

Article

eIF2 α phosphorylation in response to nutritional deficiency and stressors in the aquaculture fish, *Rachycentron canadum*

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Abstract: The present study investigates the response of the marine fish cobia, *Rachycentron canadum*, to stressors as measured by phosphorylation of the α -subunit of the translational initiation factor, eIF2. eIF2 α is the target of phosphorylation by a family of kinases that respond to a range of physiological stressors. Phosphorylation of eIF2 α not only inhibits overall protein synthesis, but allows cells to reprogram gene expression to adapt to, and recover from, stress. The deduced coding sequence of cobia eIF2 α has 94 % identity to both zebrafish (*Danio rerio*) and human eIF2 α sequences with identical phosphorylation and kinase docking sites. The present study uses a cobia cell line, Cm cells, derived from muscle, as well as cobia larvae to investigate the response of cobia eIF2 α to various stressors. In Cm cells, phosphorylation of eIF2 α is increased by nutrient deficiency, leucinol, and ER stress, consistent with the activation of the eIF2 kinases, GCN2, and PERK. In cobia juveniles, diet and water temperature affect the phosphorylation state of eIF2 α . We conclude that evaluation of eIF2 α phosphorylation could function as an early marker to evaluate diet, aquaculture stressors and disease in cobia and may be of particular use in the optimization of conditions for raising cobia larvae and juveniles.

Keywords: eIF2 α , eIF2 α -kinases, diets, stressors, *Rachycentron canadum*, cobia cells

1. Introduction

R. canadum is a perciform pelagic fish species found in warm-temperate to tropical waters of the West and East Atlantic Ocean, throughout the Caribbean, and in the Indo-Pacific off India, Australia and Japan. It is eurythermal, tolerating a wide range of temperatures, from 1.6 to 32.2 °C. It is also euryhaline, living at salinities of 5 to 44.5 ppt. Cobia is sold commercially and commands a relatively high price for its firm texture and excellent flavor. However, no designated wild fishery exists because it is a solitary species. It is currently farmed in aquaculture (1–3) and exhibits rapid growth in both off-shore cage systems as well as marine recirculating aquaculture tanks (4). Cobia seed stock used for large scale commercial aquaculture production comes exclusively from hatcheries and fingerling production is still an industry-wide bottleneck despite improvements in cobia larviculture (3; 5). Problems exist in larvae/fingerling culture, including high mortality due to stress and disease in the larval stage and the reliance on unsustainable fish meal diets. Expansion of cobia farming requires the development of sensitive assays to monitor stress during larval culture as well as the optimization of plant-based diets to increase production and sustainability (6; 7). Diet studies are both time consuming and expensive and could be facilitated by the development of faster indicators of diet quality. Similarly, a sensitive indicator of stress has the potential to assist in optimization of larviculture conditions.

Phosphorylation of Ser51 of the α -subunit of the translational initiation factor eIF2 is a key cellular response to wide range of environmental stresses such as nutrient deficiency, hypoxia, endoplasmic reticulum stress and viral infection in eukaryotes from protists to vertebrates (8–10). There are five eIF2 α -specific kinases in vertebrates (11), five in teleost fish and amphibians, four in tetrapods (12–14) that can phosphorylate eIF2 α . Each of these is activated by different stressors. HRI (EIF2AK1) is stimulated by heme depletion in erythroid cells, but also responds to oxidative stress, osmotic shock, and heat shock (15–17). HRI is the only eIF2 α kinase activated by arsenite in erythroid cells (15; 18) and is the major eIF2 α kinase responsive to heat shock. PKR (EIF2AK2) is stimulated by viral infection (19; 20), but also plays a more general role in cellular physiology (21); it can be activated in response to signals as diverse as oxidative and ER stress (22–24), as well as cytokine and growth factors signaling (22; 25) and has been implicated in the pathology of obesity (26; 27). PERK (EIF2AK3) is an endoplasmic reticulum (ER) transmembrane protein activated by the accumulation of misfolded proteins in the ER, a phenomenon termed the unfolded protein response (UPR) or ER stress (28). Changes in Ca^{2+} levels within the ER negatively affect the ability of the chaperone protein, binding immunoglobulin protein (BiP), also known as 78 kDa glucose-regulated protein (GRP-78), to maintain PERK in its inactive state (29). This allows diverse signals such as inhibition of the SERCA calcium pump, glucose deprivation, and high levels of fatty acids reduce the ER luminal calcium concentration (30; 31). GCN2 is the primary responder to nutritional deprivation and is the only eIF2 α -kinase conserved among plants and metazoans. It is activated by reduced amino acid levels in yeast and mammalian cells through uncharged tRNAs and the ribosomal P-stalk (32–35) and can also be activated by glucose deprivation (36). GCN2 is also involved in a variety of organismal functions in vertebrates such as long-term memory formation and feeding behavior (37). These four members of the eIF2 α kinases share extensive homology in their kinase catalytic domains and phosphorylate eIF2 α at the same serine residue (Ser51) (reviewed (11)). Teleost fish and amphibians also have a PKR-like kinase, PKZ, that is more closely related to the kinase domain of PKR compared to other three known eIF2 α kinases and, like PKR, is involved in the innate immune response (12; 14; 38). Phosphorylation of eIF2 α on Ser51 or its equivalent reduces the rate of protein synthesis overall. However, phosphorylation of eIF2 α increases the recruitment of a subset of mRNAs resulting in the transient activation of an alternate program of gene expression that enables the cell or organism to respond to a range of stressors (9; 39).

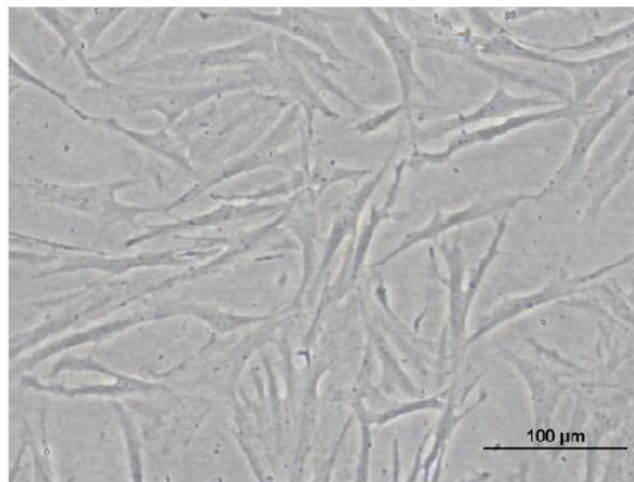
With the known relationship between eIF2 α phosphorylation and nutritional status and food choices in mammals, it was of interest to determine whether eIF α phosphorylation could be used as an early marker to evaluate diets and aquaculture conditions in fish. Studies were initiated in zebrafish, a tractable model system, to lay the groundwork for looking at fish of more interest to aquaculture. Zebrafish ZFL cells respond to a variety of stressors that activate a range of eIF2 α -kinases (Liu et al m/s in preparation). However, it was of more interest to see if cells from a marine species of aquaculture interest, such as cobia, would respond in the same way. A number of cell lines have been described from economically important marine species. These include cell lines from groupers (40; 41), tilapia (42), seabass (43), red sea bream (44), turbot (45), flounder (46; 47), sea perch (48) and gilthead seabream (49; 50). The isolation of two cobia cell lines, Cb and Cf from brain and fin, respectively, was previously reported and characterized, but the lines were subsequently lost (51). In this study we report the establishment and use of a new cell line from cobia muscle, Cm cells. To facilitate studies on Cm cells and cobia, we have successfully cloned cobia eIF2 α cDNA using degenerate primers based on eIF2 α sequences from other fish species and completing the coding sequence using 5' & 3' RACE. The current work demonstrates that recombinant cobia eIF2 α can be phosphorylated by stress-activated eIF2 α -kinases and that Cm cells respond to activators of eIF2 α -kinases by increasing eIF2 α phosphorylation. Furthermore, preliminary studies show that changes in eIF2 α phosphorylation can arise from nutritional deficiencies and temperature changes in cobia

juveniles. These results suggest that eIF2 α phosphorylation could provide a useful indicator to monitor cobia with regard to aquaculture conditions, as well as response to diets and disease.

