogen at $-20\,^{\circ}C$ until analysis. Lipids were extracted in chloroform–methanol according to Parrish (1998) using a modified Folch procedure (Folch, Lees & Sloane Stanley 1957).

Lipid classes were determined using thin-layer chromatography with flame ionization detection (TLC/FID) with a Mark V Iatroscan (Iatron Labratories, Tokyo, Japan), as described by Parrish (1987). Extracts were spotted on silica gel-coated Chromarods and a four-stage development system was used to separate lipid classes. The first separation consisted of 20-min developments in 99:1:0.05 hexane–diethyl ether–formic acid. The second separation consisted of a 40-min development in 80:20:1 hexane–diethyl ether–formic acid. The third separation consisted of 15-min developments in 100% acetone and the last separation was a 35-min development in 70:35:3.5 chloroform–methanol–water. After each separation, the rods were scanned and the four chromatograms were combined to form one complete chromatogram using T-data scan software (RSS Inc., Bemis, TN, USA). The signal detected in millivolts was quantified using lipid standards (Sigma, St. Louis, MO, USA).

Total lipids as well as neutral and polar lipids were analysed for fatty acid composition. Total lipids were separated into neutral and polar lipids using column chromatography (Yang 1995; Budge 1999). Lipid extracts were applied to the top of the column and the neutral lipids were eluted with 3 mL of 99:1:0.5 chloroform–methanol–formic acid. The remaining polar lipids were removed using 6 mL of methanol.

Fatty acid methyl esters (FAMEs) were prepared by transesterification with 10% boron fluoride (BF_3) in methanol at $85\,^{\circ}C$ for 1 h. A Varian model 3400 gas chromatography equipped with a Varian 8100 autosampler was used for fatty acid analysis. The column was an Omegawax 320 column, 30 m, 0.32 mm i.d., 0.25 μm film thickness (Supelco, Bellefonte, PA, USA). Hydrogen was used as the carrier gas and the flow rate was set at $2\,mL\,min^{-1}$. The column temperature profile was: $65\,^{\circ}C$ for 0.5 min, hold at $195\,^{\circ}C$ for 15 min after ramping at $40\,^{\circ}C\,min^{-1}$, and hold at $220\,^{\circ}C$ for 0.75 min after ramping at $2\,^{\circ}C\,min^{-1}$. The injector temperature increased from 150 to $250\,^{\circ}C$ at $200\,^{\circ}C\,min^{-1}$. Peaks were detected by flame ionization and the detector was held at $260\,^{\circ}C$. Fatty acid peaks were integrated using Varian Star Chromatography Software (version 4.02) and identification was made with reference to known standards (PUFA 1 and 37 Component FAME Mix, Supelco Canada, ON).

Statistical analysis

Differences in size between MP and NP fish were analysed using a *t*-test ($\alpha = 0.05$). The lipid composition of MP and NP fish was compared with a two-way ANOVA using lipid class and body zone as explanatory variables. In all cases there was a significant interaction between body zone and lipid class. Thus, differences between NP and MP fish were compared separately for each body zone using a Bonferroni-corrected pairwise comparison (family error rate of $\alpha = 0.05$). Residuals vs. fitted values were examined to check for normality and heteroscedasticity. Some percentage data were arcsine square root transformed in order to meet these assumptions. Principal components analysis and cluster analysis were used to simplify this multivariate data set by transforming correlated variables into a set of uncorrelated principal components (Minitab, version 10.5). This technique was employed using

15 fatty acid and three lipid class variables from the eyes, bodies and heads on MP and NP fish. Two coordinates were described that accounted for the largest and second largest variance among the samples. This allowed a display of the major trends within the data set without significant loss of total original variation.

Results

Live food

Rotifers had 106.0 µg lipid per mg dry weight while *Artemia* had higher levels of lipid per dry weight at 165.4 µg mg^{-1} (Table 2). The major lipid class in both rotifers and *Artemia* was triacylglycerol (TAG), which accounted for 44.9% and 51.5% of total lipids respectively. Rotifers contained 19.8% saturated fatty acids (SFAs), 24.8% multiunsaturated fatty acids (MUFAs) and 50.9% polyunsaturated fatty acids (PUFAs), whereas *Artemia* had higher percentages of SFAs (26.4%) and MUFAs (30.1%) and lower proportions of PUFAs (42.0%). The major PUFA in rotifers was DHA (28.8%), while this fatty acid was present at low levels in *Artemia* (4.0%). The major PUFA in *Artemia* was 18:3n-3, which accounted for 17.5% of total fatty acids.

