

Nucleotide Enrichment of Live Feed: A Promising Protocol for Rearing of Atlantic Cod *Gadus morhua* Larvae

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Abstract We investigated the effect of two commercial nucleotide products (NT1 and NT2), administered through live feed, on growth and stress tolerance of Atlantic cod larvae. Expression of genes related to muscle growth (*igf-1*, *igf1r*, *igf-2*, *fst*, *fgf6*, *myod*, and *myhc*) and nucleotide metabolism (*uox*, *hprt*, *ndk*, and *uck*) was evaluated during larval development. In addition, the expression of genes related to stress (*hif-1 α* , *hif-2 α* , *hif-3 α* , and *mb*) was studied after an air exposure stress test. The enrichment of rotifers with nucleotides did not reveal any difference in nucleotide profiles, the exception being the RNA level of the NT1-enriched group that was significantly higher than the unenriched rotifer. Unenriched *Artemia* showed poor nucleotide profiles compared to enriched *Artemia* since 5' UMP, 5' GMP, and 5' AMP were observed only in the nucleotide groups. At 38 days post-hatch (dph), NT1 group had significantly higher dry weight (3.1 ± 0.1 mg) than the control (CON; 2.3 ± 0.1 mg). The treatments did not produce any significant differences in the expression of the key myogenic genes. Among the genes associated with nucleotide metabolism, *ndk* was down-regulated in NT1 at 38 dph. In the air exposure test, survival was significantly higher in the CON (77 ± 6 %) than in NT1 (48 ± 3 %) and NT2 (50 ± 3 %). After air exposure, *mb* was expressed at lower levels in NT2 group, *hif-2 α* was induced in NT1 group, and *hif-3 α* was upregulated in all groups. Our findings indicate that the improvement in the nucleotide profile of *Artemia* upon

nucleotide enrichment could eventuate in the rapid growth of larvae.

Keywords Atlantic cod larvae · Genes · Metabolism · Muscle growth · Nucleotide · Stress

Introduction

Marine larvae production predominantly relies on live feeds such as rotifers and *Artemia*. Cultivated zooplankton species are nutritionally deficient with respect to essential *n*-3 highly unsaturated fatty acids, certain amino acids, vitamins, and minerals (reviewed by Conceição et al. (2010)). Therefore, there is a need to enrich the live feeds with nutrients and functional additives such as nucleotides. Nucleotides have been considered as semi-essential nutrients for fish and mammals (Sánchez-Pozo and Gil 2002; Li and Gatlin III 2006).

Nucleotides are the monomeric units of the nucleic acids, deoxyribonucleic (DNA) and ribonucleic acid (RNA), which play important roles in several essential physiological and biochemical functions including encoding genetic information, mediating energy metabolism and signal transduction (reviewed by Cosgrove (1998)). In mammals, under certain conditions—including diseases, limited nutrient intake, or fast growth—the endogenous supply (de novo synthesis or salvage pathway) of nucleotides will not be enough for the cells to function normally (Uauy et al. 1994; Lopez-Navarro et al. 1996; Carver 1999). Dietary supplementation with nucleotides, nucleosides, or nucleic acids can stimulate the immune system, brain, and lipid metabolism and promote liver and gut maturation as well as growth performance (reviewed by Sauer et al. (2011)). However, the requirements of nucleotide in animals are not well understood (Sauer et al. 2011).

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There is a dearth of information on the nucleotide composition of live feeds and the nucleotide requirements of fish larvae. Studies on juvenile fish have shown that nucleotide supplementation can improve growth performance, immune response, disease resistance, stress tolerance, and gastrointestinal physiology and morphology (Burrells et al. 2001; Lin et al. 2009; Cheng et al. 2011; Tahmasebi-Kohyani et al. 2011; Welker et al. 2011; Yousefi et al. 2011). High growth in rainbow trout (*Oncorhynchus mykiss*) fed nucleotides could be related to increase in expression of muscle metabolic proteins including glyceraldehyde-3-phosphate dehydrogenase, creatine kinase, nucleoside diphosphate kinase (*ndk*), and triosephosphate isomerase, as well as troponin-T-1 (Keyvanshokoo and Tahmasebi-Kohyani 2012). However, it is unknown if exogenous nucleotides affect the expression of genes involved in muscle growth or stress tolerance (Li and Gatlin III 2006; Tahmasebi-Kohyani et al. 2011). In teleost, muscle growth is regulated by the somatotrophic axis, which includes the upstream growth hormone (*gh*) and the downstream insulin-like growth factors (*igf-1* and *igf-2*) that are produced by hepatic cells and released into the blood in response to *gh* (reviewed by Florini et al. (1996)). At the muscle level, growth is regulated by various genes including myogenic regulatory factors, growth factor-like members of the fibroblast growth factor family, and the transforming growth factor- β (TGF- β) (reviewed by De-Santis and Jerry (2007)).

Busch et al. (2010) demonstrated that Atlantic cod larvae fed natural zooplankton had higher growth compared to that achieved with rotifers and *Artemia*. We hypothesized that enrichment of cultured live feed with nucleotides could affect the growth of Atlantic cod larvae positively. We tested two commercial nucleotide products as enrichment to live feeds, which were eventually administered to cod larvae. Nucleotide composition in rotifers and *Artemia* pre- and post-enrichment and the effects of enrichment on growth and stress tolerance of Atlantic cod larvae from first feeding to metamorphosis were also investigated. In addition, the expression of selected genes related to muscle growth, nucleotide metabolism, and stress during the larval development was analyzed to assess the molecular effects of nucleotide enrichment.

Materials and Methods

Nucleotide Products

The tested commercial nucleotide products are currently employed as functional additives in aquafeeds. There is only limited information on their application as enrichment materials in live feeds. Both products, available as fine powder, are derived from yeast. Their nucleotide and nucleic acid

contents are not much different. In this study, the products are referred to as NT1 and NT2 and their identities are not revealed to avoid potential conflict of interests.

Larval Rearing and Live Feed Preparation

All procedures in the study were performed in accordance with the national guidelines detailed in the “Norwegian Regulation on Animal Experimentation” act.

Atlantic cod embryos were produced at the National Breeding Station (Tromsø, Norway) and transported to the University of Nordland (UiN), Bodø, Norway. After hatching, larvae were stocked in 100-l tanks at a density of 80 larvae/l under gentle aeration. Temperature was gradually increased from 6 °C at hatching to 12 °C at day 26 post-hatch. Water exchange was progressively increased from two volume exchanges/day to ten exchanges/day after day 27. The photoperiod was kept constant (900 lx, LD 24:0). Seawater was taken from an inlet at a depth of 50 m, filtered through a Bernoulli filter to remove particles over 20 μ m, skimmed, and aerated.

Three dietary treatments were applied in four randomly assigned replicate tanks. Larvae were fed rotifers until 24 days post-hatch (dph) and later *Artemia* enriched with commercial media (Multigain—Biomar; Myre, Norway: Phosphonorse—Eximo; Tromsø Norway, 70:30 w/w), which were supplemented with either one of the nucleotide products (NT1 or NT2) or commercial media alone (control, CON).

Rotifers (*Brachionus* sp.) were grown on super fresh Chlorella-V12 (Pacific Trading Aquaculture Ltd, Wicklow, Ireland) in batch cultures. Every morning, the required amount of rotifers was rinsed prior to enrichment. Rotifers were distributed into three enrichment tanks of 30 l at a density of 1,500 individuals/ml for 5 h at 23 °C. NT1 and NT2 groups received the respective nucleotide product at 0.5 g/l. After enrichment, the rotifers were rinsed, transferred to coolers, and kept at 8 °C.

Dry *Artemia* cysts (*Artemia salina* strain Franciscana, INVE Aquaculture, Dendermonde, Belgium) were hatched in 50-l plastic bags at 29–30 °C under high light intensity and vigorous aeration. Hatched *Artemia* nauplii were rinsed three times with seawater. *Artemia* (600–700 individuals/l) was enriched for 16 h in 15-l plastic bags with the commercial enrichment medium alone (CON) or in combination with either one of the nucleotide products (1 g/l). They were harvested in a 150- μ m filter and gently rinsed with seawater before transfer to respective coolers (4–5 °C) prior to feeding.

