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ORIGINAL ARTICLE

Effect of Early Introduction of Microencapsulated Diet to Larval Atlantic Halibut, *Hippoglossus hippoglossus* L. Assessed by Microarray Analysis

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Abstract An experimental microdiet prepared using an internal gelation method was used to partially replace the traditional live feed (*Artemia*) for larval Atlantic halibut, *Hippoglossus hippoglossus* L. Three trials were conducted with microdiet introduced at 20, 32, and 43 days post first feeding and larvae were sampled at approximately 2, 13, 23, and 33 days after microdiet introduction in each trial. The success of feeding was assessed by morphometrics and histological analysis of gut contents. Microdiet particles were readily consumed after a period of adaptation and provided an adequate source of nutrients with no significant increase in mortality in the microdiet-fed group compared to the control group. However, growth was limited and there was an increased incidence of malpigmentation of the eye and skin. Subtle changes in underlying digestive and developmental physiology were revealed by microarray analysis of RNA from control and experimental fish given microdiet from day 20 post first feeding. Fifty-eight genes were differentially expressed over the four sampling times in the course of the trial and the 28 genes with annotated functions fell into five major categories: metabolism and biosynthesis, cell division and proliferation, protein trafficking, cell structure, and stress. Interestingly, several of these genes were involved in pigmentation and eye development, in agreement with the phenotypic abnormalities seen in the larvae.

Keywords Microencapsulated diet · Larvae · Atlantic halibut · Microarray

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Introduction

Atlantic halibut (*Hippoglossus hippoglossus* L.) shows good potential for cold-water aquaculture (Berg 1997; Mangor-Jenson et al. 1998) and is highly valued for food (Blanchard and Scarratt 2002). High mortality during the early stages of feeding remains as one of the major bottlenecks for the development of the halibut aquaculture industry. Low survival of larvae is often linked to nutrient deficiencies and their poor performance on start feeding diets. At the early stages of development, halibut larvae are

fed live food organisms such as copepods, rotifers, and *Artemia* (Hamre et al. 2002; Evjemo et al. 2003; Lie and Lambertsen 1985; Solbakken et al. 2002; Berg 1997; Mangor-Jenson et al. 1998; Ronnestad et al. 1998). These organisms show wide variations in food quality and nutrient composition. Generally, *Artemia* and rotifers are enriched with emulsions based on formulations that contain highly unsaturated fatty acids and specific micronutrient supplements to improve the nutritional quality of live feed organisms. In the past three decades, significant research efforts have been directed towards replacing live food organisms with dry microdiets; however, they have achieved limited success in improving the survival and growth of several cold-water marine fish larvae. Microencapsulated diets have recently been studied as alternatives to live organism feeds for sea bream (Yúfera et al. 2000, 2003, 2005), Dover sole (Applebaum 1985), and Senegalese sole (Yúfera et al. 2003, 2005). However, little information is available for early weaning of larval Atlantic halibut (Hamre et al. 2001; Naess et al. 2001).

Microencapsulation, a technique whereby stable particles with minimal nutrient leaching are produced, has been used for the production of larval diets. These diets improve the delivery of essential nutrients to the larvae, minimize nutrient leaching, and prevent subsequent water quality problems (Jones et al. 1974; Langdon 2003). Digestibility of the capsules, ability to retain nutrients, nutrient load in the encapsulated particle, durability, and minimal use of toxic substances are key qualities in the design of microencapsulated diets (Yúfera et al. 2005). Previous studies with microencapsulated diets showed low growth of fish larvae due to inadequate diet formulation and inappropriate particle structure. In many cases, good larval growth is only achieved with microbound diets when cofeeding with live prey takes place (Langdon 2003). It is possible that adding feeding stimulants, enzymes, or nutrients in live feed could improve the utilization of artificial diets (Kolkovski et al. 1997). For Atlantic halibut, a microbound diet was only effective if introduced 20 days post hatching (Hamre et al. 2001). However, a microencapsulated diet was able to substitute for live food during the early stages of larval rearing in gilthead sea bream larvae, although again only limited growth was achieved (Yúfera et al. 1999). The need for properly formulated feed with correct nutrient balance is necessary to increase feed utilization and improve larval growth.

Different forms of microencapsulated diet for larval fish have been developed in past years (Langdon 2003; Yúfera et al. 1999; Önal and Langdon 2005). Each one has advantages that must be considered in finding equilibrium between stability to prevent excessive micronutrient leaching before being ingested and sufficient digestibility for larval fish with immature digestive tracts. Recently, Yúfera et al. (2005) developed a method for producing microparticles by internal gelation whereby sodium alginate reacted with a calcium solution to produce calcium alginate. This method allows the use of practical ingredients and environmentally friendly reagents during the preparation. The microdiets elaborated using this method are able to support growth in larval fish (Yúfera et al. 2005).

Eye development, eye migration, skeletal development, successful metamorphosis, and pigmentation are crucial aspects in the growth of halibut larvae and are all affected by diet (Vilhelmsson et al. 2004; Hamre et al. 2003; Naess and Lie 1998; Gara et al. 1998; Lewis et al. 2004; Lewis-McCrea and Lall 2007; Hamre et al. 2005; Solbakken et al. 2002; Hamre et al. 2002; Bell et al. 2003). Few studies have employed microarray analysis to investigate the growth of fish in response to dietary changes and none have yet been performed on flatfish. A small-scale nutrigenomic study investigated changes in gene expression in Atlantic salmon liver resulting from partial replacement of fish oil with rapeseed oil (O.-Jordal et al. 2005) and a more comprehensive microarray analysis of the effect of ration on growth hormone transgenic Atlantic salmon has been performed (Rise et al. 2006). Three studies have investigated changes in gene expression during larval development of sea bass (Darias et al. 2008), sea bream (Sarrapoulou et al. 2005), and Atlantic halibut (Douglas et al. 2008).

The present study seeks to determine whether a microencapsulated diet produced by internal gelation (referred to as the microdiet) can be successfully used to partially replace the traditional live feed (*Artemia*) for larval Atlantic halibut, *H. hippoglossus* L. The success of feeding was assessed by morphometrics and histological analysis of gut contents. A custom Atlantic halibut microarray representing over 9,000 genes (Douglas et al. 2008) was used to assess whether the expression of genes involved in digestion and absorption of macronutrients was altered in the larvae as a result of the introduction of the microdiet.