Larval size

At the end of the experiment, only 11% of the fish were NP, and NP and MP fish weighed on average 0.6 g and 0.4 g respectively (Fig. 1a). Normally pigmented fish had an average body depth of ~15 mm, and MP fish were on average 14 mm (Fig. 1b). Figure 1(c) shows that the standard length of NP fish (35 mm) was significantly higher than that of MP fish, 32 mm ($t_{1,28} = 2.2$, $P = 0.04$).

Table 2 Lipid composition of rotifers and *Artemia* enriched with Algamac for 18 h

	Rotifers	*Artemia*
Lipid per dry weight (µg mg^{-1})	106.0 ± 13.5	165.4 ± 14.2
Per cent of total lipid		
Triacylglycerols	44.9 ± 2.4	51.5 ± 3.3
Sterols	10.7 ± 7.6	6.8 ± 2.8
Phospholipids	26.3 ± 4.6	20.4 ± 5.5
% total fatty acids		
Σ SFA	19.8 ± 0.4	26.4 ± 1.4
Σ MUFA	24.8 ± 0.4	30.1 ± 1.2
18:3n-3	0.3 ± 0.0	17.5 ± 2.0
AA	3.3 ± 0.0	2.0 ± 0.3
EPA	3.9 ± 0.1	8.0 ± 1.0
DHA	28.8 ± 0.6	4.0 ± 0.2
Σ PUFA	50.9 ± 1.0	42.0 ± 0.6

Data are mean ± SEM, $n = 6$.

Larval lipid class composition

Approximately 23% of the dry body weight of MP and NP fish was lipid (Table 3). The major lipid class

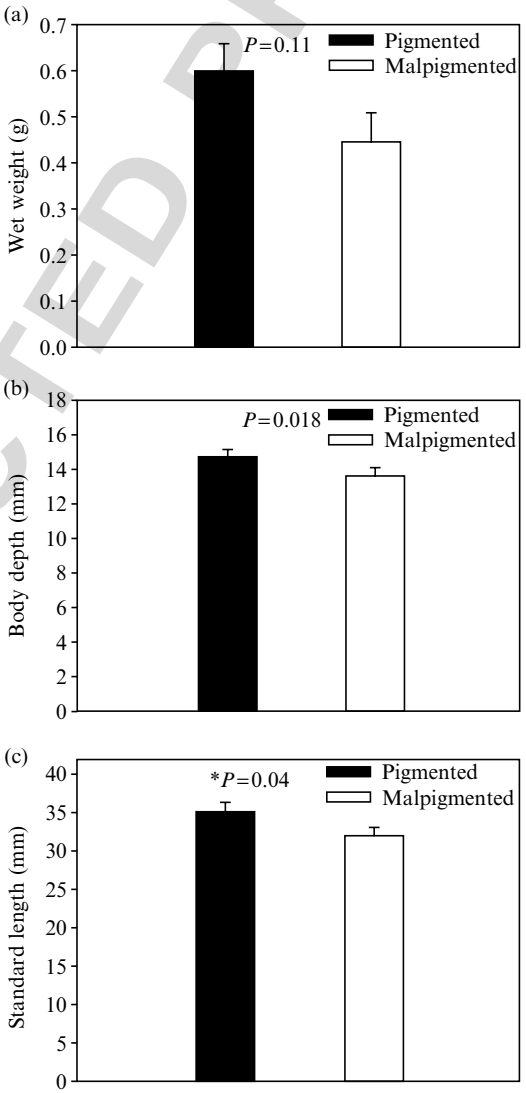

Figure 1 (a) Wet weight, (b) body depth and (c) standard length of NP fish compared with MP fish at the end of week 12. Data are mean ± SEM ($n = 15$ larvae per group, significance for *t*-test was $\alpha = 0.05$, $t_{1,28}$).

in both MP and NP fish was TAG, although more total lipid was composed of TAG in NP fish (77.1%) than in MP fish (70.3%) ($P = 0.001$). Neutral lipids accounted for more of the total lipid in NP than in MP individuals ($P = 0.002$), and MP fish had a higher percentage of phospholipid than NP fish ($P = 0.01$). Phosphatidylcholine (PC) was the major phospholipid in both types of fish, accounting for 5.8–8.4% of the total lipids.