The feeding trial started at 2 dph when rotifers (5,000 individuals/l) and green water (approximately 3–4 million cells/ml *Isochrysis* sp.) were introduced simultaneously into the experimental tanks every 6 h using automatic feeders until 24 dph. Subsequently, the transition to *Artemia* feeding

was carried out, over a period of 5 days, by gradual replacement of rotifers with *Artemia*. Until 38 dph, *Artemia* was offered to all the groups at a rate of 3,000 individuals/l at 5-h intervals.

Nucleotide Analysis

Rotifers and *Artemia*, enriched as mentioned above, were collected and washed on a 60- μ m mesh. Samples were patted dry from underneath the mesh using a paper towel and stored at -80°C until analysis. Prior to analysis, samples were freeze-dried for 72 h. Nucleotides were separated and quantified by reversed-phase HPLC following the description of Werner (1991). The analyses were kindly performed by the analytical unit of Biorigin A/S in Quata, Brazil.

Larval Sampling and Growth Measurement

For the dry weight and standard length (SL) determination, 20 and 15 larvae samples per tank, respectively, were taken at 9, 16, 23, 30, and 38 dph using a fine mesh dip net. Initial sample (30 larvae) was collected at 2 dph. To determine the dry weight, larvae were anesthetized in tricaine methanesulfonate (MS222, Argent Chemical Laboratories Inc, Redmond, WA, USA), rinsed in distilled water, transferred to pre-weighed capsules, and dried for 48 h at 60°C . Thereafter, the capsules were weighed to the nearest $0.1\text{ }\mu\text{g}$ on a microbalance (Mettler Toledo UMX2, Columbus, OH, USA). To measure the SL, larvae were photographed under an Olympus SZXIZ stereoscope using a Olympus Colour-View IIIu camera (Olympus, Soft Imaging Solutions, GmbH, Oslo, Norway), and the images were analyzed using the Cell P software (Olympus Soft Imaging Solutions, GmbH, Oslo, Norway). SL was calculated from the tip of the nose to the end of the notochord. The daily percentage increase in larval weight (% SGR) was calculated according to Ricker (1958):

$$\% \text{SGR} = (\exp^g - 1)100$$

where growth coefficient g is:

$$g = (\ln W_2 - \ln W_1) / (t_2 - t_1)$$

and W_1 and W_2 are dry weights at days t_1 and t_2 , respectively.

All samples were collected at a designated time, prior to morning feeding. For quantitative PCR assays, pooled samples of ten, three, and two individuals from each tank were taken at 16, 30, and 38 dph, respectively, as described above. Larvae were anesthetized, rinsed in distilled water, and transferred into 1.5-ml cryotubes. The tubes were snap-frozen in liquid nitrogen and later stored at -80°C until RNA extraction.

Stress Tests

Stress tests were conducted on fish that were starved for 12 h, maintained in 5-l containers, and kept under controlled oxygen conditions at a temperature of 12°C and salinity of 35 ‰. Initial samples (unstressed fish) for PCR assays and cortisol analyses were collected from each treatment, immediately anesthetized, and processed as described above. To evaluate the viability of fish from each treatment at the end of the feeding trial (38 dph), 50 fish per tank were exposed to (1) salinity stress: fish were exposed to 50 ‰ salinity (NaCl dissolved in filtered seawater) for 30 min in 5-l aerated containers and survival was determined, (2) temperature stress: fish were exposed to 15°C for 1 h and survival was determined, and (3) air exposure stress: fish were held out of water for 45 s and survival was determined at 30 min after finishing the test. For all stress tests, larvae were considered dead if they were opaque, the bodies were bent, and they did not respond to a gentle touch to the tail. During the survival evaluation, pooled samples of two to three individuals (five pools per tank) were collected for quantitative PCR assays and cortisol analyses.

Cortisol Analysis

Whole-body cortisol from larvae was extracted using the procedures described by Hiroi et al. (1997) and King and Berlinsky (2006). Frozen cod larvae were thawed, weighed, and placed in a glass test tube. Samples of pooled larvae (two to three fish) weighing 30–40 mg were homogenized in ice-cold PBS (25 mg/ml) for 30 s using a Sonic Vibra Cell sonicator (Sonic & Materials Inc, Danbury, CT, USA). The homogenate was extracted twice with 3 ml of diethyl ether (Sigma-Aldrich, St. Louis, MO, USA). For each extraction, tubes were vigorously vortexed for 1 min and centrifuged at 2,700 rpm for 5 min to separate the aqueous and ether layers. The bottom layer was frozen in dry ice and the ether layer was collected into a new test tube. Samples were dried in a water bath at 45°C for 1 h and kept to air-dry overnight before reconstitution.

Cortisol was quantified using a colorimetric 96-well enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA). Evaporated samples were reconstituted in 400 μl (samples collected before stress) or 900 μl (samples collected after stress tests) of enzyme immunoassay buffer, vortexed vigorously for 10 s, and kept at 4°C for 24 h. Samples were measured in duplicate according to the manufacturer's protocol. Cortisol extraction efficiency was 89 %, which was similar to that reported previously for Atlantic cod larvae (King and Berlinsky 2006).

RNA Extraction and cDNA Synthesis

RNA extraction from whole larvae was performed as reported by Fernandes et al. (2008). All samples were

treated with gDNA wipeout buffer supplied with QuantiTect reverse transcription kit (Qiagen, Nydalen, Sweden) for 8 min to remove traces of genomic DNA contamination. For all total RNA samples, the absorbance ratios A260/A280 nm ranged between 1.97 and 2.07, indicative of high-purity RNA. Approximately 1 µg of total RNA was utilized for cDNA synthesis with the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions.

In Silico Analysis, Cloning, and Sequencing

Sequences of muscle differentiation (*myod*), myoglobin (*mb*), and urate oxidase (*uox*) were retrieved from the NCBI database (<http://www.ncbi.nlm.nih.gov/>; Genbank accession numbers AF329903, EU053129, and DQ165081, respectively). For all of the remaining target genes including fibroblast growth factor 6 (*fgf6*), follistatin (*fst*), hypoxia inducible factors (*hif-1α*, *hif-2α*, and *hif-3α*), hypoxanthine-guanine phosphoribosyltransferase (*hprt*), *igf-1*, *igf-2*, insulin-like growth factor 1 receptor (*igf1r*), myosin heavy chain (*myhc*), *ndk*, and uridine-cytidine kinase (*uck*), DNA sequences from different teleost species were obtained from the NCBI database, translated, and used as probes to screen the Codgene database (<http://ri.imb.nrc.ca/codgene/>) using TBLASTN and TBLASTX algorithms. The retrieved expressed sequence tags (ESTs) were assembled with CAP3 (<http://pbil.univ-lyon1.fr/cap3.php>) and single contigs were generated. These sequences were used to design the forward and reverse primers (Table 1). When no EST was available (*igf-2* and *igf1r*), the orthologs in other teleost species were retrieved from the NCBI database. CLUSTALW alignments (<http://align.genome.jp/>) were performed and degenerate primers were designed against the most conserved regions of the sequences (Table 1). Netprimer software (<http://www.premierbiosoft.com/netprimer>) was used to estimate the melting temperature and to investigate the presence of potential dimers and hairpins. PCR reactions were performed in 25-µl aliquots containing 10×PCR buffer, 10 µM of each primer, 2.5 mM dNTPs, 1.5 mM of MgCl₂, and 0.1 µl of Taq polymerase (5 U/µl). The PCR products were analyzed by agarose gel electrophoresis, excised from gel with QIAquick gel extraction kit (Qiagen), and ligated onto a pSTBlue-1 plasmid vector (Novagen, Darmstadt, Germany) before transformation into NovaBlue GigaSinglets™ Competent Cells (Novagen) according to the protocol suggested by the manufacturer. Plasmids were extracted and purified using the QIAprep Spin Miniprep Kit (Qiagen). Cycle sequencing reactions were performed with T7 and SP6 primers using ABI prism BigDye (v.3.1) Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA) and sequenced using 3500xL Genetic Analyser (Applied Biosystems). DNA sequences were analyzed with Bioedit sequence editor (Hall 1999).