2. Materials and Methods

2.1.1 Establishment and culture of cobia cell line

A cobia juvenile (15 g weight, 10 cm total body length), hatched and cultured at the Department of Life Science and Institute of Zoology, National Taiwan University, Taiwan, was anaesthetized by cold shock in iced sea water for 5 min. The body surface was wiped with 5% bleach, followed by 70% ethanol. Muscle fragments were dissected out and minced to approximately 2 mm² in 10-cm diameter cell culture dishes. The tissue fragments were transferred separately into 50-ml beakers containing calcium and magnesium free phosphate-buffered saline (PBS), 0.25% trypsin and 0.2% ethylenediaminetetraacetic acid (EDTA) for tissue dissociation. The tissues were dissociated by stirring at 600 rpm and 25 °C for 15 min. The resulting tissue suspensions were transferred into 15 ml centrifuge tubes containing 3 ml fetal bovine serum (FBS) for trypsin neutralization, and centrifuged at 200 g at 25 °C for 10 min. Cell pellets, including non-dissociated tissue fragments, were resuspended in Leibovitz-15 medium (Gibco) supplemented with 5 % FBS, 100 IU/ml penicillin and 100 IU/ml streptomycin and lacking sodium bicarbonate and CO₂. The resuspended cells were seeded into a 25 cm² flask, incubated at 28 °C. One-third of the medium was replaced with fresh complete L-15 every 14 days until the attached cells reached 90% confluence. Attached cells were sub-cultured at a ratio of 1:2. Cells at 50th and 80th passages were cryopreserved in liquid nitrogen. Optimal growth was found at



found at 28 °C in 10 % FBS. Cells are fibroblast-like in appearance (**Figure 1**).

Figure 1. Cm Cells in culture resembling fibroblast morphology.

2.1.2 Identification of Cm cell origin

Analysis of the sequence of mitochondrial cytochrome oxidase subunit I gene (cox I) was used to verify whether the cell lines were actually derived from cobia. RNA was isolated from Cm cells and fresh cobia muscle using the PureLink® RNA Mini Kit (Ambion) following the manufacturer's protocol. cDNA was synthesized using Revertaid™ M-MULV reverse transcriptase and random hexamer primers to produce first strand cDNA. Amplification of cDNA fragments of the predicted size was confirmed by end-point PCR amplification using the primers described from FDA, (52). PCR products were separated using 1% TAE (tris-acetate-EDTA) agarose gel electrophoresis and stained with ethidium bromide. The cDNA products were recovered using a QIAquick PCR purification Kit (Qiagen) followed by sequencing. CLC and NCBI blast were used to compare sequence identity.

2.1.3 Primer design for analysis of cobia eIF2 α

Degenerate primers were designed for PCR from five closely related fish species, zebra fish, catfish, puffer fish, Atlantic salmon and rainbow trout. The RT-PCR products were cloned into the vector, pGEM-T and DNA sequence analysis was carried out on an Applied Biosystems Automated Sequencer (ThermoFisher, Waltham, MA, USA). 5' and 3' rapid amplification of cDNA ends (RACE)-PCR was used to construct the full length of cDNA sequence. RACE primers used to amplify the cobia eIF2 α cDNAs were designed by Primer 3 software. For a list of primers, see **Supplemental Table S1**

2.1.4 Preparation of RNA from Cm cells and cobia muscle and cDNA synthesis

Total RNA was extracted from cobia cell line, liver and muscle using the PureLink® RNA Mini Kit (Ambion, ThermoFisher, Waltham, MA, USA) following the manufacturer's protocol. The recovered RNA was spectrally analyzed for concentration at 260 nm, and for purity at both 260/280 and 260/230 ratios by Nanodrop ND-1000 spectrophotometry and by automated electrophoresis using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA). Values of >2 for 260/280 and 260/230 ratios were considered to be of sufficient purity for planned uses. Amplification of cDNA fragments of the predicted size was confirmed by end-point RT-PCR. cDNA was synthesized using Revertaid™ M-MULV reverse transcriptase (ThermoFisher, Waltham, MA, USA) and random hexamer primers to produce first strand cDNA. Total RNA (1 μ g) was used for first-strand cDNA synthesis with SuperScript™ II Reverse Transcriptase (ThermoFisher, Waltham, MA, USA) and random hexamers (QIAGEN, Germantown, MD) according to the manufacturer's instructions. The cDNA produced was diluted to represent that produced from 12.5 ng/ μ l RNA for qPCR analysis and stored at -80 °C. The quality of all cDNA preparations was assessed by end point PCR amplification using the primers described above.

2.1.5 Cobia eIF2 α cloning and generation of eIF2 α S51A constructs

Approximately 30 mg of cobia liver tissue was used as starting material for total RNA extraction for cloning. Degenerate primers were designed for RT-PCR from the closely related five fish species, zebrafish, catfish, puffer fish, Atlantic salmon and rainbow trout. The RT-PCR products were cloned into the vector, pGEM-T (Promega, Madison, WI, USA) and DNA sequence analysis was carried out on an Applied Biosystems Automated Sequencer. 5' and 3' rapid amplification of cDNA ends (RACE)-PCR was used to construct the full length of cDNA sequence. The cDNA for cobia eIF2 α , generated from RNA prepared from cobia liver and muscle tissue, was cloned into pGEMT and pCITE4a (Millipore, St. Louis, MO, USA). For cloning of eIF2 α , cDNA was prepared from RNA isolated from cobia muscle tissue, and cDNA prepared using random hexamers. All primers sets were designed to provide Nde I and BamHI recognition sites at the 5'- and 3'-ends (**Supplemental Table S1**). eIF2 α sequence was verified from five primary transformants. The open reading frame of eIF2 α was excised from pGEMT/eIF2 α using the NdeI and BamHI restriction sites and transferred into similarly digested pCITE4a. The pCITE4a constructs were used as templates for coupled transcription-translation system, using the rabbit reticulocyte TNTQuick system (Promega, Madison, WI, USA).

Generation of eIF2 α -S51A: cDNA containing the S51 to A51 mutation was synthesized by GenScript (Piscataway, NJ, USA) and cloned into pCITE4a with Nde I and BamHI recognition sites at the 5'- and 3'-ends.