Lipid accounted for ~14% of the dry weight in MP and NP fish heads (Table 3). As in the body, the major lipid class was TAG, with NP fish having 33.1% TAG and MP fish having 26.3%. Malpigmented fish contained less neutral lipid than NP animals ($P = 0.001$). Conversely, MP fish had relatively higher percentages of phospholipids ($P = 0.003$) and total polar lipid ($P = 0.002$) than NP fish.

Eyes contained less lipid per dry weight than either the bodies or heads (Table 3). MP fish had eyes with 8.5% lipid while NP fish eyes contained 8.9% lipid. The major lipid class in the eye was phospholipid, and MP fish contained higher relative amounts of phospholipid than NP fish ($P = 0.001$); however, NP fish contained higher relative amounts of neutral lipids and, in particular, of TAG ($P = 0.001$).

Fatty acid composition of juveniles

Saturated fatty acids were present in the bodies, heads, and eyes of larvae at ~20%, 25%, and 40% respectively (Fig. 2 and Table 4). The major SFA present was 16:0; however, 18:0 was present in high proportions in the polar lipids of the head and in the total lipids of the eye (Table 4). Generally, neutral lipids contained higher percentages of MUFAs and lower proportions of PUFAs than the polar lipids.

Polyunsaturated fatty acides were present in higher proportion in the polar lipids than in the neutral lipids, but this trend was not observed in the head lipids of MP fish. The major PUFA in the neutral lipids was 18:3n-3, and DHA was the major PUFA in the polar lipids of the body and head as well as in the total lipids of the eye. DHA was found at higher percentages in membranes of NP fish

Table 3 Comparison of the lipid class composition in the body, head, and eyes of NP and MP newly settled yellowtail flounder

	Body		Head		Eyes	
	MP	**NP**	**MP**	**NP**	**MP**	**NP**
Σ Lipid per dry weight (μg mg^{-1})	237.7 ± 25.4	225.6 ± 20.6	134.3 ± 15.2	153.6 ± 16.4	85.9 ± 2.9	89.0 ± 6.4
Σ Lipid per mg body zone percent of total lipids)	21.3 ± 3.0	35.6 ± 8.6	2.3 ± 0.3	3.1 ± 0.3	0.08 ± 0.01	0.08 ± 0.01
TAG	70.3 ± 1.9*	77.1 ± 1.5	26.3 ± 1.6	33.1 ± 4.0	8.9 ± 0.9*	14.8 ± 1.5
FFA	1.6 ± 0.5	2.0 ± 0.3	2.3 ± 0.6	3.6 ± 1.0	–	–
ST	5.6 ± 0.7	4.5 ± 0.5	14.2 ± 0.8	14.3 ± 1.3	15.4 ± 0.6*	18.3 ± 0.6
DAG	0.9 ± 0.9	1.3 ± 0.6	0.6 ± 0.6	4.8 ± 0.4	2.0 ± 0.1	1.5 ± 0.5
Σ NL	78.3 ± 1.4*	84.9 ± 1.1	43.4 ± 1.9*	55.6 ± 2.1	28.5 ± 1.1*	35.9 ± 1.0
AMPL	5.1 ± 0.5	4.0 ± 0.6	6.5 ± 0.3	6.0 ± 0.5	2.1 ± 0.1	1.2 ± 0.2
PC	8.4 ± 0.5	5.8 ± 0.3	29.5 ± 1.1*	20.9 ± 1.6	42.8 ± 7.4	42.5 ± 6.7
PE	5.7 ± 0.2	3.5 ± 0.3	5.2 ± 1.3	6.4 ± 1.3	4.8 ± 1.5	4.7 ± 1.4
SM	0.3 ± 0.0	0.5 ± 0.2	2.9 ± 0.5	1.7 ± 0.3	2.1 ± 0.7	0.6 ± 1.1
PS	1.0 ± 0.2	0.6 ± 0.2	6.1 ± 0.6	6.1 ± 1.7	18.3 ± 3.0	15.3 ± 7.8
LPC	0.8 ± 0.2	0.6 ± 0.1	5.0 ± 0.4	2.9 ± 0.5	3.5 ± 3.1	1.0 ± 1.0
Σ PPL	16.2 ± 1.3*	11.0 ± 0.7	48.7 ± 1.3*	38.0 ± 0.7	71.5 ± 1.2*	64.1 ± 1.2
Σ PL	21.3 ± 1.6*	15.0 ± 1.2	55.2 ± 2.0*	44.0 ± 2.2	73.7 ± 5.0*	65.4 ± 1.0