Real-Time PCR

For the target genes, specific primers for qPCR amplification were designed manually and screened for hairpins, homo-, and cross-dimers using Netprimer (Table 2). Whenever possible, primers were designed in one intron/exon border to avoid amplification of potential contaminating genomic DNA as reported (Fernandes et al. 2008). To confirm that the right product was amplified, qPCR was performed on pooled cDNA for each primer. The different products were sequenced for additional verification.

The cDNA templates were diluted 50-fold with deionized water prior to using them as templates for the qPCR reactions. Quantification of gene expression was performed by qPCR with SYBR Green chemistry (Qiagen) on a Light-Cycler® 480 (Roche, Oslo, Norway) as detailed elsewhere

Table 1 List of primers used to clone the target genes

Gene	Forward sequence (5' → 3')	Reverse sequence (5' → 3')	Accession	Size (bp)
<i>fgf6</i>	GAAGGTTATACGGAACGAGAGC	GGGCAGGAAGTGGGTGAC	HQ259083	185
<i>fst</i>	CGATCTTGACGTCCAGTACCAG	AGTTGCAAGATCCTGAGTGCT	GU938613	491
<i>hif-1α</i>	GGGGCATTGCTTCAGGG	AGTTGACTTGGTCCAGAGCTCG	JF322882	262
<i>hif-2α</i>	GATATGGAAGAAGATGAGAGGC	TAGGCTGACTGGTCCAGGG	JF322883	396
<i>hif-3α</i>	CCAGAGGAAGGTGCGGTCTCG	GCCGATGTGTTTGGAGACG	JF322884	328
<i>hprt</i>	ATGGCGACACCAGGCTCATC	TGCCTTGACTTTCTTTTGCG	JF433920	653
<i>igf-1</i>	ATGTCGAGCGCTCCTTTC	TACATGCGGTAATTCTGCC	HQ259081	542
<i>igf-2</i>	CACWCASTTTGYCACACCTG	TTGTGRYTGACDWAGTTGTCC	HQ263172	617
<i>igf1r</i>	GCCATHAARACBGTSAAACG	TCWGCCACCATRCAGTTCC	HQ263173	338
<i>myhc</i>	GTGAAGAAGAAGGAGAAGAACC	GGCGTCTGAGCAGCTCTG	HQ259082	669
<i>ndk</i>	ATGTCTGACACCGAGCGCAC	ACACCTCGTCGCTTGAACC	JF433918	418
<i>uck</i>	CCAGAGCAAGACGGAGCC	CCTCCTCCATTGGCTGTG	JF433919	719

Table 2 List of primers used for real-time PCR. Amplicon size, efficiency (E), and correlation coefficient (R^2) of the curve calibration are indicated

Gene	Forward sequence (5' → 3')	Reverse sequence (5' → 3')	Size (bp)	E (%)	R^2
<i>arp</i>	TGATCCTCCACGACGATGAG	CAGGGCCTTGCGAAGA	113	100	0.99
<i>ubi</i>	GGCCGCAAAGATGCAGAT	CTGGGCTCGACCTCAAGAGT	69	107	0.99
<i>igf1r</i>	GCTCACTGAGGAAGGAGAACTC	CAAACCTGTTGGCGTTGAGGT	114	100	0.99
<i>igf-1</i>	CGATGTGCTGTCTCTCCCG	CATAGCCCGTGGGTTTACTG	174	103	0.99
<i>igf-2</i>	GAGGATTCTATTTTCAGTCGGC	CTCCGACTTGGCTGGCTT	140	97	0.98
<i>myod</i>	GAGGACAGGAGGACACTTACTTC	GAAATCCATCATGCCGTCG	141	89	0.96
<i>myhc</i>	GGATGATCTTGAAGGTTCTCTGG	GTCCTTCTTCTTGATTTTCTCG	160	100	0.99
<i>fst</i>	CAGGGAAAGTGCAAGAAAACG	GTAGGCGTTGTTCTGCTGTT	84	112	0.98
<i>fgf6</i>	CTACAACGCCTACGAGAGCCT	GGCAGGAAGTGGGTGACG	117	107	0.96
<i>hprt</i>	CGATACAGGGAAGACAATGATG	CGTCCTCTTCACCAGCAAACCTT	94	103	0.99
<i>ndk</i>	ATGGTGTGGGAGGGTCTGAAC	GCAGTAGTCGCCCCGTGATGG	102	87	0.99
<i>uox</i>	AAAGGGGTTGAGACCATTGAG	ATGCTCAACTCCATTCTTCTCC	138	95	0.96
<i>uck</i>	CAGACATAAAGACTGGAAACACG	GATGTCCGAGTCCGTGTCC	130	91	0.98
<i>mb</i>	GACCCGTTTATTCACAGAGCAC	GCTTCTTCAGGACCGTCGC	119	107	0.98
<i>hif-1α</i>	GCTGCTGCCGTCAGACCTG	GCAGTCGTAGCGGGTGAGC	97	104	0.98
<i>hif-2α</i>	ACCAAGAAAGCCTTCCCCC	GATGTTGACCTCACAGTCGTAGC	153	103	0.98
<i>hif-3α</i>	GGTTCTACCCGAGAGCCCTG	GCCGATGTGTTTGAGACG	95	91	0.99

(Campos et al. 2010). The annealing temperature was 60 °C, except for amplification of *igf-1* and *hif-1*, which was performed at 65 °C. Specificity of the qPCR reaction and the presence of primer dimers were checked by examining the melting curves generated with a dissociation protocol from 65 to 97 °C. All samples were run in duplicate. Negative controls including samples with nuclease-free water and samples without reverse transcriptase and positive control samples were included in all real-time PCR runs. Five-point standard curves of a five-fold dilution series (1:1–1:625) from pooled cDNA were used for PCR efficiency calculation. Cycle threshold (CT) values were determined using the fit-point method using the LightCycler® 480 software with a fluorescence threshold arbitrarily set to 0.2. To calculate the mean normalized expression of the target genes, the geNorm VBA applet for Microsoft Excel was used to calculate a normalization factor based on two reference genes (Vandesompele et al. 2002). Acidic ribosomal protein (*arp*) and ubiquitin (*ubi*) were selected as reference genes in this experiment as described by Sæle et al. (2009).

Statistical Analysis

Differences among treatments (data on growth, survival, and levels of nucleotides in the live feed) were analyzed using one-way analysis of variance (ANOVA). Differences in the expression level of target genes and cortisol levels were evaluated using two-way ANOVA with dietary treatment and sampling time as factors. When the F test was significant, means were compared using Tukey's HSD test. Normality of the data and homogeneity of variance were tested before

performing ANOVA. Data expressed as percentages (survival and SGR) were arcsin-transformed. Gene expression data were log-transformed when they did not meet the assumptions of normality and/or homogeneity. Spearman's rank test was conducted to determine the correlation between gene expression and SGR. Differences were considered to be significant at the $P < 0.05$ level. Data are expressed as mean $\pm$ S.E. Statistical analyses were performed using the Statistica software (Statsoft, Tulsa, OK, USA).

Results

Live Feed Enrichment and Growth of Larvae

Unenriched rotifers showed similar nucleotide profiles as the nucleotide-enriched groups, but the NT1 treatment resulted in the highest RNA levels. NT2 treatment showed the highest level of total nucleotide, but no significant differences were observed compared to the other two treatments (Table 3). Unlike what was observed for rotifers, unenriched *Artemia* had a different nucleotide profile compared to NT1- and NT2-enriched ones. 5' UMP, 5' GMP, and 5' AMP were observed only in NT-enriched *Artemia* groups (Table 4). Guanosine and total nucleotides were significantly ($P < 0.05$) higher in NT1 enrichment, but no significant differences were seen between unenriched and NT2-fortified *Artemia*. While adenosine was detected only in unenriched *Artemia*, 3' GMP was found only in NT2-supplemented ones. RNA levels were higher in NT-enriched *Artemia*, but no differences were observed among the groups.