Methods

Experimental Feed

The microencapsulated particles were prepared by a modified method of internal gelation, as described by Yúfera et al. (2005). The mixed dietary ingredients (10% *w*/*v*; Table 1) and calcium citrate tetrahydrate 1% *w*/*v* (Aldrich, Oakville, ON, Canada) were blended into a 1.5% *w*/*v* sodium alginate solution of medium viscosity (MP Biochemicals, Santa Ana, CA, USA). Two parts of the

Table 1 Composition of the microencapsulated diet fed to larval Atlantic halibut

Ingredients	Amount (%)
Herring meal[a]	41.2
Herring roe, lyophilized[b]	15.0
CPSP-G	13.0
Squid meal	10.0
Baker's yeast	5.0
Corn starch, pregelatinized	9.8
Soy lecithin	2.0
Marine oil mixture	1.7
Vitamin premix[c]	1.3
Mineral premix[d]	1.0

[a] Herring oil was stabilized with 0.06% ethoxyquin; Comeau seafood, Saulnierville, NS, Canada; peroxide value=0.46 mEq kg^{-1} oil

[b] Prepared in the laboratory from herring roe collected from local fishery, freeze-dried, and ground to a powder

[c] Vitamin added to supply the following (per kilogram diet): vitamin A (retinol acetate), 6,000 IU; vitamin D_3 (cholecalciferol), 4,000 IU; vitamin E (DL-α-tocopheryl acetate), 400 IU; vitamin K_3 (menadione sodium bisulfite), 40 mg; thiamin (thiamin HCl), 50 mg; riboflavin, 50 mg; D-calcium pantothenate, 150 mg; biotin, 1 mg; folic acid, 15 mg; vitamin B_{12}, 0.15 mg; niacin, 200 mg; pyridoxine HCl, 20 mg; ascorbic acid (ascorbyl monophosphate), 200 mg; inositol, 400 mg; choline chloride, 200 g; butylated hydroxytoluene, 15 mg; butylated hydroxyanisole, 15 mg

[d] Minerals added to supply the following (per kilogram diet): manganous sulfate ($MnSO_4 \cdot H_2O$, 32.5% Mn), 40 mg; ferrous sulfate ($FeSO_4 \cdot H_2O \cdot 7H_2O$, 20.1% Fe), 30 mg; copper sulfate ($CuSO_4 \cdot 7H_2O$, 25.4% Cu), 5 mg; zinc sulfate ($ZnSO_4 \cdot 7H_2O$, 22.7% Zn), 75 mg; cobalt chloride ($CoCl_2 \cdot 6H_2O$, 24.8% Co), 2.5 mg; sodium selenite (Na_2SeO_3, 45.6% Se), 1 mg; sodium fluoride (NaF, 42.5% F), 4 mg

homogenized solution were emulsified in five parts of a soy lecithin (LV Lomas, Brampton, ON, Canada) and sunflower oil solution (2% *w*/*v*) for 10 min at 800 rpm in a Stir-pak laboratory mixer (Cole-Palmer Instrument; Model 4554-10) at ambient temperature. A glacial acetic acid (Caledon, Georgetown, ON, Canada) and sunflower oil solution (1/1) was added to the emulsion (4% *v*/*v* of total oil volume) and dispersed for 10 min. The microparticles settled and supernatant oil was decanted and further separated by centrifugation at 1,000 rpm for 2 min under ambient temperature (Centra CL3R; Model 120; Thermo EIC). The microparticles were stirred into an anhydrous calcium chloride solution (0.5% *w*/*v*; 0.035 mM; Panreac, Barcelona, Spain) for 8 min and settled before decanting the upper portion and then separated in a 200-µm sieve. The recovered particles were dispersed into a Tween 80 (Anachemia, Mississauga, ON, Canada) solution (1%) for 5 min, sieved, and then washed with fresh water to remove debris. The capsules were freeze-dried to produce the final microdiet.

Fish Feeding and Rearing

All fish rearing was conducted as part of a production run at Scotian Halibut Ltd., Clark's Harbour, Nova Scotia, Canada. Experimental fish were housed in parallel cylindrical tanks; each measured approximately 40 cm in width and 100 cm in depth. All halibut larvae used for the experiment were transferred from a single 7-m^3 tank with flow-through salt water (32 ppt) maintained at 11±0.2°C using a heat exchanger. The larvae were transferred at three ages: 20 days (trial 1), 32 days (trial 2), and 43 days (trial 3; 492, 628, and 706 degree days, respectively) post-*Artemia* (*Artemia salina*; Gulf Breeze Aquaculture & Seafood, Fresno, CA, USA) introduction. Approximately 200 fish were transferred to each of three replicate treatment tanks for each trial. Due to space limitations, the 7-m^3 production tank was considered the control treatment and fish were sampled from this tank at the same times as the experimental fish.

All tanks were fed *Artemia* twice daily at approximately 7 A.M. with 3×10^5 cells and at 4 P.M. with 6×10^5 cells. The experimental larvae, in addition to the *Artemia* feed, were fed the microdiet every half hour to excess. *Artemia* feed amounts were adjusted in the control tank over time according to how quickly the *Artemia* were cleared from the tank after feeding; however, the experimental larvae were fed a constant amount twice daily of *Artemia* and microdiet to excess until day 40 when *Artemia* was discontinued and larvae were fed only microdiet to excess. The microdiet was then fed for an additional 10 days. Trial 3 larvae that were transferred on day 43 were cofed *Artemia* and microdiet on the day of transfer only and then fed exclusively microdiet until day 53. Larvae in all tanks were fed a commercial dry diet (Gemma micro, Skretting, Bayside, NB, Canada) to excess from day 53 onwards.

Oxygen and temperature were measured daily in the experimental tanks from when larvae were first transferred until the end of the trial. Mortalities for experimental tanks were removed daily and counted. Mortalities for the control tank were measured daily from day 20 until the end of the trial. Feeding times for both the microdiet and the *Artemia* were also recorded daily.

Sampling and Measurement of Larvae

The control group and each replicate from the three experimental groups were sampled throughout the trial to obtain material for microarray and histological analyses. Sampling occurred at days 2 and 10–13 after transfer for all three trials. Additional sampling occurred at days 21–23 after transfer for trials 1 and 2 and also at day 33 after transfer for trial 1.

For larvae up to 30 days post first feeding, 50 individuals were sampled for RNA work and for histology. For older larvae, 20 individuals were sampled. Larvae were euthanized with an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA, USA) and placed in 50 mL tubes with twice their volume of RNALater (Ambion, Austin, TX, USA). Samples for histology were fixed overnight at 4°C by immersion in 4% *v*/*v* paraformaldehyde in Tris–HCl (pH7.8) and processed for paraffin embedding (Murray et al. 2006). Ten fixed larvae at each sampling point were measured for weight, length, and width. All fish were photographed and measured using the Nikon AZ100 microscope (Nikon Canada, Mississauga, ON, Canada) with the NIS Elements Advanced Research package V.3.0. All fish were photographed against a white background and were held flat with a microscope slide. Lengths were measured from the tip of the snout/mouth to the terminus of the body muscle not including the caudal fin rays (standard length). The widths were measured by drawing a line perpendicular to the length measure, starting the line at a point near the anus. The width measures did not include ventral or dorsal fin areas.

Microscopy

Paraffin-embedded tissues were sectioned at 7 µm, mounted on uncoated glass slides, dried briefly, and then baked overnight at 60°C to enhance adherence to the slides. For general histological examination, sections were deparaffinized, rehydrated, and then stained with hematoxylin and eosin using standard procedures. Four slides were prepared from each fish and four serial sections were examined from each for liver morphology and gut contents. Feed intake was calculated as the percent of the gut filled by food for each of four to six individuals per sampling time.

Image Capture and Analysis

The microscopy images for control and experimental individuals were captured and analyzed for the “percentage of gut filled by food” by performing area measurements using the Simple PCI 6.0 software (Simple PCI, Hamamatsu 2008).

Statistics

A one-way analysis of variance (ANOVA) was performed using SAS Version 9.1 (SAS Institute, Cary, NC, USA) to analyze the feed consumption data and calculate the significant difference between the experimental and control fish for “percentage of gut filled by food.” Statistical analysis of comparative morphometric data was performed using one-way ANOVA in Excel (Microsoft, Redmond, WA, USA).