2.1.6 Preparation of cell extracts for analysis of eIF2 α phosphorylation

Cm cells ($\sim 10^7$) in monolayers were rinsed once with ice cold PBS containing 25 mM sodium molybdate, 10 mM β -glycerophosphate, and 100 mM NaF prior to lysis in 500 μ l 25 mM HEPES-KOH, pH 7.2, 5 mM EDTA, 100 mM KCl, 0.5 % Elugent (Millipore, St. Louis, MO, USA), 10 % glycerol, 1 mM DTT, 0.5 mM microcystin (Sigma-Aldrich, St. Louis, MO, USA), and 1 tablet/10 ml protease inhibitor pill (Fisher Scientific, Waltham, MA, USA) (53). Lysates were collected and clarified by centrifugation (10,000 \times g for 5 min at 4 °C) prior to snap-freezing and storage in liquid N₂. In general, extracts contained approximately 2–3 mg/ml of protein.

2.1.7 Immunoblot analysis to detect eIF2 α phosphorylation

Cobia cell extract samples were fractionated by 15 % SDS-PAGE, electrotransferred to PVDF membranes, and subjected to immunoblot analysis using polyclonal antibody against phosphorylated Ser51 of mammalian eIF2 α (CellSignaling, Danvers, MA, USA) followed by goat anti-rabbit secondary antibody coupled to HRP and coupled with chemiluminescence. Chemiluminescence was detected using the ProteinSimple Fluorochem E (Pasadena, CA, USA) with quantification using ImageJ software. Total eIF2 α levels were determined by reprobing the stripped blot with rabbit antibody that recognizes equally the phosphorylated and unphosphorylated forms of eIF2 α . Values obtained with the anti-eIF2 α [P] antibody were normalized for the total amount of eIF2 α present in the sample.

2.1.8 *In vitro* phosphorylation of cobia eIF2 α

35S-radiolabeled eIF2 α and the nonphosphorylatable variant were produced in the reticulocyte TnT cell-free transcription/translation system (Promega, Madison, WI, USA) by incubation at 30 °C for 30 min, after which they were supplemented with x units of purified mammalian recombinant HRI (EIF2AK1), PKR (EIF2AK2), PERK (EIF2AK3) or GCN2 (EIF2AK4) followed by incubation at 30 °C for an additional 10 min. Microcystin (0.5 μ M) was included to inhibit phosphatase activity. Each sample was diluted in 10 volumes isoelectric focusing sample buffer (9.5 M urea, 1% Pharmalyte, pH 4.5–5.4, 1% Pharmalyte, pH 5–6 (Sigma-Aldrich, St. Louis, MO, USA), 5% CHAPS ((Sigma-Aldrich, St. Louis, MO, USA)), 50 mM DTT)) prior to analysis. Samples were subjected to vertical slab isoelectric focusing (VSIEF) (54), using a narrow pH range of 4.5–6 in the presence of 8.8 M urea, 3 % acrylamide, 1 % CHAPS, 5 % Pharmalyte (pH 4.5–6), and 50 mM DTT. Proteins were electrophoresed at 2 mA/gel with a 1200 voltage limit for 16 h, using reverse polarity with 0.01 M glutamic acid at the anode and 0.05 M histidine at the cathode. Proteins were transferred to PVDF and 35S-labeled protein was visualized using Typhoon (Amersham Biosciences, Piscataway, NJ, USA).

2.1.9 *Experimental fish and systems*

This study was carried out in accordance with the guidelines of the International Animal Care and Use Committee of the University of Maryland Medical School (IACUC protocol # 0610015). Juvenile (~2 g) cobia, *R. canadum*, were obtained from the Virginia Agricultural Experiment Station, Virginia Tech, Hampton, VA. Juveniles were maintained at 27 °C in a marine recirculating system at the Institute of Marine and Environmental Technology's Aquaculture Research Center, Baltimore, MD. Diet formulations are given in **Supplemental Table S2**.

2.1.10 *Preparation of cobia liver samples*

Samples of liver from cobia juveniles maintained on a range of diets and at different temperatures were collected, snap-frozen in liquid N₂ immediately and stored at -80°C for later analyses. Tissue extracts were homogenized using lysing matrix D (MP Biomedicals, Solon, OH) (3 lysis buffer: 1 tissue ratio). Tissue was disrupted using FastPrep-24 5G (MP Biomedicals, Solon, OH) for 40 sec, kept on ice for 5 min and microcentrifuged at 12,000 g for 15 min at 4 °C. Supernatants were snap-frozen in liquid N₂ immediately and stored at -80°C for later analyses. Phosphorylation of eIF2 α was determined by immunoblot analysis of SDS-PAGE fractionated proteins as described (54).

3. Results

3.1.1 Identification of Cm cell origin

The identification of Cm cells as cobia derived was confirmed by sequence comparison of *cox 1* cDNA from Cm cells and fresh muscle. Using each of two primer sets, the PCR-amplified *cox 1* cDNA amplicons from Cm cells and fresh muscle were of the same predicted sizes; ~ 650 bp band after agarose gel electrophoresis. DNA sequencing and analysis demonstrated that *cox 1* cDNAs amplified from fresh cobia muscle tissue and from Cm cells were identical. Moreover, all *cox 1* cDNA sequences amplified from RNA from Cm cells or fresh cobia muscle were 99%~100% identical with (GenBank accession # FJ154956.1) and cobia cytochrome oxidase subunit 1 (COI) gene (GenBank accession # KJ202194.1) (Figure 2). These data confirmed that the origin of the established Cm cells is from cobia.