Table 3 Nucleotide content (% dry weight) in unenriched (CON) and enriched (NT1 and NT2) rotifers

	CON	NT1	NT2
Protein	58.493±0.177 ^a	59.122±0.161 ^a	58.498±0.236 ^a
RNA	5.867±0.102 ^b	6.333±0.136 ^a	6.320±0.160 ^{a,b}
Guanine	N.D.	N.D.	N.D.
Adenine	N.D.	N.D.	N.D.
Guanosine	0.246±0.046 ^a	0.172±0.053 ^a	0.264±0.052 ^a
Adenosine	0.033±0.009 ^a	0.030±0.005 ^a	0.049±0.024 ^a
5' CMP	N.D.	N.D.	N.D.
5' UMP	N.D.	N.D.	N.D.
5' GMP	0.008±0.002 ^a	0.007±0.001 ^a	0.009±0.002 ^a
5' IMP	0.011±0.002 ^a	0.015±0.007 ^a	0.017±0.004 ^a
5' AMP	N.D.	N.D.	N.D.
3' GMP	0.024±0.006 ^a	0.056±0.029 ^a	0.097±0.052 ^a
2' GMP	0.005±0.001 ^a	0.005±0.002 ^a	0.006±0.002 ^a
3' AMP	0.017±0.003 ^a	0.037±0.017 ^a	0.099±0.064 ^a
Σ nucleotides	0.304±0.065 ^a	0.317±0.058 ^a	0.541±0.167 ^a

All values ($n=6$) are presented as mean ± S.E.

N.D. not detectable, CMP cytidine monophosphate, UMP uridine monophosphate, GMP guanosine monophosphate, IMP inosine monophosphate, AMP adenosine monophosphate

^{a,b} Different superscripts in the same row indicate significant differences among treatments ($P<0.05$)

Table 4 Nucleotide content (% dry weight) in unenriched (CON) and enriched (NT1 and NT2) *Artemia*

	CON	NT1	NT2
Protein	56.130±0.879 ^a	59.273±0.558 ^a	58.980±1.615 ^a
RNA	8.522±0.216 ^a	9.048±0.242 ^a	9.060±0.408 ^a
Guanine	N.D.	N.D.	N.D.
Adenine	N.D.	N.D.	N.D.
Guanosine	0.096±0.015 ^b	0.276±0.085 ^a	0.157±0.028 ^{a,b}
Adenosine	0.033±0.001	N.D.	N.D.
5' CMP	N.D.	N.D.	N.D.
5' UMP	N.D.	0.099±0.041	0.053±0.003
5' GMP	N.D.	0.060±0.018	0.023±0.005
5' IMP	N.D.	N.D.	N.D.
5' AMP	N.D.	0.131±0.075	0.047±0.019
3' GMP	N.D.	N.D.	0.022±0.008
2' GMP	0.017±0.002 ^a	0.016±0.006 ^a	0.011±0.002 ^a
3' AMP	N.D.	N.D.	N.D.
Σ nucleotides	0.206±0.045 ^b	0.522±0.19 ^a	0.300±0.036 ^{a,b}

All values ($n=5$) are presented as mean ± S.E.

N.D. not detectable, CMP cytidine monophosphate, UMP uridine monophosphate, GMP guanosine monophosphate, IMP inosine monophosphate, AMP adenosine monophosphate

^{a,b} Different superscripts in the same row indicate significant differences among treatments ($P<0.05$)

During the first 4 weeks of rearing, there were no dry weight differences between treatments. However, at the end of the experiment (38 dph), significant differences were observed. NT1 larvae had significantly higher dry weight ($P=0.01$; 3.1 ± 0.1 mg) than CON (2.3 ± 0.1 mg), while no significant differences were observed between larvae from NT2 (2.7 ± 0.1 mg) and CON groups (Fig. 1a). The differences in SL were not significant, the respective larval lengths being NT1— 12.8 ± 0.2 mm, NT2— 12.6 ± 0.2 mm, and CON— 12.2 ± 0.1 mm at 38 dph (Fig. 1b). Larvae from the NT1 group showed a higher SGR ($P=0.01$; 11.99 ± 0.36 %) than CON (11.04 ± 0.32 %) group for the overall experimental period (2–38 dph ; Table 5). All larval groups had lower SGR in the second week of the experiment, ranging from 5.9 to 8.1 % per day, but no significant differences were seen between treatments. On the other hand, increased growth rates were obtained after *Artemia* introduction in the last 2 weeks of experiment when the SGR varied from 12.6 to 16.8 among the groups, but no significant differences were found.

Sequence Analysis

The amplicon size of partial sequences isolated for Atlantic cod and the GenBank accession numbers are shown in Table 1. The deduced amino acid sequence conservation was generally high (Table S1 in “Electronic supplementary material”). As expected, amino acid sequences were more conserved between fish species compared to mammals and birds. CLUSTALW multiple alignments of Atlantic cod amino acid sequences with other orthologous proteins revealed conservation across different taxa of domains typical of each protein (Figs. S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, and S12 in “Electronic supplementary material”). For example, in *fgf6* protein alignments, the fibroblast growth factor domain and the five heparin binding sites were observed (Fig. S1 in “Electronic supplementary material”). *Myhc* showed the highest identity values, reaching 95 % of identity with deduced amino acid sequences of seabass (*Dicentrarchus labrax*), tiger pufferfish (*Takifugu rubripes*), and zebrafish (*Danio rerio*) (Table S1 and Fig. S2 in “Electronic supplementary material”). In contrast, *hif-1α*, *hif-2α*, and *hif-3α* showed the least conservation across the fish species examined, with 54, 44, and 64 % identity, respectively (Table S1 and Figs. S3, S4, and S5 in “Electronic supplementary material”).

Expression of Genes Related to Muscle Growth

Overall, there were no statistically significant differences in gene expression among the three different treatments at a particular time point (Fig. 2). Expression patterns varied

Fig. 1 Growth parameters of Atlantic cod larvae reared on different live feeds (CON, NT1, and NT2). **a** Dry weight (μg) of whole body ($n=20$), **b** standard length (mm; $n=15$). Data are expressed as mean $\pm$ S.E. Different letters indicate significant differences among treatments ($P<0.05$)