RNA Extraction

For larvae transferred at day20 (trial 1), RNA was isolated from five whole individuals by homogenizing in 1.5 ml Trizol (Invitrogen, Burlington, ON, Canada) according to the manufacturer's recommendation. For control samples, fish were randomly taken from the three replicate samples at each of the sampling times and processed as above, giving a pooled control for each sampling time. After rough quantitation, 100 µg of each RNA sample was cleaned using the RNeasy kit (Qiagen, Mississauga, ON, Canada) following the RNA Cleanup protocol. A 2-µl aliquot of each eluted sample was quantified using a NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and RNA integrity was determined using a Bioanalyzer 2100 (Agilent Technologies, Mississauga, ON, Canada). The RNA integrity number generated for all RNA samples was over 8.5.

Microarray Experiments

Since early weaning was the focus of this study, microarray experiments were performed for larvae from trial 1 only. cDNA was prepared from 1 µg of total RNA from each of the three replicates at each time point (days2, 13, 23, and 33 after sampling) using the Array 900 kit (Genisphere, Hatfield, PA, USA) and labeled with Alexa Fluor™ 647 dye (see Fig. 1). This was hybridized to the Atlantic halibut oligonucleotide array, which contains 9,277 unique features from Atlantic halibut printed in quadruplicate (Douglas et al. 2008), with control cDNA prepared from 1 µg of a pooled RNA from fish randomly sampled from the production tank at the same time points and labeled as above with the Alexa Fluor™ 546 dye. The formamide-based hybridization buffer was used and hybridization was performed overnight at 43°C. Capture reagent (2.5 µl) was added in 30 µl of hybridization buffer and the microarray incubated at 52°C for 4 h.

Microarrays were scanned at 543 and 633 nm using a ScanArray® 5000XL Microarray Acquisition scanner (Packard Bioscience, Billerica, MA, USA) at a resolution of 10 µm. Laser power was set at levels between 80% and 100% and photomultiplier tube settings were set at values ranging from 70% to 95% to adjust Alexa 555 and Alexa 647 channels on individual slides. Spot intensities were measured using the SpotReader version 1.3 (Niles Scientific, Portola Valley, CA, USA) software, and the CSV files loaded into the ArrayPipe (Hokamp et al. 2004) server at the Institute for Marine Biosciences.

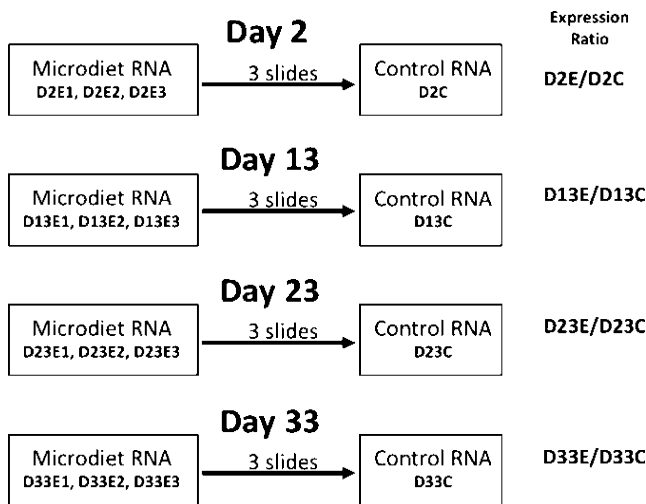

Fig. 1 Experimental design for gene expression analysis of fish from trial group 1 fed microdiet at 20 days post first feeding. RNA from experimental fish sampled at D2, D13, D23, and D33 is shown in *boxes on the left* and RNA from control fish sampled at the same times is shown in *boxes on the right*. Replicates for experimental samples are designated E1, E2, and E3. Pooled control RNA samples are designated C

Markers (blanks and those containing *Arabidopsis* controls) were flagged and the remaining spots were corrected for background using the "limma normexp BG correction" option. Background-corrected spots were normalized using the "limma loess (subgrid)" option and data from the quadruplicate spots were merged (using median log2 ratios). The "limma eBayes mod *t*-test (within group)" was applied to test for significant spots and the medians of the ratios across triplicate arrays from each time point were then calculated. Spots with p values <0.05 and fold changes less than −2 or greater than +2 were retained for further analysis. Significance analysis of microarrays (SAM; Tusher et al. 2001) was also performed for each set of triplicate arrays in order to select differentially expressed genes that were statistically significant.

Gene Ontology (GO) analysis (Ashburner et al. 2000) was performed using MatLab® (v. 7.0, The MathWorks, Natick, MA, USA) functions that emulate the functionality of GO::TermFinder (Boyle et al. 2004; Flight and Wentzell 2009). GO terms for 3,927 of the 9,277 unique genes on the microarray were obtained from http://www.pleurogene.ca. Two types of analysis were conducted. The first examined the genes at each time point (D2, D13, D23, and D33) that were found to be differentially expressed with a p value <0.05 in comparison to all the genes that passed filtering for the time point. The second analysis examined the low p value genes that passed filtering at three or more time points and compared them with all the genes that passed filtering at three or more time points. Multiple testing correction was implemented using the false discovery rate (FDR), and significant GO terms were those with an FDR <5%. The analysis was carried out using GO terms from biological process (P), molecular function (F), and cellular component (C).

Quantitative Real-Time PCR Experiments

First-strand cDNA was prepared from 1 µg of total RNA from each of the samples used for microarray analysis using the Superscript III First-Strand Synthesis Super Mix (Invitrogen). Primers for candidates to be validated: glutathione *S*-transferase (gstA1), DNA polymerase delta subunit 3 (polD3), replication protein A3 (rpa3), titin (ttn1), and peripherin (prph) and two housekeeping genes elongation factor 1A1 (ef1A1) and ribosomal protein S4 (rps4) were designed based on the Atlantic halibut expressed sequence tag (EST) corresponding to the microarray spot, using PrimerQuest^{SM} software (http://www.idtdna.com/Scitools/Applications/Primerquest/) and synthesized by Integrated DNA Technologies (Toronto, ON, Canada). Amplicon sizes were approximately 140–160 bp and the optimum T_m was 55°C (Table 2). All quantitative real-time polymerase chain reactions (qRT-PCR) were performed in a 20-µl total reaction volume (18 µl master mix and 2 µl PCR product/cDNA template). The master mix contained 6.4 µl H_2O, 0.8 µl of each primer (0.4 µM final concentration), and 10.0 µl of the SYBR Green Mix (Roche Applied Science, Laval, PQ, Canada). The following cycling conditions were used: (1) denaturation, 5 min at 95°C; (2) amplification repeated 40 times, 10 s at 95°C, 10 s at 55°C, and 15 s at 72°C with ramp rate of 4.4°C/s, 2.2°C/s, and 4.4°C/s, respectively; (3) melting curve analysis, 1 min at 95°C and 1 min at 55°C with ramp rate of 4.4 and 2.2°C/s, respectively, then up to 95°C at a rate of 0.1°C/s; (4) cooling, 10 s at 40°C with ramp rate of 2.2°C/s. Each sample was analyzed in duplicate and reactions were performed in a Light Cycler (Roche Applied Science). Crossing point values were compared and converted to fold differences by the relative quantification method using the Relative Expression Software Tool (REST©) 384 v. 2 (Pfaffl et al. 2002) with ef1A1 and rps4 as the reference genes.