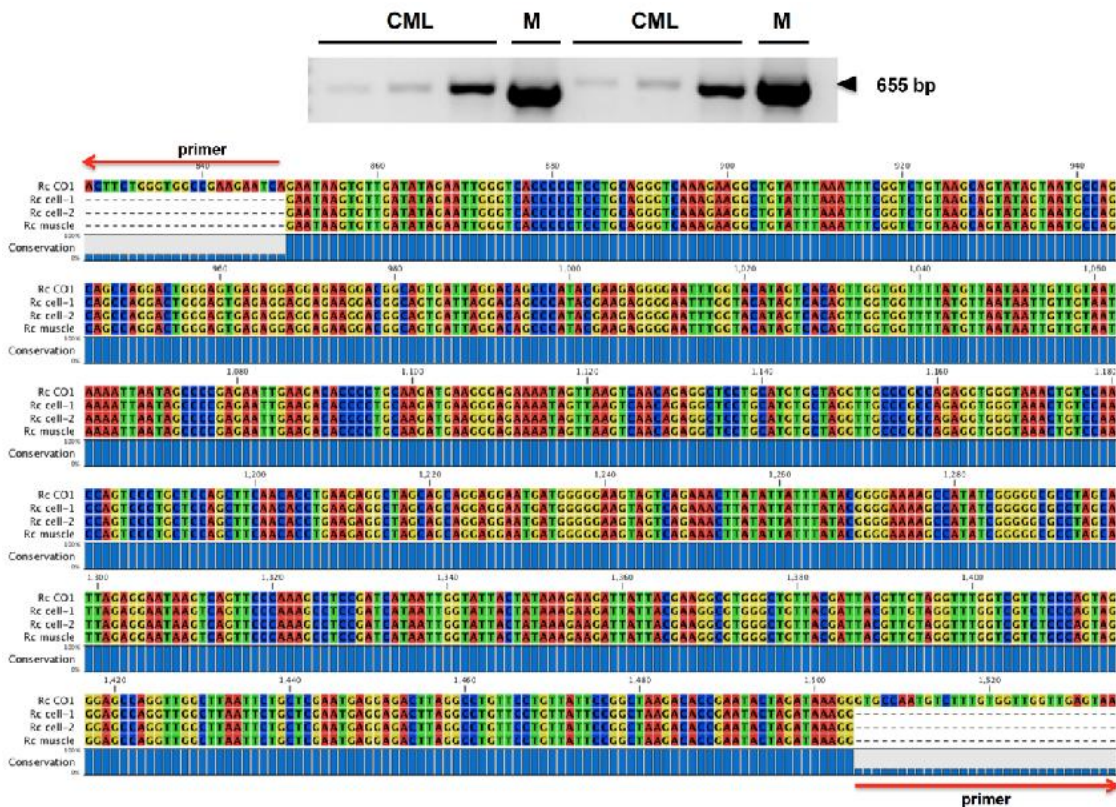


Figure 2. Identification of cobia cell origin

Upper panel shows PCR-amplified DNA from cobia muscle cell line (Cm) and cobia muscle (M) using primer pairs specific for cobia cytochrome oxidase subunit 1 (COI) gene, and primers given in Supplemental Table S1. Amplicons were fractionated by 1 % agarose gel electrophoresis. Multiple alignments of amplified cobia COI gene from cobia muscle cell line and cobia muscle in comparison with cobia cytochrome oxidase subunit 1 (COI) gene (GenBank accession # KJ202194.1) by the ClustalW program were show below. The red arrows indicate the primer position.

3.1.2 Cobia have two eIF2 α transcripts but identical coding sequences

Two transcripts of eIF2 α are found in cobia, although the coding sequence for each is identical (accession # KJ513464). Compared to human and zebrafish eIF2 α , the cobia coding sequence is 94 % identical at the amino acid level.

Table 1: Characteristics of cobia eIF2 α

eIF2 α	Gene	ID#	#aas	%ID	MW(Da)	pI	Chrom	Location	Accession #
<i>R. canadum</i> eIF2 α	eif2s1		315	94	35.97	5.02			KJ513464
<i>D. rerio</i> eIF2 α -a	eif2s1a	321607	315	94	36.13	4.97	17	NC_007128.6	NM_199569.1
<i>D. rerio</i> eIF2 α -b	eif2s1b	321564	315	94	36.17	4.98	20	NC_007131.6	NM_131800.2
<i>H. sapiens</i> eIF2 α	EIF2S1	1969	315	100	36.1	5.01	14	14q23.3	NM_004094

Table 1 shows the percent identities in amino acid composition, predicted molecular weights and isoelectric points. The predicted isoelectric points and molecular weights of the eIF2 α s are in good agreement with those of human and zebrafish eIF2 α . The cobia eIF2 α is predicted to be 35.97 kDa, respectively, compared with human eIF2 α which has a predicted molecular weight of 36.1 kDa. The deduced isoelectric points are also very similar, 4.84 for cobia eIF2 α compared to 5.02 for human eIF2 α . **Figure 3** shows the multiple alignment of the N-terminal 120 amino acids of cobia eIF2 α compared to human and zebrafish eIF2 α s. This covers the phosphorylation site and kinase docking domain. (A multiple alignment of the complete amino acid sequences are provided as supplementary **Figure S1**). Residues in the phosphorylation loop region surrounding the eIF2 α phosphorylation site (Ser-51 in human eIF2 α) are identical. Similarly, residues in the PKR docking site (residues 79–83) of cobia eIF2 α are identical to those in the human and zebrafish sequences, suggesting it is a good substrate for eIF2 α -specific protein kinases. In addition, Ser-48, Ile-55, Leu-84, Arg-88 and Val-89, residues critical for interaction with the α -, β -, and δ -subunits of the guanine nucleotide exchange factor, eIF2B (55), are present in cobia eIF2 α indicating that the interaction between both eIF2 α s and eIF2B is also conserved. The first α -helix of human eIF2 α is known to be involved in interactions that fix the orientation of the helical domain with the β -barrel (56). In mammalian eIF2 α , this region contains a cysteine residue that is involved in a disulfide bridge and is thought to stabilize the interaction of the two domains. This residue is not found in non-vertebrate eIF2 α s and appears to be a characteristic of vertebrate eIF2 α s.

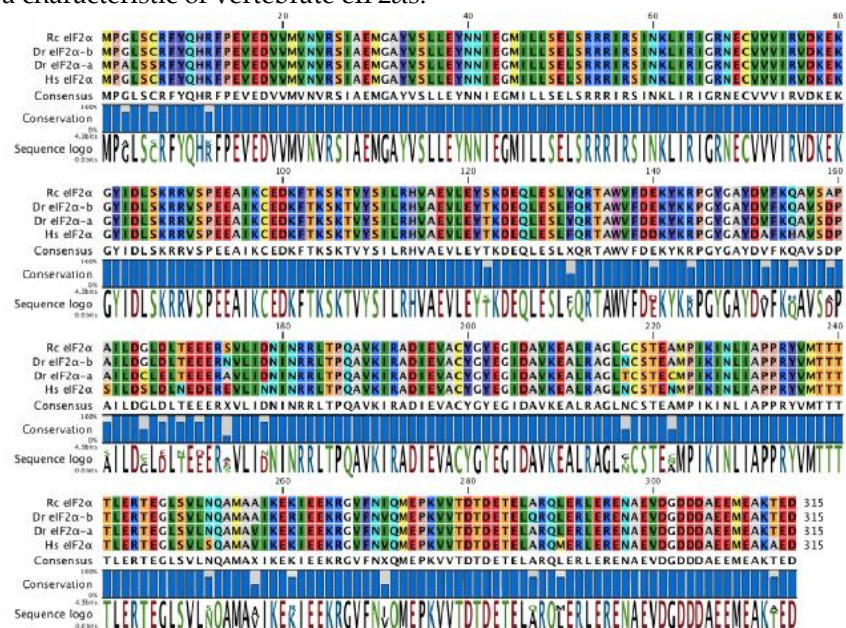


Figure 3. Multiple alignment of eIF2 α from cobia, zebrafish and human

ClustalW alignment of the N-terminal 120 residues of eIF2 α from cobia, *Rachycentrum canadum*, in comparison with zebrafish eIF2 α s and human.

Each of the four eIF2 α -kinases, HRI, PKR, PERK and GCN2 can be activated in mammalian cells by a variety of stressors (reviewed (10)). Thapsigargin is an inhibitor of endoplasmic reticulum Ca²⁺ ATPase that blocks the ability of the cell to pump calcium into the sarcoplasmic and endoplasmic reticula which causes these stores to become depleted (57). This causes endoplasmic reticulum (ER) stress and activates the unfolded protein response, including the activation of PERK (58; 59). GCN2 is activated by amino acid and glucose starvation (36; 60). Leucinol is the alcohol formed by total reduction of the carboxylic acid group of leucine. It is an inhibitor of leucyl-tRNA synthetase and so can mimic leucine deficiency in cells (61). N-methylprotoporphyrin (NMPP) is a potent inhibitor of ferrochelatase (K_i~10 nM), the terminal enzyme of the heme-biosynthetic pathway (62) and is able to activate the heme-sensitive kinase HRI (63). Polyinosinic:polycytidylic acid (poly I:C) is a synthetic dsRNA that is used experimentally to model viral infections *in vivo* (reviewed (64) and is used *in vivo* and *in vitro* to activate PKR.