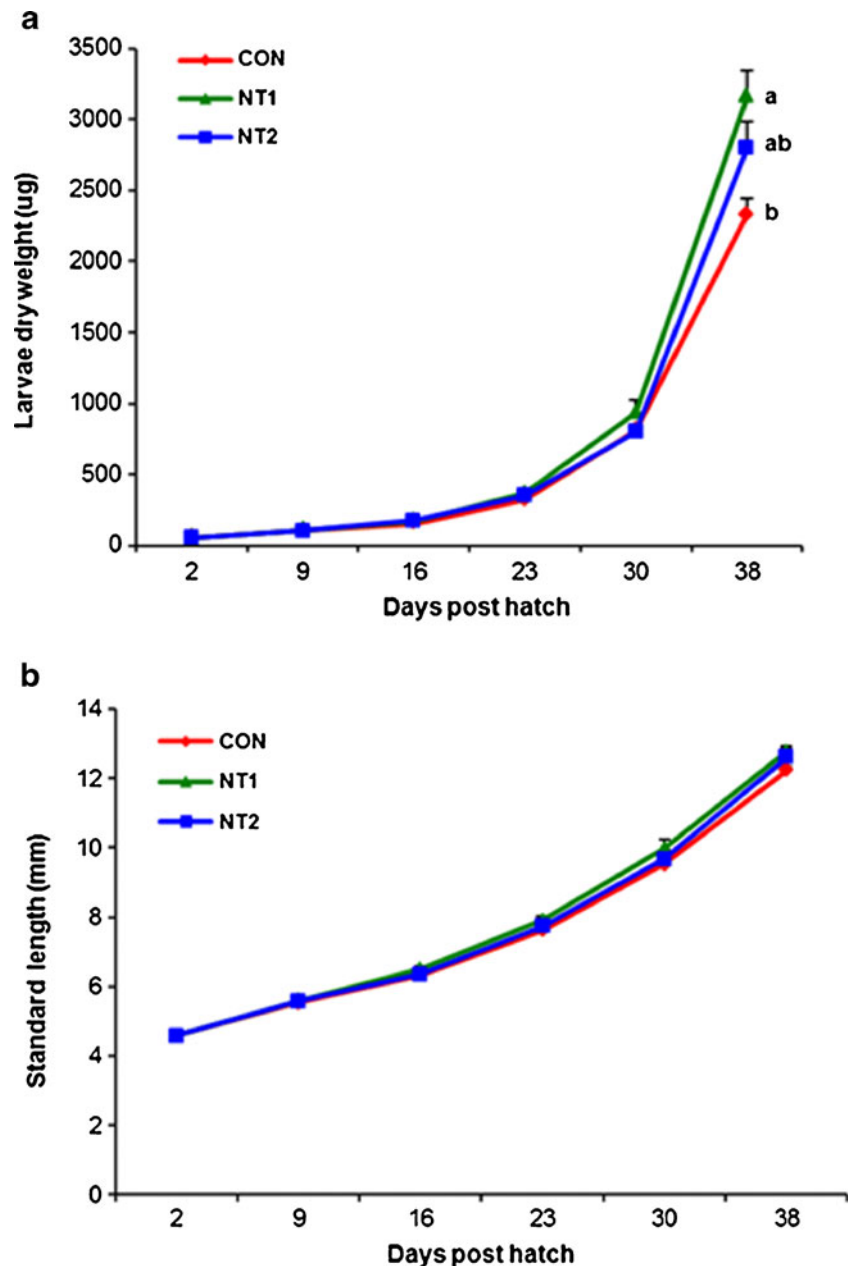


Table 5 Specific growth rate (% SGR) of Atlantic cod larvae during the periods 2–9, 9–16, 16–23, 23–30, and 30–38 dph

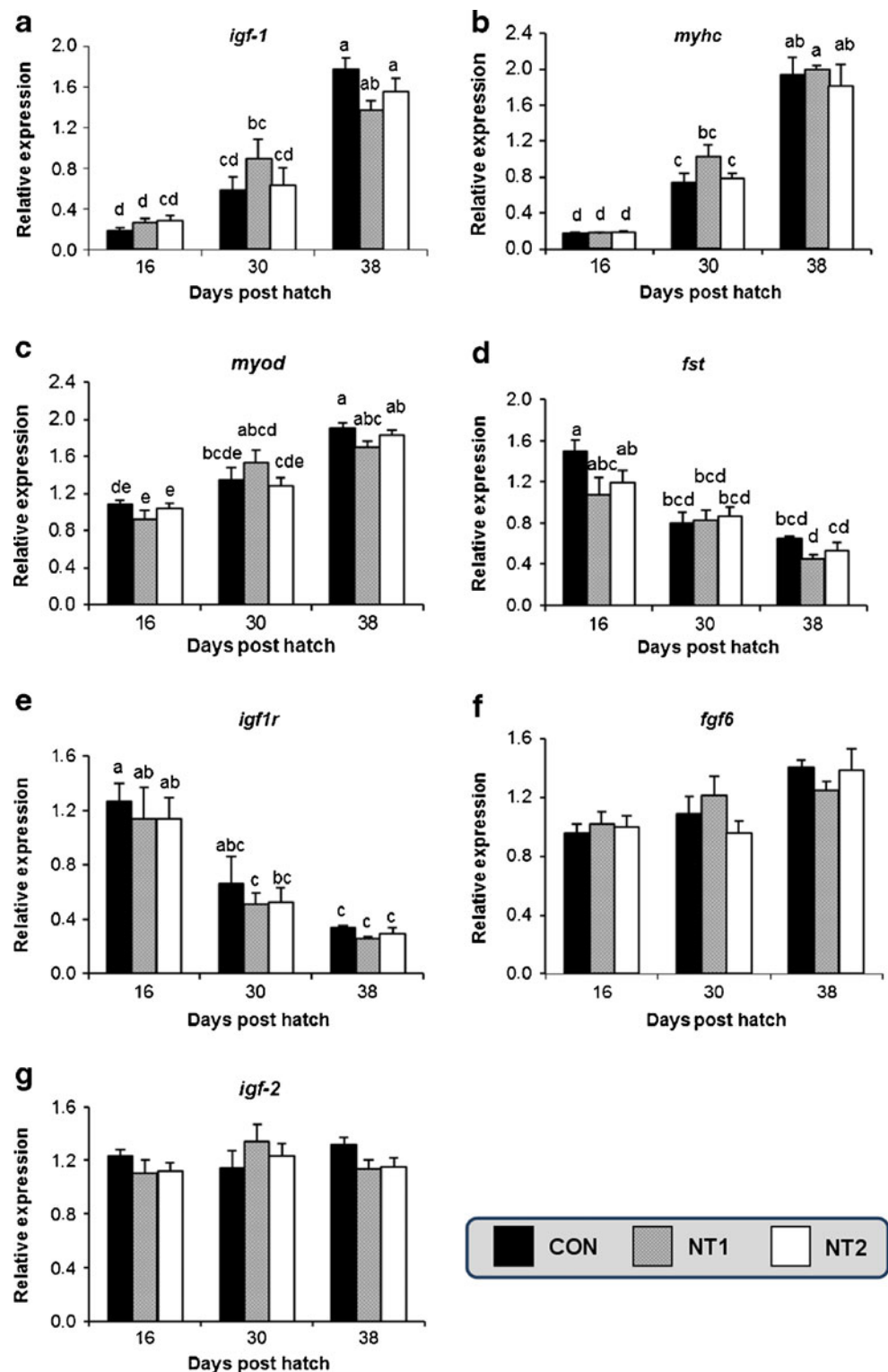
Days post-hatch	CON	NT1	NT2
2–9	9.65 $\pm$ 0.54 ^a	10.71 $\pm$ 0.59 ^a	9.73 $\pm$ 0.68 ^a
9–16	5.98 $\pm$ 1.08 ^a	6.47 $\pm$ 0.41 ^a	8.13 $\pm$ 0.84 ^a
16–23	11.05 $\pm$ 1.75 ^a	11.45 $\pm$ 0.59 ^a	10.20 $\pm$ 0.77 ^a
23–30	14.30 $\pm$ 1.69 ^a	14.32 $\pm$ 0.77 ^a	12.64 $\pm$ 0.74 ^a
30–38	14.11 $\pm$ 0.75 ^a	16.63 $\pm$ 0.71 ^a	16.83 $\pm$ 0.71 ^a
Overall 2–38	11.04 $\pm$ 0.32 ^b	11.99 $\pm$ 0.36 ^a	11.60 $\pm$ 0.43 ^{a,b}

Data are expressed as mean $\pm$ S.E.

^{a,b} Different superscripts in the same row indicate significant differences among treatments ($P<0.05$)

among the tested genes across the rearing period. *Igf-1* increased more than five-fold in all treatments from 16 to 38 dph (Fig. 2a). *Myhc* increased approximately ten-fold in all treatments during the same period, while *myod* increased approximately 1.8-fold in all groups (Fig. 2b, c). *Fst* was down-regulated approximately two-fold in all groups from 16 to 38 dph, while *igf1r* decreased approximately four-fold in all treatments in the course of the experiment (Fig. 2d, e). *Fgf6* and *igf-2* mRNA levels remained stable (Fig. 2f, g). The transcript levels of *myhc*, *igf-1*, *myod*, and *fgf6* positively correlated with SGR ($R=0.94$, 0.93 , 0.91 , and 0.84 , respectively), whereas *fst* and *igf1r* negatively correlated with SGR ($R=-0.95$ and -0.94 , respectively).