*ANOVA, $P < 0.05$ (family error rate). MP fish were significantly different from NP fish within a given body zone. TAG, triacylglycerol; FFA, free fatty acid; ST, sterol; DAG, diacylglycerol; NL, neutral lipid; AMPL, acetone mobile polar lipid; PC, phosphatidycholine; PE, phosphatidyethanolamine; SM, sphingomyelin; PS, phosphatidylserine; LPC, lysophosphatidylcholine; PPL, phospholipid; PL, polar lipid. Data are mean ± SEM, $n = 5$.

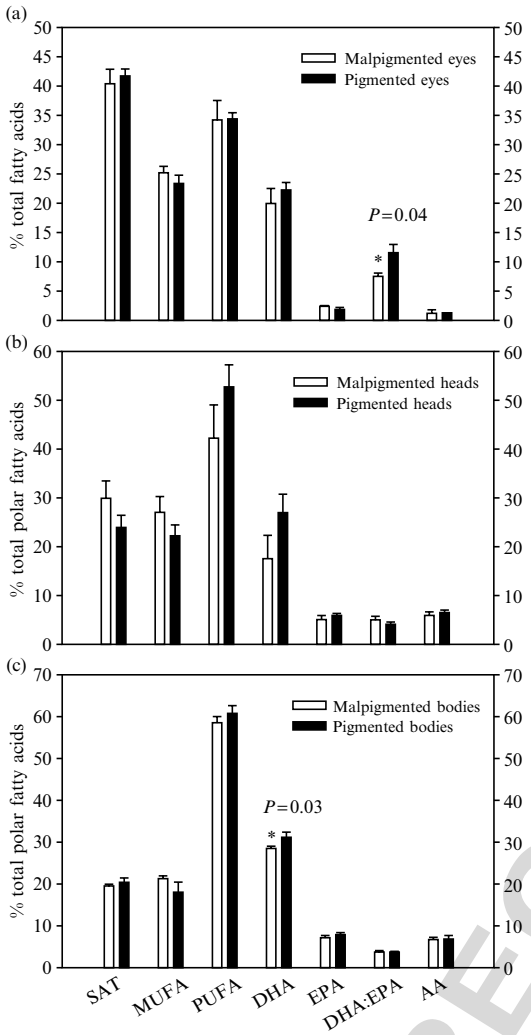

Figure 2 Summary of fatty acid composition in different body zones of newly settled MP vs. NP yellowtail flounder. (a) Eye total lipid. (b) Head polar lipid. (c) Body polar lipid. *Significant difference between NP and MP fish. Data are mean ± SEM ($n = 5$). *ANOVA, $P < 0.05$ (family error rate). MP fish were significantly different from normally pigmented fish.

than in MP fish. However, this relationship was significant only in the polar lipid fraction of the body (Fig. 2c, $P = 0.03$). EPA was present equally in the neutral and polar lipids, and this resulted in a higher DHA/EPA ratio in the polar lipids than in the neutral lipids of the head and bodies. The total lipids of the eye contained low proportions of EPA, which resulted in a high DHA/EPA ratio in the eye. This ratio was significantly higher in the eyes of NP fish (11.7:1) than in MP fish (7.6:1) (Fig. 2a, $P = 0.04$).