The response of Cm cells to these known activators of eIF2 α -kinases was examined, as shown in **Figure 4**. Cm cells treated with known activators of eIF2 α -kinases; incubation in nutrient limiting medium or in the presence of leucinol (4 μ M) for activation of GCN2; thapsigargin (1 μ M) for activation of PERK; poly(I):poly(C) (50 μ g/ml with 200 μ g/ml DEAE dextran) for activation of PKR and NMPP (2 μ M) for activation of HRI. Cells were harvested at various time points, as determined by preliminary experiments, and analyzed by immunoblotting.

3.1.4 Effects of nutrient deficiency and leucinol on Cm cells

Nutrient deficiency and leucinol treatment were used to determine whether GCN2 can be activated in cobia cells. Reducing the Cm cell L-15 culture medium to 30 % in phosphate buffered saline (PBS) for 24 h reduced both amino acid and serum levels. After 24 h, eIF2 α phosphorylation was increased (**Figure 4, Panel A, left**) consistent with the activation of GCN2. When leucinol (4 mM) was included in the complete culture medium, an increase in eIF2 α phosphorylation could be seen within 1 h and continued to increase over 24 h (**Figure 4, Panel A, right**). This also strongly suggests activation of GCN2 and is consistent with the response of mouse ES cells to leucinol (65). Mouse ES cells also respond very rapidly to leucinol treatment. However, Gcn2^{-/-} mouse ES cells failed to show any induction in eIF2 α phosphorylation during the first 3 h of leucine deprivation demonstrating that the effect of leucine deprivation on eIF2 α phosphorylation could be attributed to GCN2.

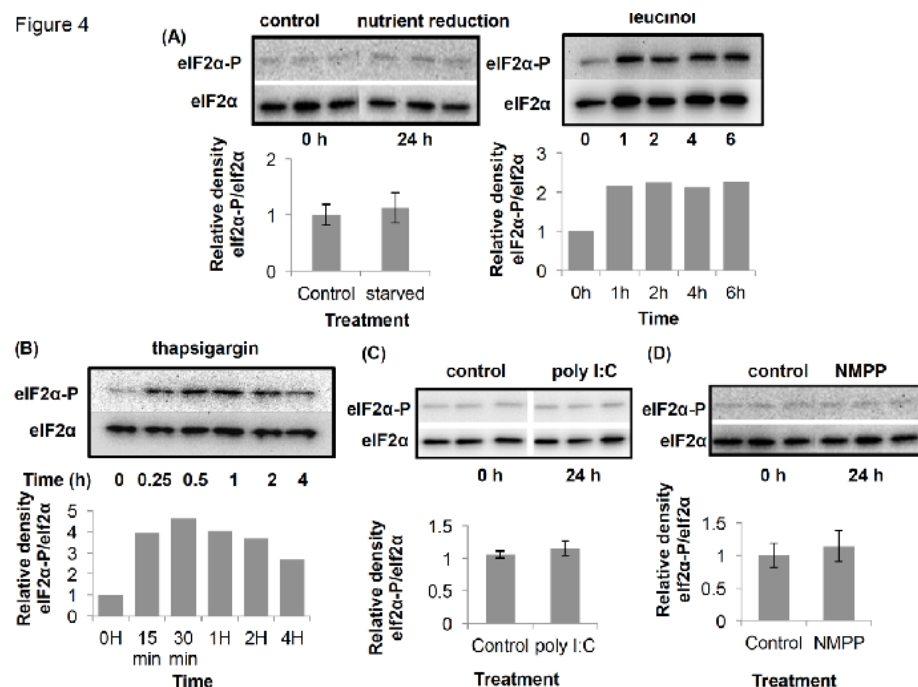


Figure 4. Response of cobia muscle cells to activators of eIF2-kinases

Cobia muscle cells treated with known activators of eIF2 α -kinases: (A) Nutrient reduction cells were maintained in 30% L-15 cell culture medium in PBS for 24 h; (A) leucine (4 mM), for up to 24 h, for activation of GCN2; (B) thapsigargin (1 μ M), for up to 2 h, for activation of PERK; (C) PIC (50 μ g/ml), for 24 h, for activation of PKR; (D) NMPP (2 μ M), for up to 24 h, for activation of HRI. Proteins in cell extracts were fractionated by SDS-PAGE and eIF2 α was visualized by chemiluminescence following electro-transfer to PVDF, and probed with antibody to phosphorylated eIF2 α and (after stripping) antibodies to total eIF2 α . Phosphorylated and total eIF2 α was visualized by chemiluminescence following electro-transfer to PVDF. Chemiluminescence was detected and image generated (upper panels) using the ProteinSimple Fluorochem E, with quantification using ImageJ to estimate level of phosphorylated eIF2 α (lower panels).

3.1.5 Effects of thapsigargin on Cm cells

Thapsigargin is non-competitive inhibitor of the sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA; 66). eIF2 α phosphorylation increases quickly in Cm cells on incubation with thapsigargin (1 μ M). These results indicate that PERK can be activated in cobia cells.

3.1.6 Effects of poly I:C on Cm cells

Cm cells were treated with 50 μ g/ml poly I:C and 200 μ g/ml DEAE dextran. **Figure 4, panel C**, shows that treatment for 24 h showed almost no eIF2 α phosphorylation level difference between control and treatment group. This is in contrast to the effect of poly I:C in rainbow trout RTG2 cells and zebrafish ZFL cells (53). These results could indicate that PKR is not expressed in Cm cells, is not activated, or that cobia eIF2 α is not a good substrate for PKR.

3.1.7 Effects of N-methylprotoporphyrin on Cm cells

N-methylprotoporphyrin (NMPP) is a transition-state analogue and potent inhibitor of ferrochelatase, is used to induce heme deficiency and has been shown to increase eIF2 α phosphorylation in rat hepatocytes (63). Cobia Cm cells were treated with NMPP (2 μ M) for up to 24 h. Unlike the effect in rat hepatocytes which respond rapidly to NMPP, Cm cells show no increases in eIF2 α phosphorylation after 24 h (**Figure 4, panel D**). These results indicate that HRI may not be expressed in Cm cells, is not activated or that cobia eIF2 α is not a good substrate for HRI.

3.1.8 Cobia eIF2 α can be phosphorylated by eIF2 α -kinases *in vitro*

Although Cm cells responded to activators of GCN2 and PERK with increased eIF2 α phosphorylation, they did not respond to activators of PKR or HRI. We took the alternative approach of looking at the capacity of recombinant cobia eIF2 α to be phosphorylated by the kinases *in vitro* as we have previously shown for recombinant zebrafish and rainbow trout eIF2 α (53). ³⁵S-labeled cobia eIF2 α was synthesized in a reticulocyte translation system to which we later added purified mammalian eIF2 α -kinases. Phosphorylated and non-phosphorylated forms were separated by vertical slab gel isoelectric focusing (VSIEF) (**Figure 5**). This analysis showed that cobia eIF2 α can be phosphorylated by eIF2 α -kinases and that PKR and HRI are either not well expressed or not activated by poly I:C or NMPP in Cm cells.