Fig. 2 Transcript levels of *igf-1* (a), *myhc* (b), *myod* (c), *fst* (d), *igf1r* (e), *fgf6* (f), and *igf-2* (g) at 16th, 30th, and 38th day post-hatch in the whole body of Atlantic cod larvae fed on different live feeds (CON, NT1, and NT2). Expression levels of target genes were quantified by qPCR and normalized using the geometric average of *ubi* and *arp*. Data are expressed as mean $\pm$ S.E. ($n=8$). Different letters indicate significant differences ($P<0.05$)

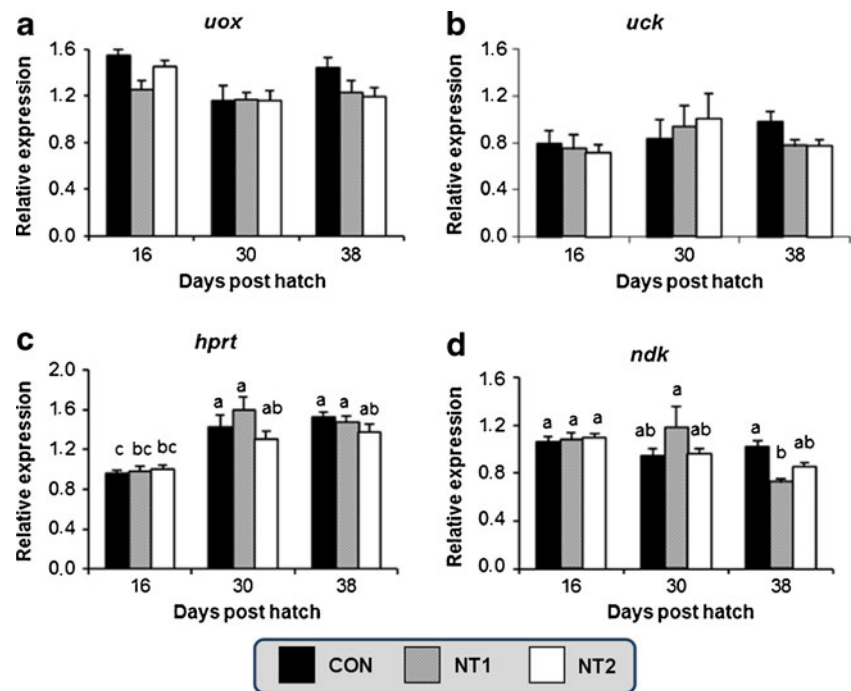


Expression of Genes Related to Nucleotide Metabolism

No differences were observed for *uox* and *uck* mRNA levels among the groups as well as across the sampling time points (Fig. 3a, b). *Hprt* expression was also similar among the groups but increased approximately 1.5-fold from 16 to 30

dph in all treatments when *Artemia* was introduced as live feed and remained high in all groups at 38 dph (Fig. 3c). *Ndk* expression was significantly lower in NT1 group at 38 dph, while no differences were observed between NT2 and CON groups (Fig. 3d). *Hprt* expression positively correlated with SGR ($R=0.76$).

Fig. 3 Transcript levels of *uox* (a), *uck* (b), *hprt* (c), and *ndk* (d) at 16th, 30th, and 38th day post-hatch in the whole body of Atlantic cod larvae fed on different live feeds (CON, NT1, and NT2). Expression levels of target genes were quantified by qPCR and normalized using the geometric average of *ubi* and *arp*. Data are expressed as mean $\pm$ S.E. ($n=8$). Different letters indicate significant differences ($P<0.05$)



Survival, Cortisol Levels, and Expression of Genes After Stress Tests

In the salinity and temperature stress tests, survival was more than 88 % for all treatments and no significant differences were observed among treatments. However, in the air exposure test, survival was significantly higher ($P<0.001$) in CON group (77 ± 6 %) than in NT1 (48 ± 3 %) and NT2 (50 ± 3 %) groups. Regarding the cortisol levels, no differences were observed among treatments prior and after the stress tests (Fig. 4). All three fish groups had the highest cortisol levels when subjected to air exposure compared with the other two stressors. Considering the results related to survival and cortisol levels, the expression of *mb*, *hif-1 α* , *hif-2 α* , and *hif-3 α* were evaluated after the air exposure test. No induction of expression was observed for *mb* and *hif-1 α* in all treatments (Fig. 5a, b). However, after the air exposure test, *mb* was expressed at lower levels in NT2 group compared to CON and NT1 groups. For all treatments, *hif-3 α* was upregulated approximately two-fold, while *hif-2 α* was only induced in NT1 treatment (Fig. 5c, d).

Discussion

Live Feed Enrichment and Growth

Use of baker's yeast *Saccharomyces cerevisiae* for rotifer production is a common practice in a number of hatcheries

(Conceição et al. 2010). This product contains a high concentration of nucleotides (Maloney 1998), which benefits the growth of marine fish larvae. Our results demonstrate that the nucleotide profile in control rotifers was similar to NT-enriched rotifers, except for RNA levels. Moreover, growth rates were similar among treatments during this stage. A reason for the similar profile in rotifers among treatments might be the low dose of nucleotides (0.5 g/l) used for rotifer enrichment. Unlike in the case of rotifers, in *Artemia* the nucleotide profile differed between treatments, and the total amount of nucleotides in control *Artemia* was lower as compared to NT1- and NT2-enriched *Artemia*. Also, NT1 larvae were significantly (34 %) heavier than CON larvae at 38 dph when the experiment was terminated. Therefore, nucleotide enrichment could enhance the growth of cod larvae during *Artemia* stage.

In the present study, no differences in SL were observed among treatments. According to Galloway et al. (1999), SL is not a suitable variable to describe differences in growth between cod larval groups since larvae with low somatic growth rates prefer to reach a higher size to facilitate swimming. The SGR of Atlantic cod larvae are generally found within the range 4.9–13.7 % per day (Galloway et al. 1999; Baskerville-Bridges and Kling 2000; Kortner et al. 2011). In our study, SGR of fish from the three treatments did not differ when larvae were fed rotifers. However, when larvae were fed *Artemia*, the SGR reached 16.6 and 16.8 % in the treatments NT1 and NT2, respectively, possibly due to the improved nucleotide profile of *Artemia*. Busch et al. (2010) demonstrated that Atlantic cod larvae fed natural zooplankton

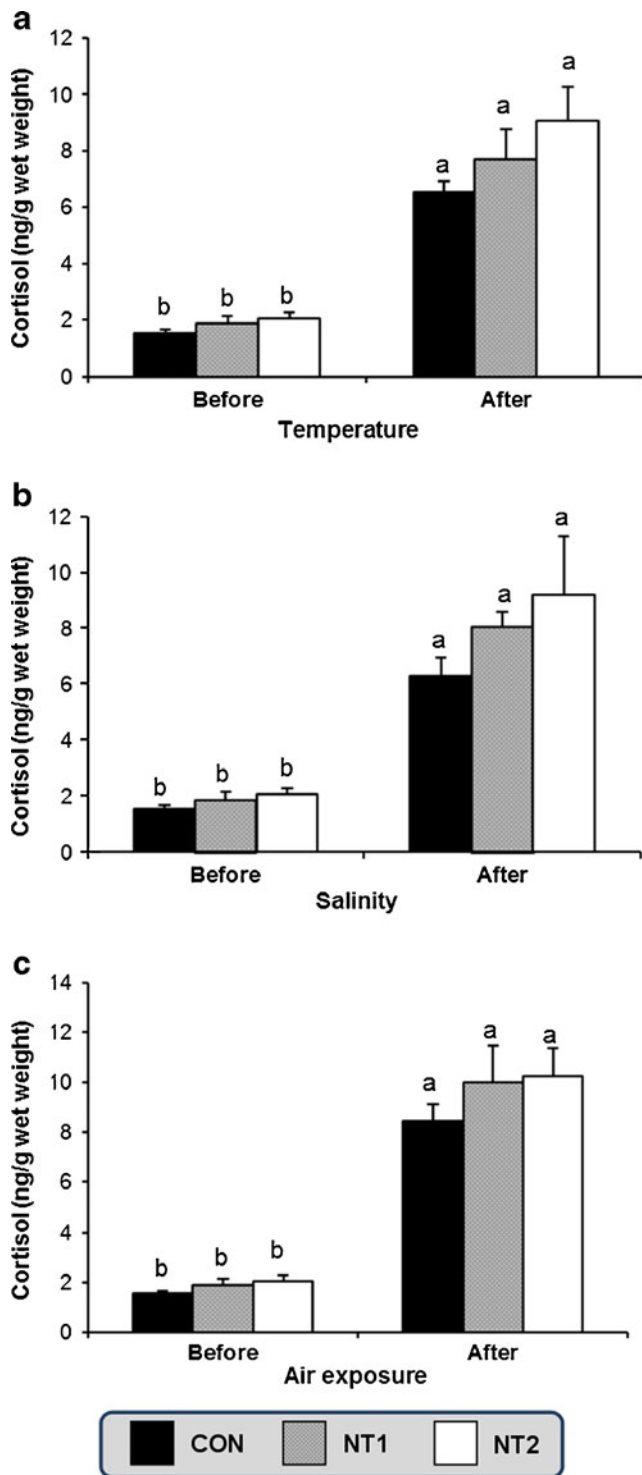


Fig. 4 Cortisol levels in whole-body homogenates of Atlantic cod larvae that were reared on different live feeds (CON, NT1, and NT2) and subjected to different stress tests at 38 days post-hatch. **a** High temperature, **b** high salinity, **c** air exposure. Pre- and post-exposure data are shown as mean $\pm$ S.E. ($n=6$). Different letters indicate significant differences ($P<0.05$)

have a high growth potential since larvae reached 9.7 mm at 19 dph. In our study, larvae reached this size only at 30 dph,