Principal components analysis

Principal components analysis allowed the description of the majority of the variance in the data set using 15 fatty acid and three lipid class variables. Figure 3(a) shows the coefficients plotted for the first two principal components. These principal components cumulatively accounted for 77% of the total variance. The first PC accounted for 64% of the variance and separated lipid parameters into those associated with membranes and those associated with storage. Lipids associated with membranes such as phospholipids, DHA, 18:0 and 16:0 loaded negatively onto this axis. Conversely, lipid parameters associated with storage such as per cent TAG, 18:3n-3, and lipid per dry weight loaded positively onto the PC1 axis. The PC2 axis accounted for only 13% of the variance and separated lipid parameters based on the level of unsaturation. PUFAs, DHA, arachidonic acid (AA) and 22:5n-3 were positively loaded on this axis, whereas MUFAs, SFAs, 18:1n-9 and 14:0 were negatively loaded along PC2.

Figure 3(b) shows the scores for the eyes, heads and bodies of NP and MP fish plotted along PC1 and PC2. Eyes grouped together on the negative side of the axis and bodies grouped together on the positive side of PC1. The grouping of eyes on the negative segment of the axis was expected, as eyes have high levels of polar lipid present in the form of highly unsaturated phospholipids. Bodies were associated with very high levels of lipid and, in particular, TAG, which resulted in their position on the positive side of PC1. Heads were located near the origin of PC1, indicating their intermediate lipid composition, consisting of approximately equal amounts of membrane and storage lipids. Differences between body zones were greater than between NP and MP fish within a given zone, and no separation of MP and NP fish into clusters was observed.

Discussion

Size of juveniles

Normally pigmented (NP) yellowtail flounder were significantly larger than malpigmented (MP) fish. McEvoy *et al.* (1998) examined differences in both lipid composition and size between NP and MP Atlantic halibut. Differences were compared between MP and NP fish fed Super Selco-enriched *Artemia* or a copepod diet. The copepod treatment resulted in higher levels of NP fish (92%) than in the

Table 4 Neutral and polar fatty acid composition of MP and NP newly settled yellowtail flounder larvae. Data are means, $n = 5$

	Body				Head				Eyes	
	Malpigmented		Normally pigmented		Malpigmented		Normally pigmented		Malpigmented	Normally pigmented
	NL	PL	NL	PL	NL	PL	NL	PL	Total	Total
14:0	3.7	1.1	2.9	1.2	2.8	1.8	3.8	1.2	1.8	1.8
16:0	11.3	7.7	10.4	8.1	18.2	11.3	11.9	9.0	22.4	23.6
18:0	2.7	9.4	2.8	9.9	2.9	14.1	3.0	12.1	12.7	12.1
Σ SFA*	19.3	19.6	18.1	20.5	25.8	29.8	20.9	24.2	40.4	41.9
18:1n-9	17.5	10.6	17.6	9.8	16.0	16.6	16.9	13.2	14.9	12.9
18:1n-7	5.5	4.8	6.4	3.6	5.7	5.7	5.9	4.9	4.7	5.6
16:1n-7	5.9	1.8	4.3	1.8	5.1	2.2	5.9	1.7	3.4	3.5
20:1n-9	3.7	2.7	3.2	2.7	3.1	2.9	3.6	2.7	0.8	0.6
Σ MUFA†	36.4	21.7	33.2	18.6	31.2	27.9	34.0	22.9	25.4	23.6
18:2n-6	6.4	3.6	5.8	4.2	5.2	3.6	5.8	3.6	2.2	1.9
18:3n-3	13.8	3.5	17.6	3.5	12.5	2.8	12.3	2.4	2.7	2.3
20:4n-6 (AA)	1.8	7.0	1.9	7.2	2.8	6.1	2.9	6.7	1.4	1.3
20:5n-3 (EPA)	7.0	7.3	6.8	8.0	7.2	5.2	8.1	6.2	2.5	2.0
22:5n-3	1.3	1.8	1.2	1.9	1.0	1.2	1.4	1.3	0.6	0.7
22:6n-3 (DHA)	9.6	28.7	9.7	31.5	9.9	17.7	10.2	27.2	19.2	22.8
Σ PUFA‡	44.3	58.8	48.7	60.9	43.0	42.3	45.1	52.9	34.3	34.5
DHA/EPA	1.4	4.0	1.4	3.9	1.4	3.4	1.3	4.4	7.6	11.7
DHA/AA	5.2	4.1	5.1	4.3	3.5	2.9	3.7	4.1	14.2	17.2
EPA/AA	3.9	1.0	3.6	1.1	2.6	0.9	2.8	0.9	1.8	1.5