Results

Microdiet and Larval Performance

The microdiet consisted of particles of 50–200 µm that were of an irregular shape (Fig. 2). The diet sank relatively quickly to the bottom of the tanks and sometimes clouded the tanks even with regular cleaning. In a visual comparison of surface area of the fish at the conclusion of the trial, the

Table 2 Primer sequences for genes-of-interest selected for qRT-PCR analysis

Gene	Sequence (5′ > 3′)	Product size (bp)
Glutathione *S*-transferase (gstA1)	F: AGTGCCGTGTGTTTATACCTGGAG R: ACTGAGTTGAGTTTCTCGGTGAGG	137
Peripherin 1 (prph)	F: TACATGCGTTCAGCGTCACCTA R: ACTCCTGGTTGATGGCTTCAGA	145
Replication protein A3, 14 kDa (rpa3)	F: AAAGCGTTCACTGTGTCGGATG R: TCGCAGCATGTTGTATGTCGTG	150
Similar to titin isoform N2-B (ttn1)	F: AAAGCGTTCACTGTGTCGGATG R: CTTCTCGCAGCATGTTGTATGTCG	154
DNA polymerase delta subunit 3 (polD3)	F: ACAAGGTGTCCGTTGTCAGAGA R: TCACAGCATCGTAGTCCACACT	144
Elongation factor 1A1 (ef1A1)	F: AAGAGGACCATCGAGAAGTT R: GTCTCAAACTTCCACAGAGC	141
Ribosomal protein S4 (rps4)	F: GCCAAGTACAAGCTGTGCAA R: AGGTCGATCTTGACGGTGTC	138

Elongation factor 1A1 and ribosomal protein S4 were selected as reference genes

control fish were 100% larger when compared to the fish fed the microdiet (Fig. 3). Measurements of weight, length, and width also showed significant (ANOVA; $p<0.05$ or 0.001) differences in growth with the exception of the trial 3 fish and the first sampling point in trial 1 (Fig. 4). The experimental fish appeared to be developmentally delayed; by the end of the trial, eye migration in the control group was completed but fish in the experimental groups had either not yet started eye migration or had not completed it. Differences in pigmentation were also apparent; in the control tank, <25% of fish were malpigmented, whereas in the experimental tanks, 75% were malpigmented. Of these, 25% were nonpigmented and 50% were abnormally pigmented ("orange" fish). The bodies of these "orange" fish were light brown to orange and their eyes were a lighter hue than the normal black seen in the control fish.

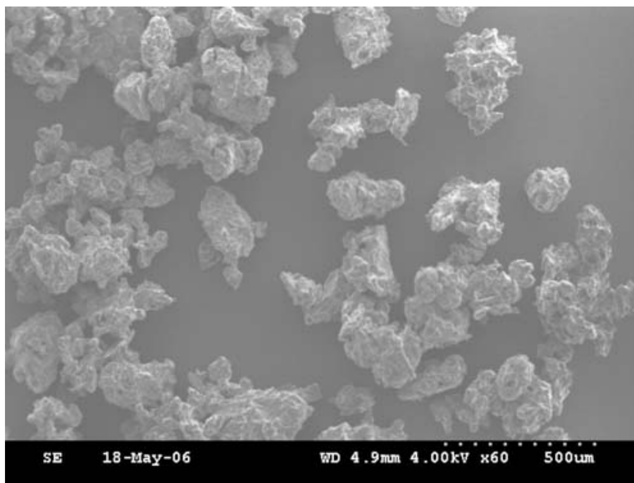

Fig. 2 Freeze-dried microencapsulated diet produced as described in the "Methods" section. Scale bar (*dots*) represents 500 µm

Mortalities varied between tanks; however, differences in mortalities between treatments were insignificant (ANOVA; $p>0.05$). Mean mortalities were 32 (SD=1.4), 46.5 (SD=30.4), and 44 (SD=0) for trials 1, 2, and 3, respectively. In the control tank, which contained many more fish than the experimental tanks, there were only 20 mortalities by the end of the trial.

Feed Consumption

The percentage of filled intestine as determined by image analysis of sections of gut from the larval samples is shown in Table 3. For all three trials, there was a significant difference between the control and experimental fish for the first 10 days with the former exhibiting a full gut and the latter only a partially filled gut (Table 3 and Fig. 5). However, for the larvae in trials 1 and 2, the experimental larvae appear to have adjusted to the microdiet after time as there was no significant difference in the amount of gut filled with food compared to control larvae at the later sampling times.

Gene Expression Changes

Of the 39,936 spots on the Atlantic halibut microarray, between 3,600 and 8,900 passed quality control for each array (Table 4). After calculating the median log2 ratios of the quadruplicate spots, approximately 2,000–4,000 data points were retained from each array. Following similar analysis of the triplicate arrays, approximately 1,500–2,300 spots were detected for each time point. In order to restrict our analysis to the highest quality data, only spots detected on two or more of the arrays were retained from each of the four time points. Of these, approximately 250–300 had a *p*

Fig. 3 Phenotypic differences between experimental and control fish at the conclusion of the trial. *a* Trial group 2 transferred at 32 days post first feeding; *b* trial group 3 transferred at 43 days post first feeding; *c* control. Note the size difference and pigmentation. Larvae are not available from trial group 1 transferred at 20 days post first feeding as they were all required for RNA extraction due to their small size

value of <0.05 at each of the four time points and approximately two thirds were greater than twofold upregulated or downregulated. Fifty-eight genes were in common among the significantly differentially regulated genes in three or more time points (Tables 5 and 6). The gene showing similarity to trypsin was most closely related to a trypsin from sponge and the signal probably arose from either the *Artemia* live prey or, less likely, a component of the microdiet. For this reason, it was excluded from further analysis, as were those with no annotation.

SAM generated gene lists containing genes only present in all three of the replicate arrays. For microarrays corresponding to larvae sampled at D2, D13, D23, and D33 after transfer, there were 37, 54, 80, and 65 genes, respectively, that were significant by SAM. All of these genes were also present in the ArrayPipe results with the exception of three genes (23998—unknown, 22338—similar to ubiquinol–cytochrome *c* reductase complex 11-kDa protein, and 33342—similar to collagen type X, alpha 1) that were present only in the D23 SAM list and one gene (25531—unknown) that was present only in the D33 SAM list.

The differentially expressed genes that were present in gene lists from at least three out of the four time points could be grouped into five main classes according to their functions: metabolism and biosynthesis, protein trafficking, structural proteins, stress proteins, and those involved in cell division and proliferation (Tables 5 and 6). GO term analysis showed that the majority of these 26 annotated genes were significantly enriched in GO terms corresponding to metabolic processes when compared to all of the genes that