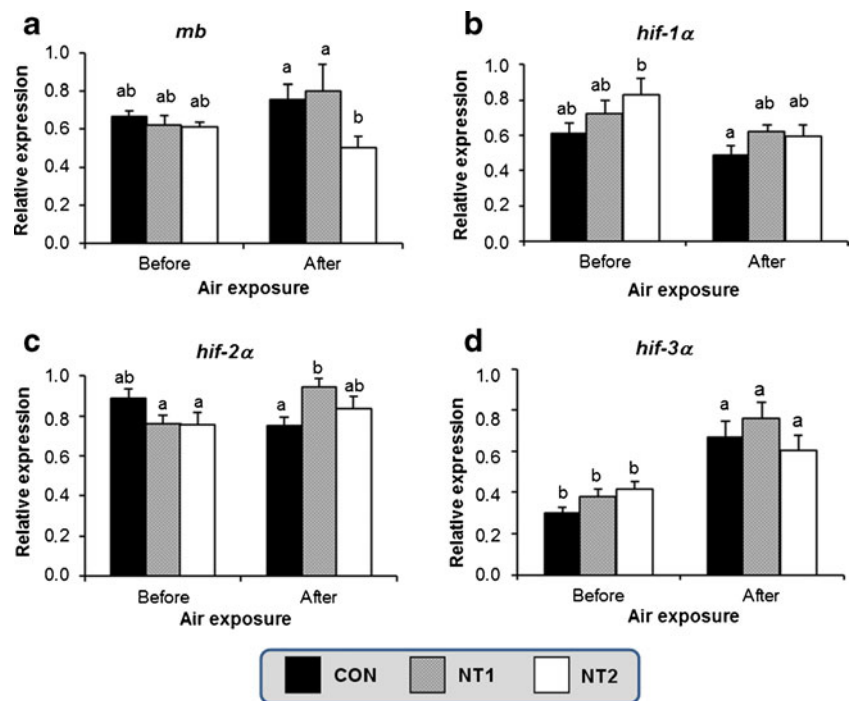
indicating that rotifer feeding alone may not be adequate for cod larvae.

Expression of Genes Related to Muscle Growth

In the present study, genes related to the different events of myogenesis were selected. The genes *igf-1*, *igf-2*, and *igf1r* are involved in the regulation of somatotrophic axis. *Myod* regulates muscle differentiation, while *fgf6* affects the proliferation of myogenic cells, muscle differentiation, and muscle phenotype. *Fst* antagonizes the activity of several members of TGF- β superfamily, and *myhc* is important for myotube formation in the last step of myogenesis (reviewed by De-Santis and Jerry (2007)). The expression of the aforementioned seven genes was not affected by nucleotide treatment despite the difference in growth performance at 38 dph. Juveniles of rainbow trout fed diets containing different levels of plant protein for 12 weeks showed differences in growth performance and in the diameter of white muscle fibers (Alami-Durante et al. 2010a). However, similar to our study, the expression of genes involved in myogenesis, including *myod*, myogenin (*myog*), proliferative cell nuclear antigen, and *myhc*, was not different between dietary treatments. In Senegalese sole (*Solea senegalensis*), high levels of dietary lipids affected the growth performance negatively, and among the 15 muscle growth-related genes analyzed, only *fgf6*, *igf-1*, muscle regulatory factor 4 (*mrf4*), and myosin light chain 2 (*mylc2*) were differentially regulated (Campos et al. 2010). Rainbow trout juveniles fed diets that contained different levels of carbohydrates and energy for 12 weeks showed alterations of *myod*, myogenic factor 5 (*myf5*), and *myog* expression in white muscle, but no significant modification was observed in growth performance (Chapalamadugu et al. 2009). In the same way, juveniles of rainbow trout fed diets containing different plant protein mixtures for 12 weeks demonstrated changes in the expression of *myod* and fast-*myhc* in white muscle, though there were no alterations in growth performance (Alami-Durante et al. 2010b). Based on all these findings, there is no clear relationship between differences in growth performance and alteration in the expression of the myogenic genes studied. Moreover, specific differences in the expression of genes involved in muscle growth might exist in different fish species due to the complexity of myogenic events besides nutrient-induced as well as age-specific alterations.

Studies on the transcription of *igf-1*, *igf-2*, and *igf1r* during larval development in several fish species demonstrated developmental stage-based expression patterns (Eliès et al. 1999; Greene and Chen 1999; Ayson et al. 2002; Peterson et al. 2005; Funes et al. 2006; Ponce et al. 2008). In the present study, the transcription of *igf-1* also increased more than five-fold, while *igf1r* decreased approximately four-fold in all groups during larval development (Fig. 2).

Fig. 5 Transcript levels of *mb* (a), *hif-1 α* (b), *hif-2 α* (c), and *hif-3 α* (d) in the whole body of Atlantic cod larvae fed on different live feeds (CON, NT1, and NT2) and subjected to air exposure. Expression levels of target genes were quantified by qPCR and normalized using the geometric average of *ubi* and *arp*. Pre- and post-exposure data are shown as mean $\pm$ S.E ($n=6$). Different letters indicate significant differences ($P<0.05$)



However, the expression of *igf-2* remained stable in all groups in the course of the experiment. In the redbanded seabream (*Pagrus auriga*), the pattern of *igf-1* and *igf-2* expression during larval development (Ponce et al. 2008) was similar to that observed in the present study. In Senegalese sole, transcripts of *igf-1* increased during larval development, while the expression of *igf-2* that was higher during pre-metamorphosis declined during metamorphosis (Funes et al. 2006). Our results indicate that *igf1r* expression was similar to those reported by Eliès et al. (1999) in turbot, where the *igf1r* was primarily expressed during early development and decreased during middle to late development. Therefore, these results indicate that *igf-2* is required throughout the development as opposed to *igf1r* that is necessary during early larval development and *igf-1* during the transition from larval to juvenile stages.

Fgf6 plays an important role in prolonged muscle hyperplasia in trout due to its consistent expression from embryos to adult stage (Rescan 1998). This gene seems to participate in the continuous proliferation of myogenic cells, leading to the recruitment of new fibers during fish growth (Rescan 2005). Our data are in agreement with those mentioned earlier since the *fgf6* mRNA levels were stable during larval development of Atlantic cod, indicating that hyperplasia might play an important role on somatic growth up to metamorphosis.

In Atlantic cod, *myod* expression was investigated during embryonic development (Hall et al. 2003). The transcripts were primarily identified in the presomitic mesoderm and its expression extended until after complete formation of somites at 50-somite stage. However, the pattern of *myod* expression after hatching is unknown in Atlantic cod and

such information for other species is limited. In tiger pufferfish, the canonical splice variant of *myod1* was found to increase circa 1.7-fold between hatching and first feed stages (Fernandes et al. 2007). Similarly, in the present study, Atlantic cod *myod* expression increased approximately 1.8-fold from 16 to 38 dph in all treatments, and a positive relationship was observed between SGR and the transcript levels of *myod*. This change in expression could be associated to an intense myoblast proliferation which mainly contributes to hyperplasia during larval growth in rainbow trout as pointed out by Johansen and Overturf (2005).