*Includes ai-15:0, 15:0, i-17:0, ai-17:0, 17:0, and 20:0.
†Includes 18:1n-5, 20:1n-7, 22:1n-11, 22:1n-9, and 24:1.
‡Includes 16:2n-4, 16:3n-4, 16:4n-3, 18:3n-3, 18:4n-3, 20:2n-6, 20:3n-6, 20:4n-3, and 22:4n-6.

Artemia-reared group (66%). Fish in the copepod treatment group were also significantly larger. However, NP and MP fish within the *Artemia* treatment showed no differences in size.

Conversely, a similar study by Estevez and Kanazawa (1996) found that MP fish were significantly larger than NP fish at day 50 pos thatch. This increased size was hypothesized to result from differences in visual function in NP and MP fish. Further, MP fish were reported to grow faster as a result of their 'better vision under bright illuminations'. The light intensity used (600–1000 lux) was thought to be too stressful for the NP fish and thus caused a relative reduction in growth. A similar level of light intensity was used here with no adverse effects observed on the growth of NP yellowtail flounder.

Purchase, Boyce and Brown (2000) examined the effects of three different photoperiods on the growth and survival of MP and NP yellowtail flounder older than 1 year (5.0 cm standard length) and found no differences in growth between NP and MP fish. This is different from the data presented here. However, our study examined the size of newly settled larvae, whereas Purchase *et al.* (2000) used fish older than 1 year. Thus, differences in age may be a possible explanation for these variable results.

Lipid class composition of juveniles

Lipid accounted for ~ 23% of the dry weight in the body in both NP and MP fish. Whalen (1999) compared the lipid composition in wild juvenile (1+ year class) yellowtail flounder with that of cultured juveniles. Cultured fish were found to have much higher levels of lipid per dry weight in both the liver (14%) and muscle (8.4%) than did wild fish (3.6% and 1.3% respectively). In this study, TAG was the main lipid class in the body of MP and NP juveniles, accounting for 70% and 77% of the total