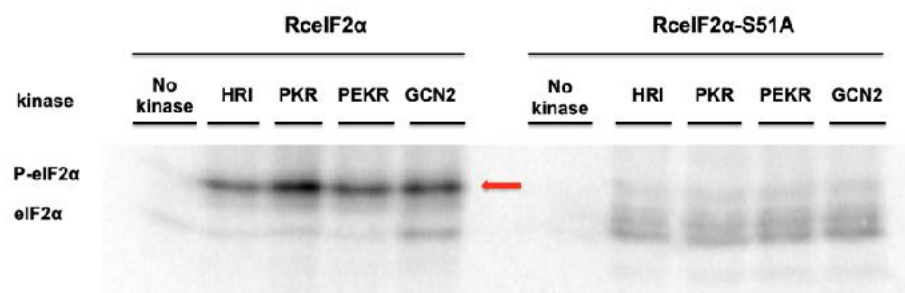


Figure 5. *In vitro* phosphorylation of cobia eIF2 α by purified recombinant mammalian eIF2 α -kinases

³⁵S-radiolabeled cobia eIF2 α , eIF2 α -S51A were synthesized in the reticulocyte TnT cell-free transcription/translation system by incubation at 30 °C for 30 min after which they were supplemented with x units of purified mammalian recombinant HRI

(EIF2AK1), PKR (EIF2AK2), PERK (EIF2AK3) or GCN2 (EIF2AK4) followed by incubation at 30 °C for an additional 10 min. Microcystin (0.5 μ M) was included to inhibit phosphatase activity. Each sample was diluted in 40 volumes isoelectric focusing sample buffer prior to analysis. Samples were subjected to vertical slab isoelectric focusing (VSIEF) to separate phosphorylated and non-phosphorylated forms, using a narrow pH range of 4.5–6. Proteins transferred to PVDF and visualized using the Typhoon imager. The red arrow indicates the position of phosphorylated eIF2 α .

3.2. eIF2 α phosphorylation in cobia juveniles

3.2.1. Comparison of eIF2 α phosphorylation in fish fed a fish meal versus an all plant protein diet

In order to be useful in aquaculture situations, it was necessary to demonstrate that eIF2 α phosphorylation responds to nutritional differences and stress, not just in Cm cells, but in fish. During the current investigation, the Place lab was conducting comparisons of different dietary formulations for cobia, focusing on the formulation of plant protein diets. We took advantage of this by sampling fish from a few of the diet trials. Our investigation was not intended to be an exhaustive analysis of eIF2 α phosphorylation under all dietary regimes, but samples of fish were taken to ask if changes in eIF2 α phosphorylation could be documented. For instance, the performance of cobia juveniles maintained for 12 weeks on an all plant protein diet, PP, was compared with those on a mixed fish meal/plant protein diet, FM (6). The all plant protein diet was supplemented with menhaden oil to provide essential omega-3 fatty acids, but not with taurine, known to be essential in cobia diets (6; 7). However, the PP diet did contain 0.02 % taurine. The complete formulations are given in **Supplemental Table S3**. The specific growth rate (SGR) in cobia juveniles on the FM diet for 8 weeks was 4.72 ± 0.02 , compared with 0.57 ± 0.12 for fish on the PP diet. The phosphorylation of eIF2 α in livers of these fish at the end of the feeding trial is shown in **Figure 6**. Higher eIF2 α phosphorylation levels can be seen in the fish fed the plant protein diet without taurine supplementation. In fact, the level of eIF2 α phosphorylation is as high as that observed in juveniles maintained without feeding for 7 days.

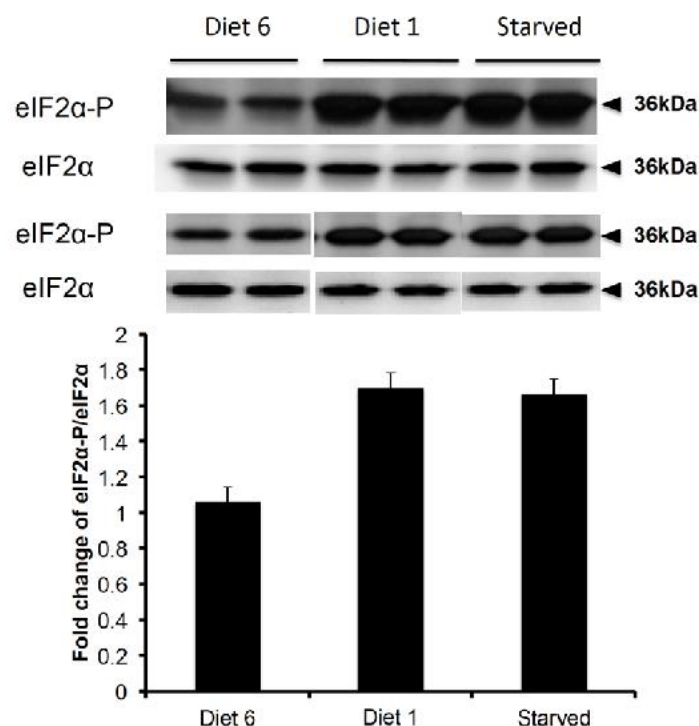


Figure 6. Comparison of eIF2 α phosphorylation in cobia fed a fish meal versus plant protein diets

Cobia juveniles maintained for 12 weeks on an all plant protein diet, PP, were compared with animals fed a mixed fish meal/plant protein diet, FM (6) and unfed fish. The complete formulations are given in Supplemental Table S2. Proteins in tissue extracts were fractionated by SDS-PAGE and transferred to PVDF. Phosphorylated and total eIF2 α were visualized by enhanced chemiluminescence, using antibody to phosphorylated eIF2 α and (after stripping) antibodies to total eIF2 α , as described in legend to **Figure 4**. The bar diagrams in lower panels represent the quantification of the blots corresponding to the respective lanes in the upper panel.

3.2.2. Comparison of eIF2 α phosphorylation in fish fed a diet with partial replacement of fish meal/poultry by-product/soy protein concentrate with a non-GMO soybean cultivars

Although fish meal can be replaced at least in part by soybean concentrate, most commercially available soybean products are from genetically modified organisms (GMOs), the use of which is not universally accepted. Schillinger Genetics, Inc. has developed multiple cultivars of soybean with potential as fishmeal replacements in diets for aquaculture. Cultivars have been developed with reduced levels of the anti-nutritional factors raffinose, stachyose, and trypsin inhibitors. A diet was formulated, 3010-50, to replace 50 % of protein supplied by fishmeal with a cooked, solvent-extracted soybean meal formulation 3010 from Schillinger Genetics Inc. Poultry meal, wheat flour, soy protein concentrate, corn, and fish oil were all varied to maintain the 3010-50 diet isonitrogenous, isolipidic, and isocaloric qualities compared with the reference diet, FM, and to contain similar amino acid profiles. The diets differ significantly in menhaden and poultry meal, soy protein concentrate, NPF1-3010, menhaden oil. The 3010-50 diet is also supplemented with 0.15 % taurine. The performance characteristics of 3010-50 in a 12-week trial were compared with that of the reference diet, FM, containing 45.5 % fishmeal (7). The complete formulation is given in Supplemental Table S4. Both diets gave very good performance characteristics; 3010-50 gave slightly better performance characteristics than FM, with an SGR of 3.45 ± 0.08 , CF of 0.715 ± 0.04 compared to SGR of 3.29 ± 0.08 , CF of 0.637 ± 0.637 (7). This is reflected in the level of eIF2 α phosphorylation which is lower in fish fed the 3010-50 diet (**Figure 7**). Note that levels of phosphorylated eIF2 α are low in each condition, with the ratio of phosphorylated to total eIF2 α in the reference diet is equivalent to that observed in the FM diet (Diet 6) in **Figure 6**.