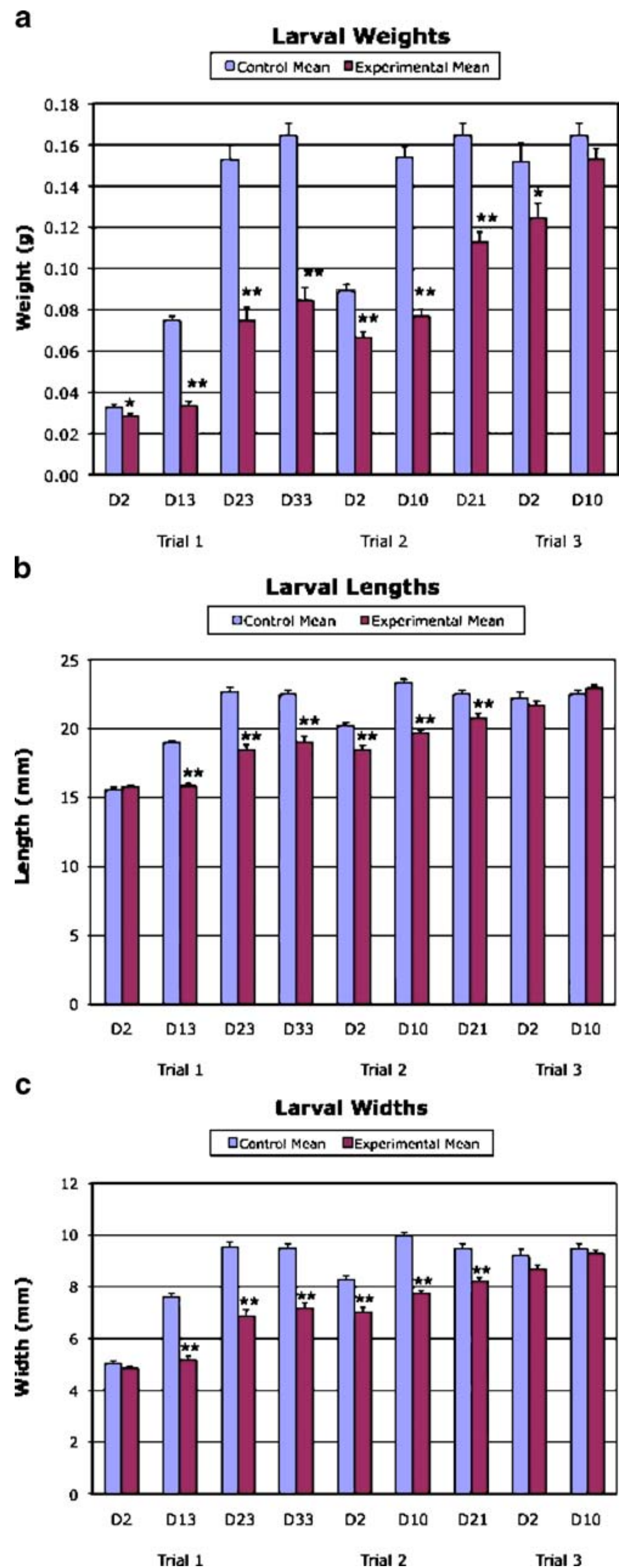

Fig. 4 Morphometrics of experimental and control fish from trials 1, 2, and 3 at different sampling times. **a** Weight, **b** length, **c** width. Mean values with standard errors are shown. $*p<0.05$; $**p<0.001$

Table 3 Average percent filled intestine of experimental (Exp) and control (Con) Atlantic halibut larvae at different sampling times (in days) after transfer (trials 1, 2 and 3) and introduction of microencapsulated diet

Day of transfer	Sampling time after transfer	Average percent filled intestine (Exp)	Average percent filled intestine (Con)	*p* value
20 (trial 1)	D2	21.83 (154)	82.50 (67)	$1.63e^{-06}$
20 (trial 1)	D13	62.50 (75)	87.50 (43)	$3.73e^{-03}$
20 (trial 1)	D23	51.17 (787)	69.67 (462)	0.229
20 (trial 1)	D33	51.25 (806)	83.75 (6)	0.063
32 (trial 2)	D2	64.00 (48)	89.25 (6)	$4.74e^{-04}$
32 (trial 2)	D10	63.33 (216)	91.75 (6)	$5.59e^{-03}$
32 (trial 2)	D21	64.12 (304)	66.25 (456)	0.869
43 (trial 3)	D2	42.5 (841)	87.5 (7.5)	$4.57e^{-03}$
43 (trial 3)	D10	30.00 (390)	85.83 (24)	$5.23e^{-05}$

Variance is shown in brackets. Significant *p* values are italicized

D day after transfer

passed filtering (Table 7). When the differentially expressed genes were compared at each of the four sampling times (Table 8), significant enrichment in GO terms associated with metabolic processes was evident at D2 after transfer, but at D23, the enrichments were associated with ion transport and muscle physiology. No significant enrichments were seen at D13 or D33.

Five genes-of-interest were selected for verification by qRT-PCR: two genes that were upregulated at all time points (gstA1 and prph) and three genes that were downregulated at all time points (polD3, rpa3, and ttn1). The choice of housekeeping genes ef1A1 and rps4 as reference genes was based on their apparent stability throughout Atlantic halibut larval development (Infante et al. 2008). Although the magnitude of the changes in the expression of the genes-of-interest was not the same as in the microarray experiments, qRT-PCR analyses confirmed that glutathione *S*-transferase was significantly upregulated at D23 and peripherin at D2. DNA polymerase delta subunit 3 was not found to be significantly downregulated by qRT-PCR, whereas replication protein A3 and titin were significantly downregulated at D2 and D13.

Discussion

The microdiet appeared to be successfully consumed (assessed by percent of gut filled) after an initial period of adaptation. This occurred whether the diet was introduced at 20 or 32 days post first feeding. For the later introduction at 43 days post first feeding, the larvae did not consume the microdiet well (42.5% and 30% filled guts after 2 and 10 days, respectively). Since the larvae were switched to

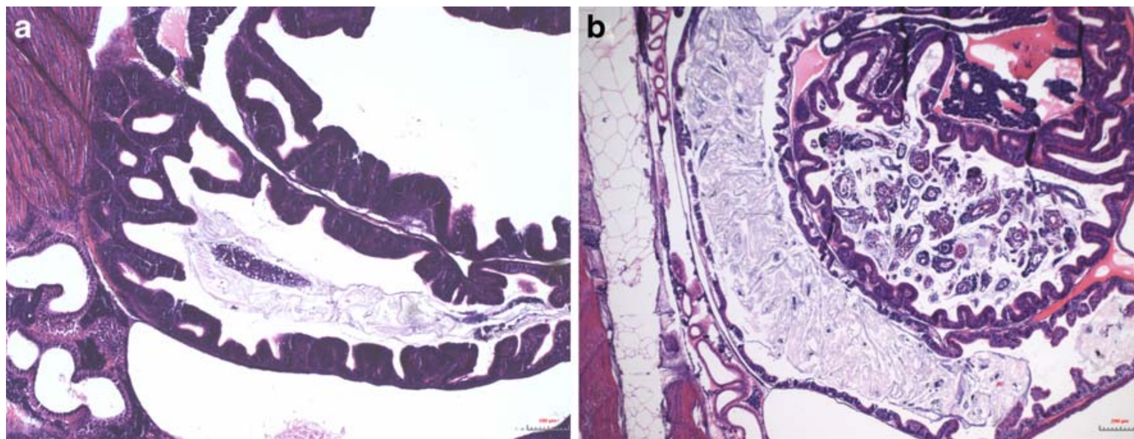

Fig. 5 **a** Representative cross-section of the intestine of larval Atlantic halibut transferred from trial 1 and sampled 2 days after the introduction of the microdiet. **b** Representative cross-section of the intestine of larval Atlantic halibut from the production tank at the same time. Images are representatives of 16 sections analyzed for each of four to six individuals per sampling time. Scale bar is 200 μ. Note the difference in food contents of the gut

Table 4 Spots identified as significantly ($p<0.05$) differentially expressed (greater than twofold or less than −2-fold) in three replicate arrays at each sampling time during trial 1

Sampling time after transfer	Replicate array	Bkgd corrected/ normalized	Merged Rep spots	Merged Rep arrays	$p<$ 0.05	>2 up	>2 down	Common genes
D2	1	6,720	2,622					
D2	2	6,903	2,725	2,001	242	57	72	
D2	3	3,965	1,824					
D13	1	5,719	2,236					
D13	2	5,578	2,195	1,779	281	89	59	
D13	3	5,045	2,301					58
D23	1	4,566	1,973					
D23	2	3,626	1,323	1,557	299	71	126	
D23	3	8,902	3,079					
D33	1	6,483	2,582					
D33	2	7,349	2,670	2,344	314	169	92	
D33	3	7,489	2,777					

D day after transfer, *Bkgd* background, *Rep* replicate

the commercial diet after 10 days, it was impossible to determine if they would have adapted to the microdiet.