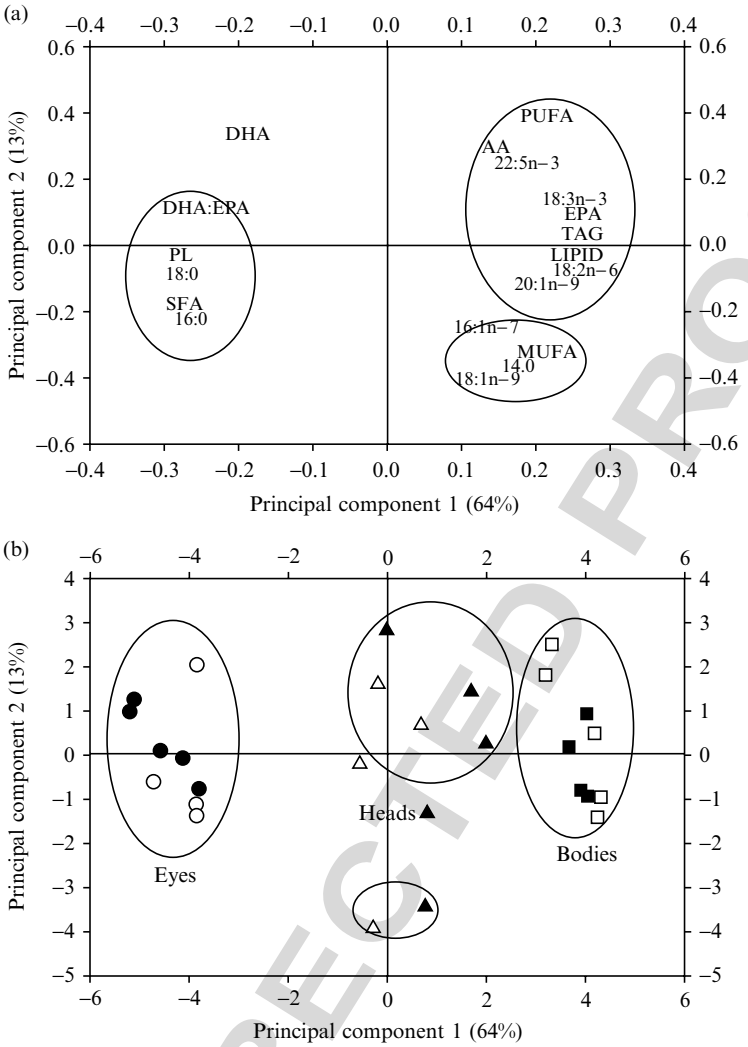

Figure 3 Principal components analysis (PCA) of lipid data from eyes, heads and bodies of MP and NP juvenile yellowtail flounder. Five samples of MP and NP fish tissue were analysed from each body zone. The fatty acid parameters used were: 14:0, 16:0, 16:1n-7, 18:0, 18:1n-9, 20:1n-9, 20:4n-6 (AA), 20:5n-3 (EPA), 22:5n-3, 22:6n-3 (DHA), DHA:EPA, Σ SFA, Σ MUFA, Σ PUFA, TAG, PPL and lipid per dry weight ($\mu g\,mg^{-1}$). Groups were determined by cluster analysis. (a) Lipid parameter coefficients for PC1 and PC2. (b) Eyes, heads and bodies scores for PC1 and PC2.

lipid respectively. Whalen (1999) also found that TAG was the major lipid class in the flesh of both cultured (87%) and wild fish (60%). Thus, both studies indicate that cultured yellowtail flounder use adipocytes in muscle tissue as a major storage site for neutral lipids.

Yellowtail flounder had higher levels of lipid per dry weight (23%) and TAG (~74%) than those reported for other flatfish species. Estevez and Kanazawa (1996) reported that lipids accounted for ~10% of the dry weight in newly settled Japanese flounder, with only ~16% of the total lipid presentas TAG. McEvoy *et al.* (1998) reported that newly settled Atlantic halibut contained only ~31% of the total lipids in the carcass as TAG. However, the measurements on both Japanese flounder and Atlantic halibut were taken at a younger age/developmental stage than those for yellowtail. Japanese flounder were measured on day 50 post hatch (~65 mg wet weight), whereas Atlantic halibut were measured on day 43 after first feeding (~140 mg wet weight). Here, yellowtail flounder were sampled on day 84 past first feeding when larvae weighed ~400 mg and were 100% settled.

Yellowtail flounder of a similar age/developmental stage to Japanese flounder and Atlantic halibut, cited above, were described by Copeman (2001). On day 43 after first feeding, yellowtail larvae had on average 14% of their dry weight as lipid and ~18% present as TAG. Therefore, differences in lipid composition between yellowtail analysed in this study and other flounder species can probably be explained by variation in the age at sampling. Further, days 43–84, which represent the 6 weeks following the first observations of settling behaviour, is a time during which yellowtail flounder dramatically increase their neutral lipid storage (Copeman 2001).

Comparisons between the different body zones showed that more lipid per dry weight was present in the bodies (~230 $\mu g\,mg^{-1}$) than in the head (~143 $\mu g\,mg^{-1}$) or eyes (~88 $\mu g\,mg^{-1}$). Most of this increase in lipid can be accounted for by increased percentages of TAG in the body (~73%) compared with the head (30%) or eyes (12%). PCA analysis clearly distinguished different body zones based on the amount of storage or membrane lipids present (PC1). Multivariate statistics have previously been utilized to differente between cod (*Gadus morhua*) and haddock (*Meanogrammus aeglefinus*) eggs and between different developmental stages (Vogt, Moksness, Sporstol, Knutsen, Nordenson & Kolset 1986). Navarro, McEvoy, Amat and Sargent (1995) also applied multivariate statistics to differentiate between different body zones in larval sea bass (*Dicentrarchus labrax*) fed different diets.