In Atlantic salmon (*Salmo salar*), *myhc* expression correlated with muscle protein accretion (Hevrøy et al. 2006). However, recent studies in rainbow trout and Senegalese sole showed no correlation between *myhc* expression in white muscle and growth rate or protein gain (Alami-Durante et al. 2010b; Campos et al. 2010). Our results are similar to those of Hevrøy et al. (2006) since a positive relationship was found between *myhc* expression and SGR. During the larval development of Atlantic cod, *myhc* expression increased more than ten-fold in all groups from 16 to 38 dph, but there did not exist a treatment effect. In trout, expression of *myhc* during development was related to hyperplastic growth since during the larval stage fish muscle grows mainly by increasing its fiber numbers (Gauvry and Fauconneau 1996). In Atlantic cod, too, the number of fibers increased in the white muscle throughout larval development (Galloway et al. 1999). Therefore, the increased *myhc* expression in the present study might also be related to hyperplastic growth.

Fst is an activin binding protein that antagonizes the activity of several members of the TGF- β superfamily including

myostatin, a potent negative regulator of myogenesis (Hill et al. 2002; Welt et al. 2002). To our knowledge, only in sea bream (*Sparus aurata*) was *fst* and myostatin expression evaluated simultaneously during larval development (Funkenstein et al. 2009). Their observations until 9 dph indicated the highest *fst* expression at 4 dph, which decreased subsequently, while myostatin expression remained unchanged. In the current study, the expression of *fst* decreased during larval development irrespective of the treatments, and a negative relationship was noted between *fst* and SGR.

Galloway et al. (1999) described the development of axial musculature in Atlantic cod larvae until 31 dph. They showed that the number of small fibers increased throughout larval development and high somatic growth rates during this period were associated with an increased contribution of hyperplasia to axial muscle growth. The expression patterns of *myod*, *fgf6*, and *myhc* from 16 to 30 dph noted in the present study also support these findings. However, information related to the muscle morphology of Atlantic cod from 31 dph to juvenile stage (20–30 mm; Falk-Petersen 2005) is scanty. In our study, the transcript levels of *myod*, *fgf6*, and *myhc* from 30 to 38 dph also followed the same pattern of expression as observed during earlier time points, suggesting that hyperplasia is responsible for muscle growth even at the later stages. However, the contribution of hypertrophy to muscle growth is expected to take over hyperplasia when Atlantic cod reaches its juvenile stage.

Expression of Genes Related to Nucleotide Metabolism

In the present study, the expression of four enzymes related to purine and pyrimidine metabolism, *uox*, *uck*, *hpri*, and *ndk*, were analyzed. Among them, *uox* (EC 1.7.3.3) plays an important role in the degradation of purines and its activity and expression have been evaluated in Atlantic salmon (Andersen et al. 2006). Juvenile salmon fed diets rich in nucleic acids had significantly higher liver *uox* enzyme activity, whereas mRNA levels were the same as in the control diet (Andersen et al. 2006). In our study also, *uox* expression was similar among the groups as well as during larval development. The differences in mRNA levels and enzyme activity were attributed to rapid mRNA degradation or, alternatively, post-transcriptional control of activity (Andersen et al. 2006).

In rainbow trout, the increased protein levels of *ndk* were attributed to the enhanced rate of cell proliferation in fish fed dietary nucleotides (Keyvanshokoo and Tahmasebi-Kohyani 2012). In the present study, *ndk* expression remained stable during larval development. However, *ndk* expression was lower in NT1 and NT2 groups at 38 dph, but only NT1 group showed a significant difference compared to CON group. The differences observed between the two studies might be related to post-transcriptional control of *ndk* similar to that described for *uox* (Andersen et al. 2006).

Moreover, differences in the nucleotide composition of the two products may have affected the expression of *ndk*.

Hprt is a constitutively expressed housekeeping gene among most tissue and cell types (reviewed by Jiralerspong and Patel (1996)). However, Walsh et al. (1990) demonstrated that nucleotide availability increased the *hpri* expression in murine intestinal epithelial cells. In our study, no differences were observed among treatments, which could indicate the absence of nucleotides such as guanine in the live feed. Interestingly, *hpri* expression increased in all treatments, significantly only in NT1 and CON, after *Artemia* introduction, and a positive relationship was observed with SGR. According to Walsh et al. (1992), an immediate transient synthesis of *hpri* mRNA is required prior to DNA replication during the gap 1 (G1) phase in proliferating stem cell population. Therefore, increased *hpri* expression after *Artemia* introduction might be related to intense cell proliferation, which is confirmed by high values of SGR.

Survival, Cortisol Levels, and Expression of Genes After Stress Tests

Beluga sturgeon (*Huso huso*) juveniles fed diets containing graded levels of nucleotides had a lower cortisol level after crowding stress compared to control group (Yousefi et al. 2011). Similarly, juvenile catfish offered nucleotide-enriched diets had cortisol concentrations significantly lower than the control group when subjected to crowding stress (Welker et al. 2011). On the contrary, in an earlier study on juvenile red drum (*Sciaenops ocellatus*), exogenous nucleotides did not inhibit the cortisol response caused by confinement stress (Li et al. 2005). In the present study also, the nucleotide treatments did not inhibit cortisol release in any of the three different stress tests. Further, nucleotide supplementation negatively affected survival after the air exposure stress test. The increasing metabolic cost of feeding during the development of Atlantic cod larvae (McCollum et al. 2006) and faster growth of the nucleotide-fed groups could have resulted in higher oxygen demand. The low survival observed in the nucleotide groups subjected to the air exposure stress, unlike in the temperature and salinity tests, points to the higher oxygen requirements of the nucleotide groups. Therefore, Atlantic cod larvae fed nucleotide-enriched live feed might be more susceptible to certain stressors. Li and Gatlin III (2006) have pointed out the necessity to investigate the right dosage of nucleotide supplementation since excessive levels may have negative effects on fish.

In fish, the roles of *HIFs* during acute and chronic hypoxia have been investigated in sea bass (Terova et al. 2008), rainbow trout (Soitamo et al. 2001), Wuchang bream (*Megalobrama amblycephala*) (Shen et al. 2010), Atlantic croaker (*Micropogonias undulatus*) (Rahman and Thomas 2007), and grass carp (*Ctenopharyngodon idellus*) (Law et al.

2006). However, the information is inadequate. To our knowledge, this is the first report which evaluated the three oxygen-dependent α subunits at the same time in fish after hypoxic conditions. Our results demonstrate that *hif-3 α* is regulated in a different manner than *hif-1 α* and *hif-2 α* since the transcript levels of *hif-3 α* were quickly upregulated in all treatments after air exposure. In mammals, similar results were obtained by Heidebreder et al. (2003). They suggested that this subunit could constitute a more rapidly reacting component of the response to tissue hypoxia or that *hif-3 α* might respond in a more sensitive way than *hif-1 α* and *hif-2 α* . Prior to the air exposure test, the expression of *HIFs* and *mb* were the same among treatments, demonstrating that oxygen homeostasis was normal for all groups. However, after the stress test, treatment-specific responses were observed since *mb* was down-regulated in NT2 group, suggesting loss of cell homeostasis during hypoxia.

In conclusion, the results of this study demonstrate for the first time that nucleotide-enriched *Artemia* can benefit growth in Atlantic cod larvae. However, faster somatic growth was not supported by alteration in the expression of genes related to myogenesis. Hyperplasia is likely to be the main mechanism of muscle growth during larval development as supported by the expression patterns of *fgf6*, *myod*, and *myhc*. In addition, this study revealed that Atlantic cod larvae fed nucleotide-enriched live feed are more susceptible to certain stress as evidenced by the results of survival and cortisol levels. Our results suggest that, among the hypoxia-inducible factors, *hif-3 α* may be one of the first effectors of adaptive responses to hypoxic conditions in fish. Live feed enrichment with nucleotides is a promising approach to increase larval growth, but further elucidation of their physiological roles is necessary.

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