The larvae appeared to prefer *Artemia* since the microdiet was not consumed initially as well. However, when *Artemia* was removed from the experiment at day 40 and the larvae were fed the microdiet only, the tanks were clear of uneaten particles. Histological examination of the gut of experimental larvae showed that the microdiet was physically digestible. Therefore, even with the exclusion of *Artemia* in their daily diet, the microparticles provided an alternate diet on which the larvae could survive. Fernández-Díaz et al. (1994), working with a protein microencapsulated diet, reported that fish larvae clearly prefer live prey to inert diets.

Mortalities of the experimental groups were somewhat higher than the control group, although this was not statistically significant. For example, trial 3, the last to be fed the experimental diet, had twice as many mortalities as the control group. This could be because they did not have time to adjust from the longer preceding period on the *Artemia* diet and were only given 1 day to adjust before they were switched to the microdiet. Trial 1 had a longer weaning period to switch from *Artemia* and adjust to the new microdiet and had the least mortalities. In fact, an early cofeeding period seems to be beneficial for accepting and processing inert diets, allowing better growth performance when weaning starts (Engrola et al. 2009; Curnow et al. 2006; Cañavate and Fernández-Díaz 1999; Rosenlund et al. 1997). In future studies, if the microdiet is administered, it may be beneficial to allow a few weeks for the fish to adjust to a new diet.

Lower feed consumption by larvae could have been due to the small particle size. The diet contained particles 50–200 µm in size; however, a more appropriate size range would have been 400 to 600 µm. The small particle size may have prevented the larvae from ingesting the diet due to its low visibility; it simply could have been ignored or not recognized as a food item. Alternatively, additional energy utilized to capture the small feed particles by larvae may have limited energy available for their growth and development.

Another explanation for the reduced growth in trial fish could be the density of the microdiet, which quickly sank to the bottom of the tank. Since halibut optimally feed within the water column, any particles that were not consumed in the brief time sinking through the water column collected on the bottom of the tank. Crucial micronutrients may also have leached from the microdiet prior to intake. If the microdiet had been suspended in the water column for a longer period of time, it may have been more available to the larvae. Changes to the physical properties of the microdiet to make it more buoyant should improve the results.

Although the diets were formulated to provide adequate level of nutrients, low bioavailability of certain nutrients as well as the leaching of nutrients may have limited growth of the experimental fish compared to fish fed live food organisms. As seen in Fig. 4, when fish were fed microdiet at early ages (trials 1 and 2), they exhibited reduced weight, length, and width compared to control fish. A similar observation was reported for sea bream larvae when their normal feed was substituted with the microdiet (Yúfera et al. 2005). The experimental halibut also appeared developmentally delayed, such as in eye migration and pigmentation, compared to the control group. This is likely due to small size, perhaps caused by nutrient deficiency or limited consumption of microdiet.

Table 5 Common genes (26 annotated) significantly differentially expressed at three or more time points

Spot ID	Class and gene description	Gene name	D2 ratio	D13 ratio	D23 ratio	D33 ratio
Metabolism and biosynthesis						
25124	Aldolase A fructose-bisphosphate	aldoaa	2.40	0.61	0.48	nd
26528	Glyceraldehyde-3-phosphate dehydrogenase	gapdh	1.67	0.57	0.54	nd
29338	Glutamine-fructose-6-phosphate transaminase2	gfpt2	1.37	1.59	1.79	nd
25716	Similar to DB83 protein (transmembrane protein 33)	tmem33	nd	15.76	14.95	12.95
24645	Similar to 1-acylglycerol-3-phosphate *O*-acyltransferase	agpat9	2.83	5.61	2.64	4.42
27053	Similar to vertebrate phosphodiesterase 3A, cGMP-inhibited	pde3A	1.43	1.87	3.48	nd
Protein trafficking						
32878	Similar to golgi membrane protein SB140	yip1	34.69	17.58	nd	18.89
33188	Similar to Bet1 homolog Golgi vesicular membrane trafficking protein p18	bet1	22.71	22.83	22.01	22.28
Structural proteins						
26020	Collagen type I, a3	col1a3	0.71	0.54	0.25	nd
25564	Similar to collagen, type IX, alpha 1	col9A1	0.12	0.15	0.14	0.45
23405	*Similar to titin isoform N2-B, connectin*	ttnl	0.50	0.13	0.08	0.08
30296	Warm-temperature-acclimation-related 65-kDa protein-like (hemopexin domain)	wap65-2	nd	2.29	1.85	0.59
32651	BetaA2-2-crystallin	cryba2b	1.68	7.23	nd	0.64
29133	Similar to es1 protein	es1	0.56	2.72	2.05	nd
27459	*Peripherin 1*	prph	25.50	22.38	26.04	12.15
Stress proteins						
28450	*Glutathione S-transferase, alpha*	gstA1	11.46	15.39	13.63	6.10
28618	Hepcidin precursor type II		0.60	14.57	nd	0.04
Cell division and proliferation						
33896	Replication protein A3, 14 kDa	rpa3	0.01	0.10	0.50	0.36
23084	*DNA polymerase delta subunit 3*	polD3	nd	0.70	0.48	0.45
24367	Basic transcription factor 3	btf3a	0.21	0.53	0.35	nd
23849	Similar to transmembrane protein 85, proliferation inducing gene 17	tmem85	0.08	0.66	0.09	0.42
22232	Similar to mitotic spindle assembly checkpoint protein MAD2A	mad2l1	1.65	0.57	0.49	nd
30073	Lens epithelium-derived growth factor	ledgf	nd	0.59	0.63	0.61
26658	Zinc finger, A20 domain containing 2	zfand5b	nd	6.71	6.71	4.66
32867	SFRS protein kinase 1	srpk1	4.05	33.69	5.90	8.52
32013	*Eukaryotic translation elongation factor 1B2*	eef1b2	1.64	1.35	1.79	nd

Those genes that were tested by qRT-PCR are italicized

nd no significant data in the microarray

In order to study changes in larval gene expression, we compared larvae fed control diets with those fed microdiet. Since the control fish were reared in the production tank in optimal conditions throughout the trial, they were at a more advanced developmental stage than their experimental counterparts, especially in the later sampling times. This is evident in the morphometric data for trial 1 presented in Fig. 4. Although this may be seen as a limitation in our study of gene expression changes, most larval feeding trials are conducted in this manner since, unlike with juveniles, it is difficult to separate larval development changes from treatment effects. Rather than compare gene expression changes sequentially over time in the control fish and corresponding sequential changes in the experimental fish, we chose to compare gene expression changes in the control fish relative to the experimental fish at each sampling time with the caveat that developmental differences may preclude absolutely accurate comparisons. Every precaution was taken by the experienced hatchery staff to minimize handling stress.