Fatty acid composition of juveniles

Both NP and MP fish preferentially retained PUFAs in their polar lipid rather than their neutral lipid, and this trend was observed in the body, head and eyes. Among the highly unsaturated fatty acids, DHA and AA were found in higher proportions in the polar lipid, whereas EPA was found equally in neutral and polar fractions. Increased retention of DHA relative to EPA in the polar lipids of larval fish has also been observed in gilthead seabream (*Sparus aurata*) and turbot (*Scopthalmus maximus*) larvae (Rainuzzo *et al*. 1994; Rodriguez, Perez, Diaz, Izquierdo, Fernandez-Palacios & Lorenzo 1997). This trend across species and body zones emphasizes the important specialized role of DHA in normal membrane structure and function. Interestingly, the proportion of PUFAs in the eye was lower than that in the body or head. This was due to reduced relative proportions of 18:2n-6, 18:3n-3, AA and EPA. As a result, DHA/EPA ratios were higher in eye tissue than in either the head or body.

The eyes contained higher levels of SFAs than did other tissues. The major SFAs present in the eyes were 16:0 and 18:0, and the major PUFA present was DHA. Bell & Dick (1993) described the molecular species composition of the phospholipids present in the eyes of juvenile herring. In the PC fraction of the eye. the phospholipid molecular species 16:0/22:6, 18:0/22:6 and 22:6/22:6 accounted for 30%, 10% and 15% PC respectively. Similarly, Bell & Dick (1991) described the molecular species present in the retinal phospholipids of Atlantic cod and found that 16:0/22:6, 18:0/22:6, and 22:6/22:6 accounted for, respectively, 23%, 9% and 30% of the PC fraction. Thus, the proportions of fatty acids present in the eye suggest that the molecular species composition of yellowtail flounder eyes is similar to that reported for other marine fish species.

A significantly higher percentage of DHA was found in the body polar lipids from NP (31.5%) fish than in body polar lipids from MP fish (28.7%); however, this trend was not significant in comparisons of head and eye tissue. Similarly, Estevez and Kanazawa (1996) reported significantly higher proportions of DHA in the polar lipids of the head and eyes in NP Japanese flounder than in MP fish. McEvoy *et al*. (1998) found higher absolute amounts of DHA in the eyes of NP Atlantic halibut than in MP fish. We observed a higher DHA/EPA ratio in NP fish eyes than in MP fish eyes. This was not observed in Japanese flounder or Atlantic halibut as EPA was also found in higher relative and absolute levels in the eyes of MP juveniles.

In yellowtail flounder, Japanese flounder, and Atlantic halibut, differences between NP and MP fish were related to higher relative or absolute amounts of DHA in neural tissues, particularly in the eye. Further, Bell & Dick (1993) correlated the levels of di-DHA phospholipids in the eye with the appearance of rods in the retina of juvenile herring. Rod outer segment membranes have been found to be particularly high in di-DHA phospholipids in other vertebrates (Stinson, Wiegand & Anderson 1991).

Kanazawa (1993) proposed a nutritionally based hypothesis for the occurrence of abnormal pigmentation in hatchery-reared flatfish. He stated that vitamin A, DHA and phospholipids were important in the formation of rhodopsin in the eye and that a lack of rhodopsin impaired vision. Further,

impairment of vision was hypothesized to cause a deficiency in neural stimulation (central nervous system) and thus also hormonal stimulation (melanophore-stimulating hormone), both of which are essential for the formation of melanophores. However, a lack of DHA-rich phospholipids may impair not only visual membrane function, but also general neural function and, in particular, melanophore cell membrane function.

Conclusions

Normally pigmented fish were significantly larger than MP fish at the time of 100% settlement. There were differences between the lipid composition of different body zones and between NP and MP fish within these zones. NP fish had a higher percentage of their lipid as TAG, whereas MP fish contained relatively higher amounts of phospholipids. Normally pigmented fish showed higher levels of DHA in the polar lipid of the body and higher DHA/EPA ratios in the eyes. Higher relative and absolute amounts of DHA in the neural tissues of NP fish have been reported for other marine species.

These data tend to support previous hypotheses regarding the importance of DHA in visual and thus neural and hormonal development. However, behavioural and histological studies are required to determine whether DHA actually affects visual function during the 'pigmentation window'. Further, nutrition has yet to be directly linked to any of the mechanisms involved with pigmentation development, such as the level of rhodopsin in the eye, the numbers of rods in the retina or the amount of melanophore-stimulating hormone in larvae.

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