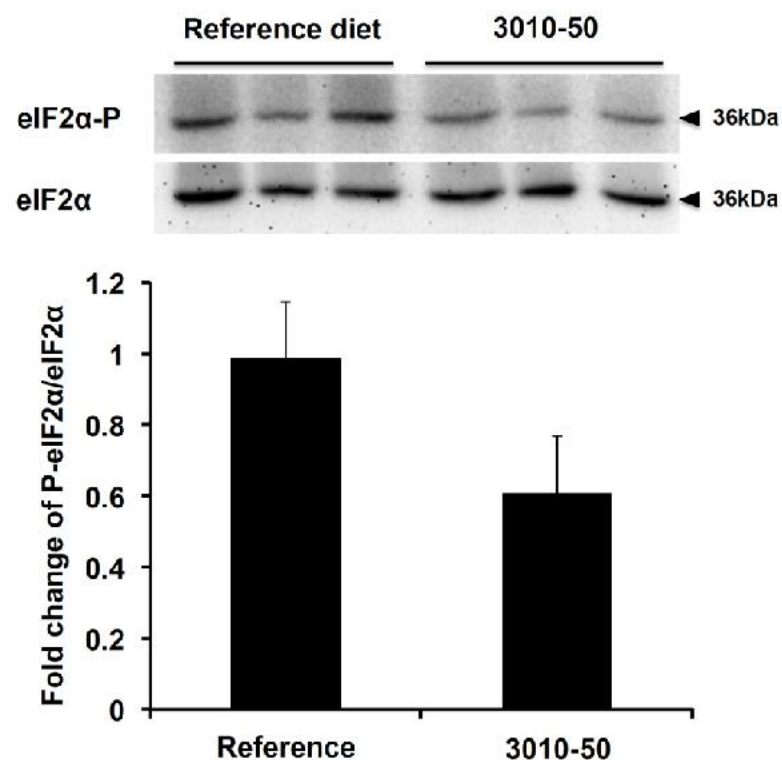


Figure 7. Comparison of eIF2 α phosphorylation in cobia juvenile fed reference diet versus a diet with soy protein concentrate using a non-GMO soybean cultivars (3010-50)

Juvenile cobia keeps in tank fed a diet with partial replacement of fish meal, poultry by-product (reference diet) and soy protein concentrate with a non-GMO soybean culti-

vars (3010-50) and diet formula given in Table S3. Proteins in tissue extracts were fractionated by SDS-PAGE and transferred to PVDF. Phosphorylated and total eIF2 α were visualized by enhanced chemiluminescence, using antibody to phosphorylated eIF2 α and (after stripping) antibodies to total eIF2 α , as described in legend to Figure 4. The bar diagrams in lower panels represent the quantification of the blots corresponding to the respective lanes in the upper panel. 3.2.3. eIF2 α phosphorylation during "cold banking" and acclimation

"Cold banking" is a carefully employed technique to slow down fish growth rate. It is especially effective with fingerlings, when an investigator is trying to stagger fish production. A comparison was made of "cold banked" juveniles, maintained at 20 °C and fed a maintenance ration 5 days a week at 1.5 % body weight (bw) daily for 6 weeks prior to acclimation, with cold-banking parameters based on Holt et al. (5). Acclimation to 27 °C and 5 % bw feeding daily was established over the course of two weeks, increasing 1 °C per day and 0.5 % bw per day for one week, followed by maintenance at 27 °C for one week. eIF2 α phosphorylation was compared in the cold-banked and acclimated fish in **Figure 8**. The level of eIF2 α phosphorylation was higher in the fish acclimated to 27 °C. This surprised us since cold shock activates PERK in mammals (67). However, a similar response is seen in human livers kept at cold temperatures for transportation (68). Cold transportation temperatures activate the IRE-1 pathway component of ER stress early. In contrast, at colder temperatures, ATP levels are lower, so that PERK is not activated (requires autophosphorylation), and levels of eIF2 phosphorylation is low. However, this portion of the ER stress response happens as a second phase once the liver is re-perfused and the temperature increased.

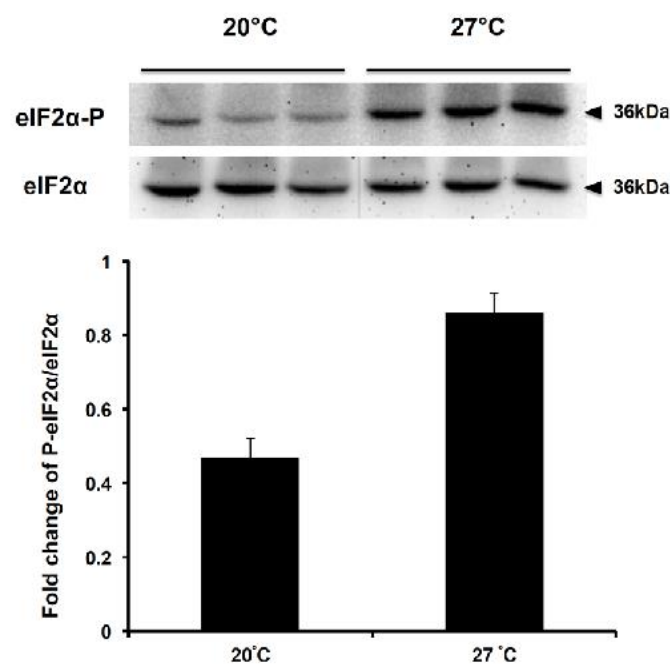


Figure 8. Effects of cold banking on eIF2 α phosphorylation level

Effects of water temp change on eIF2 α phosphorylation level in cobia liver. Juvenile cobia keeps in tank that water temperature holds at 20 °C and 27 °C. Proteins in cell extracts were fractionated by SDS-PAGE and transferred to PVDF. Phosphorylated and total eIF2 α were visualized by enhanced chemiluminescence, using antibody to phosphorylated eIF2 α and (after stripping) antibodies to total eIF2 α , as described in legend to **Figure 4**. The bar diagrams in lower panels represent the quantification of the blots corresponding to the respective lanes in the upper panel. Y-axis is the relative ratio of eIF2 α phosphorylation compare to total eIF2 α .