Our previous microarray study of sequential development of Atlantic halibut larvae under control conditions indicated that the major gene expression changes seen between larvae at days 21 and 64 post hatching were related

Table 6 Common genes (31 unannotated) significantly differentially expressed at three or more time points

Spot ID	Class and gene description	D2 ratio	D13 ratio	D23 ratio	D33 ratio
Unknown					
26900	similar to *Platichthys flesus* cDNA	nd	7.25	2.85	7.86
24743	similar to *Platichthys flesus* cDNA	nd	0.55	0.33	0.29
22286	Unknown	9.31	4.71	6.80	6.05
22495	Unknown	0.03	0.07	0.18	nd
23894	Unknown	0.05	0.05	0.09	0.17
24705	Unknown	0.51	0.33	nd	0.16
25273	Unknown	nd	0.21	0.17	0.17
25322	Unknown	nd	0.57	0.22	0.56
25423	Unknown	0.00[a]	0.00[a]	0.01	0.01
25817	Unknown	0.17	nd	0.12	0.52
26258	Unknown	0.15	0.41	0.13	0.22
27507	Unknown	4.46	3.07	nd	0.26
29255	Unknown	nd	0.00[a]	0.01	0.01
29542	Unknown	nd	0.41	0.50	0.41
29572	Unknown	27.60	24.19	6.04	5.58
29586	Unknown	nd	1.93	1.81	1.80
30344	Unknown	2.14	2.77	2.66	2.85
30656	Unknown	0.05	0.16	0.16	0.30
31066	Unknown	0.01	0.01	0.03	0.04
32064	Unknown	0.34	0.55	0.35	nd
32115	Unknown	0.23	0.39	0.33	0.27
32981	Unknown	0.63	0.35	0.14	nd
33063	Unknown	0.17	0.21	0.10	nd
33643	Unknown	2.55	7.15	nd	3.83
33961	Unknown	0.34	0.32	nd	0.27
34273	Unknown	15.76	22.45	6.93	6.08
34707	Unknown	22.39	6.01	14.00	36.95
35166	Unknown	31.09	12.48	3.51	15.53
35406	Unknown	nd	10.67	11.32	14.29
35416	Unknown	nd	6.59	3.07	2.31
35506	Unknown	0.05	0.13	0.04	0.46
28930	Trypsin (contaminant)	5.14	4.67	9.36	4.02

Trypsin is a contaminant possibly from *Artemia* or microdiet constituents

nd no significant data in the microarray

[a] Values <0.01 but rounded to two decimal places

to digestion and muscle development (Douglas et al. 2008). Similar results were obtained in a comparison of sea bass larval development where genes involved in muscle development were expressed at later stages of development (31–43 days post hatching) and those involved in energy production were expressed early in development (Darias et al. 2008). In sea bream, genes involved in energy metabolism are also expressed early in development (Sarrapoulou et al. 2005). As discussed below, the changes we saw in response to the introduction of microdiet were mainly involved in metabolism, eye development, pigmentation, and tissue structure and gave an indication of subtle underlying physiological changes responsible for the growth and phenotypes observed.

Our analysis focused on significantly differentially expressed genes that we could track across at least three of the four time points. Of the 58 genes that satisfied these criteria, 31 were for unknown genes or ESTs that could not be annotated. Several of these unannotated genes showed very high upregulation (>20-fold) or were effectively shut off (<0.005-fold); their identification may reveal interesting processes that are sensitive to constituents of the microdiet, starvation, or stress. In fact, two of the genes that were similar to *Platichthys flesus* cDNA (Spot IDs 24743 and 26900) were from a liver cDNA library that had been selected for genes induced by chemical pollutants (Williams et al. 2006) and may be general stress proteins that are also induced by dietary stress.

Table 7 GO term analysis of 26 annotated genes that were significantly expressed with a p value <0.05 at three or more time points compared to 3,414 that passed filtering

GO term	FDR (%)	Term description
6468	0.90	Protein amino acid phosphorylation
19318	1.00	Hexose metabolic process
6006	1.11	Glucose metabolic process
44262	1.23	Cellular carbohydrate metabolic process
5996	1.25	Monosaccharide metabolic process
6066	1.33	Alcohol metabolic process
46365	1.43	Monosaccharide catabolic process
46164	1.67	Alcohol catabolic process
44275	2.00	Cellular carbohydrate catabolic process
6817	2.00	Phosphate transport
19320	2.50	Hexose catabolic process
15698	2.53	Inorganic anion transport
16052	3.33	Carbohydrate catabolic process
16310	3.63	Phosphorylation
44265	4.24	Cellular macromolecule catabolic process
6820	4.74	Anion transport
6796	4.76	Phosphate metabolic process
6007	5.00	Glucose catabolic process
6096	5.00	Glycolysis
6793	5.00	Phosphorus metabolic process

FDR false discovery rate

Most of the genes involved in metabolism and biosynthesis were upregulated in the fish fed microdiet compared to control diet. GO analysis revealed an association of the genes with metabolic processes that were enriched particularly at D2 after transfer. This may reflect their attempt to adapt to marginal nutrient intake from the microdiet needed for optimum growth. Two glycolytic enzymes, aldolase A fructose-bisphosphate and glyceraldehyde-3-phosphate dehydrogenase, were upregulated 2 days after switching to the microdiet, indicating that the larvae were attempting to derive energy from glucose stores. Glutamine-fructose-6-phosphate transaminase 2 is an isozyme of glutamine-fructose-6-phosphate transaminase 1 (Gfpt1), the rate-limiting enzyme in glucosamine-6-phosphate biosynthesis, an important pathway for cellular glucose sensing. It is expressed throughout the central nervous system, especially in the spinal cord in humans and mice and has been implicated in type II diabetes (Zhang et al. 2004). Interestingly, the isozyme Gfpt1 promotes ontogenetic melanocyte darkening (Yang et al. 2007). In our study, Gfpt2 was moderately upregulated in fish fed the microdiet. A gene encoding transmembrane protein 33, which is similar to DB83 protein, a component of the melanosome proteome (Chi et al. 2006), was also highly upregulated at all three time points for which we have data. Since malpigmentation is such an issue in the aquaculture of Atlantic halibut, possible roles of these proteins in flatfish melanocyte development should be further examined. Two enzymes involved with lipid metabolism, 1-acylglycerol-3-phosphate *O*-acyltransferase and cGMP-inhibited phosphodiesterase 3A, were upregulated at all time points tested. cGMP-inhibited phosphodiesterase 3A plays various roles in vertebrates including procoagulant activity, lipid metabolism, and signal transduction mainly through the regulation of intracellular cAMP levels (Zhang and Colman 2007), whereas 1-

Table 8 GO term analysis of differentially expressed genes with a p value <0.05 in comparison to all the genes that passed filtering at each sampling point (D2, D13, D23, and D33)

Sample time	p<0.05	Passing filter	FDR (%)	Term description	GO term
D2	241	2001	3.20	Glycolysis	6096
			3.56	Monosaccharide catabolic process	46365
			4.00	Alcohol catabolic process	46164
			4.57	Cellular carbohydrate catabolic process	44275
			4.73	Alcohol metabolic process	6066
D13	280	1779	nd	nd	nd
D23	299	1557	0	Multicellular organismal process	32501
			0	System process	3008
			0.8	Striated muscle contraction	6941
			1	Muscle contraction	6936
			1.33	Muscle system process	3012
			2.57	Inorganic anion transport	15698
			3	Phosphate transport	6817
D33	313	2344	nd	nd	nd

FDR false discovery rate, *nd* not detected

acylglycerol-3-phosphate *O*-acyltransferase is involved in regulating phospholipid biosynthesis (Gale et al. 2006).

Two genes encoding proteins involved in trafficking through the Golgi and endoplasmic reticulum (ER) were highly upregulated (approximately 20-fold) in fish fed the microdiet. Golgi vesicular membrane trafficking protein p18 (Bet1) is involved in SNARE interactions in vesicular transport (Joglekar et al. 2003) and Golgi membrane protein SB140 (yip1) is involved in the regulation of ER–Golgi traffic at the level of ER exit sites (Barrowman et al. 2003). The increased expression of these two genes may reflect a disruption in the fishes' ability to traffic newly synthesized proteins efficiently through the ER.

Genes for a number of structural proteins were also differentially regulated. Two forms of collagen, Ia3, which is found in the skin, and IXa1, which may participate in electrostatic interactions with polyanionic glycosaminoglycans in cartilage, were both downregulated. The gene encoding the titin isoform N2-B was also downregulated. Titin is a scaffold for signaling proteins in muscle and is responsible for establishing and maintaining the structure and elasticity of sarcomeres in striated muscle (Steffen et al. 2007). Warm-temperature-acclimation-related 65-kDa protein contains hemopexin-like repeats which occur in vitronectin and some matrix metalloproteinase families such as collagenases, stromelysins, and other enzymes that metabolize the extracellular matrix. It may be important in the remodeling of the extracellular matrix and play an important role during early development (Nakaniwa et al. 2005). It was upregulated at D13 and D23 but had decreased by D33. Enrichment in GO terms associated with muscle physiology among the differentially expressed genes at D23 (Table 8) confirms the effect seen on structural proteins. Taken together, the downregulation of structural proteins would result in the lower growth seen in the experimental fish compared to the control fish.

Genes for three proteins involved in eye development were upregulated. Crystallin betaA2-2 participates in ocular development and contributes to the transparency and refractive properties of the eye lens (Wang et al. 2008). It was highly upregulated at D13 following the introduction of the microdiet. A gene for a protein similar to es1 protein was also upregulated at this time. Zebrafish es1 is expressed specifically in adult photoreceptor cells and appears to be important for maintaining normal retina structure and function (Chang and Gilbert 1997). Peripherin 1 (also known as plasticin) is a neuronal intermediate filament protein that constitutes part of the cytoskeleton and is important for retinal tissue regeneration (Cameron et al. 2005; Glasgow et al. 1992). It is a marker of anterior neural induction and is expressed most strongly in the brain of the tailbud stage *Xenopus* embryos (Sharpe et al. 1989). In our study, it was highly upregulated in larvae fed the microdiet throughout all four time points. As discussed above, transmembrane protein 33 (DB83 homolog) is found in melanosomes and may impact eye pigmentation. Also, lens epithelium-derived growth factor and basic transcription factor 3 (BTF3a) have been shown to be differentially expressed during retinal development in mice (see below). These genes associated with eye development may have contributed to some of the aberrations in the eyes of larvae fed the microdiet.

The gene for glutathione *S*-transferase A1 was highly upregulated in larvae fed microdiet. This protein is responsible for detoxification of reactive electrophilic compounds, including intracellular metabolites produced endogenously as a result of cellular oxidative processes, as well as exogenous compounds such as drugs, pollutants, and pesticides. Two similar gstA genes have been isolated from a related flatfish, *Pleuronectes platessa* (Leaver et al. 1997); it was suggested that these particular glutathione *S*-transferases are involved in the detoxification of fatty acid metabolites, particularly those arising from polyunsaturated fatty acid oxidation. It is possible that oxidation products or some of the artificial components of the microdiet induced the expression of this gene. Hepcidin was highly upregulated at D13 but downregulated at other time points. Hepcidin is a bifunctional peptide involved in both innate immunity and iron homeostasis and is highly expressed in response to bacterial infection and iron overload (Ganz 2006). Interestingly, its expression was reduced in response to growth hormone transgenesis in Atlantic salmon (Rise et al. 2006), which would result in increased availability of iron for hemoglobin biosynthesis but possibly compromised innate immunity. The dramatic upregulation of hepcidin at D13 may reflect the stresses affecting the larvae in response to the microdiet and subsequent adaptation to the microdiet (with concomitant decrease in hepcidin expression) at later time points.

Most of the genes involved in replication, translation, and cell proliferation that we identified as differentially expressed were downregulated in fish fed the microdiet relative to the controls. These included DNA polymerase delta subunit 3 and replication protein A3, 14 kDa, a nuclear single-stranded DNA binding protein that appears to be involved in all aspects of DNA metabolism including replication, recombination, and repair. Also downregulated were genes encoding transmembrane protein 85 (proliferation-inducing gene 17) and BTF3a transcription factor, which is essential for post implantation embryonic development in mice (Deng and Behringer 1995) and is also differentially expressed in the outer neuroblastic layer of the developing retina (Blackshaw et al. 2004). The gene encoding a protein similar to mitotic spindle assembly checkpoint protein MAD2A, which prevents progression of the cell cycle until all chromosomes are properly

aligned at the metaphase plate (Howell et al. 2000), was also downregulated as was the gene for lens epithelium-derived growth factor, a chromatin-associated protein that has been implicated in transcriptional regulation and that provides cellular protection against stress by transactivating stress-associated genes. This protein also facilitates cellular protection against ethanol stress and plays a role in retinoic acid production (Fatma et al. 2004), which is required for the normal growth and maintenance of many cell types, including lens epithelial cells.

Two genes encoding regulatory proteins were upregulated in larvae fed microdiet. The "zinc finger, A20 domain containing 2 protein" bears the inhibitor of cell death-like zinc finger domains, which mediate the self-association of A20 and subsequent IL-1-induced NF-kappaB activation (Heyninck and Beyaert 1999). Its moderate upregulation (fourfold to sevenfold) could impact the expression of immune/stress-related genes that are affected by NF-kappaB. The gene encoding SFRS protein kinase 1, a serine/arginine (SR) protein kinase specific for the SR-rich domain family of splicing factors was massively upregulated (33-fold) at D13 and moderately upregulated at the other time points (fourfold to eightfold). It is thought to play a role in the regulation of both constitutive and alternative splicing by regulating phosphorylation and intracellular localization of splicing factors (Ma et al. 2008). The gene for eukaryotic translation elongation factor 1B2, which participates in the elongation cycle of protein biosynthesis, was only moderately affected.

Conclusion

Although not completely successful, the microdiet showed promise as a larval feed in Atlantic halibut. It was an improvement from previous experiments because the particles were readily consumed after a period of adaptation and provided sufficient nutrients at the maintenance level for larvae. Similar to other microdiets, growth was limited in the fish. Insights into the reasons for growth limitation and malpigmentation have been revealed through studies of gene expression changes. Adjustments to the method of feeding the microdiet, such as suspending the particles in the water column using aeration, may improve results. Changing the physical properties and formulation of the microdiet, such as a larger particle size and lower density, may show an improvement in the overall health and quality of larval halibut.

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