4. Discussion

The present study investigated the response of the marine fish cobia, *Rachycentron canadum*, to stressors as measured by phosphorylation of the α -subunit of the translational initiation factor, eIF2, the target of a family of protein kinases that respond to physiological stressors. The high conservation of eIF2 α throughout vertebrates allowed us to use commercial antibodies to eIF2 α and phosphorylated eIF2 α for our studies. Also in this study, a new cobia cell line, Cm, has been described and demonstrated to be of use in demonstrating the response to nutrient deficiency and ER stress. The response in Cm cells to nutrient deficiency and ER stress mimic that observed in mammalian and ZFL cells. The lack of response to poly(I:C) and NMPP most likely reflects low level expression of these kinases in the Cm cell line. However, investigation of this will depend on uncovering the sequences of cobia eIF2 α kinases to allow antibody development. The Cm cells are likely to be very useful for additional molecular studies to investigate the basis of the dietary requirements of cobia. These cells could also be used for investigation of fatty acyl elongase, a key enzyme in the synthesis of polyunsaturated fatty acids. Similarly, the Cm cells could be used to investigate the defects in the taurine biosynthetic pathway in cobia in more detail as well as responses to added taurine. Because of the lack of response to poly(I:C), it is unclear whether Cm cells will be useful for susceptibility testing for viruses such as iridoviruses and nervous necrosis virus (NVV) important viral pathogens of the fry and fingerling stages of cobia (51; 69).

Beyond investigations in Cm cells, we asked if eIF2 α phosphorylation could be used as a rapid indicator of fish physical condition. There is an accumulation of reports of the eIF2 α kinases in fish. The investigations have focused on the dsRNA activated eIF2 α kinase, PKR, rather than nutrition because of its role in defense against virus infections. PKR has been identified in zebrafish, three-spined stickleback, *Gasterosteus aculeatus*, fugu, *Takifugu rubripes*, puffer fish, *Tetraodon nigroviridis*, common carp, *Cyprinus carpio*, crucian carp, *Carassius auratus*, grass carp, *Ctenopharyngodon idellus*, fathead minnow, *Pimephales promelas*, medaka, *Oryzias latipes*, Atlantic salmon, *Salmo salar*, and Japanese flounder, *Paralichthys olivaceus* (14; 70–73). All are interferon-stimulated genes and play roles in interferon-mediated antiviral responses. It has been proposed that PKR may also form part of the adaptive response to prevent further accumulation of energy in the overfed state. PKR is normally activated by double-stranded RNA, and interestingly the RNA binding domain of PKR has been shown to be required for its activation in response to lipids (27). PERK has been identified in Chinese rare minnow, *Rhynchocypris oxycephalus* and medaka, *Oryzias latipes*, in which it is involved in ER stress (74; 75), and in zebrafish in which it is involved in the response to tributyl tin and other metal poisoning (76; 77). PERK is the other eIF2 α kinase known to be involved in the response to nutritional changes. One of the problems that can arise with unbalanced fish diets is the development of fatty liver disease (FLD) and PERK has been reported to be involved in fatty liver disease zebrafish larvae (78). HRI has only been reported so far in Japanese flounder where it responds to heat shock (79). So far, the results reported here in cobia cells represent the first report of GCN2 activation in fish, although it is implied in the effects of dietary lysine in largemouth bass, *Micropterus salmoides* (80). Since GCN2 is the primary responder to nutritional deprivation, this opens up the whole area of fish diet and eIF2 α phosphorylation. GCN2 is also involved in a variety of organismal functions in vertebrates such as feeding behavior (37; 81). To ensure an adequate supply of nutrients, omnivores choose among available food sources. This process is exemplified by the well-characterized innate aversion of omnivores to otherwise nutritious foods of imbalanced amino acid content. Brain-specific inactivation of GCN2 impairs this aversive response (37). In mice, GCN2 is rapidly activated in the mediobasal hypothalamus after consumption of a leucine-deficient diet (81). Knockdown of GCN2 in this particular region shows that GCN2 activity controls the onset of the aversive response.

5. Conclusions

Our preliminary studies have shown that in cobia juveniles, responses at the level of eIF2 α phosphorylation can be observed in response to diet and water temperature. This highlight the usefulness of monitoring eIF2 α phosphorylation in the establishment of optimum diets and aquaculture conditions and provides a means to investigate reasons underlying poorly accepted diets. This proof of principle opens the way to a more systematic investigation of responses at the level of eIF2 α phosphorylation in cobia and other species of aquaculture interest. We conclude that eIF2 α phosphorylation may be useful as an indicator of fish condition and health in aquaculture situations. The development of eIF2 α phosphorylation as a routine assay for fish health is unlikely because of level of technical skill needed to use the method usefully. Nevertheless, the monitoring of eIF2 α phosphorylation has the potential to provides a very useful tool for analysis in dissecting out where dietary deficiencies may lie and what molecular processes underlie nutritional pathologies and suboptimal aquaculture conditions.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1,

Table S1. Primer pairs used for eIF2 α -RACEprimer, cloning of cobia eIF2 α and cobia COX1 gene recognition

Primers used to amplify the cobia eIF2 α cDNAs were designed from the published coding sequences from five close related fish species, zebra fish (NM_131800.2), catfish (GU588091.1), puffer fish (CR685632.2), Atlantic salmon (NM_001140183) and rainbow trout (NM_001124296.1), selected most conserved sequence as target region and we initially amplified a ~900 bp cDNA. The complete coding sequence was assembled by 5' & 3' RACE. The degenerate primers among the above oligonucleotides incorporate a statistical mix of monomers at the positions labeled V (A, C or G), S (C or G), R (A or G), Y (C or T) or D (A, G or T) [in accordance with IUPAC convention].

Primer	Sequence (5'-3')	T _m
RceIF2 α 5'GSP1	CACACTTGATGGCCTCCTCT	56.9
RceIF2 α 5'NGSP1	TACTCGGATGACGACCACGC	59.1
RceIF2 α 3'GSP2	GGGTCTGTCTGTCCTCAACC	57.3
RceIF2 α 3'NGSP	AGAGGCTAGAGCGGGAGAAC	58.4
RceIF2 α F1	GAGGTGGAGGATGTGGTGAT	56.4
RceIF2 α F2	GAGTACAACAACATCGAGGG	52.9
RceIF2 α R1	YTCDGCTTTGGCCTCCAT	55.7
RceIF2 α R2	TGACVGCCTGTGGDGTSAG	59.7
RcBaF	GATCCTGACAGAGCGTGG	55.3
RcBaR	AGCACAGTGTTGGCGTACAG	57.9
RcFISHCOILBC_ts	CACGACGTTGTAAAACGACTCAACYAATCAYAAAGATATYGG-CAC	64.3
RcFISHCOIHBC_ts	GGATAACAATTCACACAGGACTTCYGGGTGRCCRAARAATCA	65.9
RcCOX1F	TCAACCAACCACAAAGACATTGGCAC	60.2
RcCOX1R	TAGACTTCTGGGTGGCCAAAGAATCA	59.8

Table S2. Dietary formulations for the fish meal versus plant protein diets

Ingredient (g kg ⁻¹)	FM	PP
Menhaden fish meal	345	0
Soy Protein concentrate	0	269
Corn Protein concentrate	44.3	193.4
Poultry by-product meal	118	0
Wheat Flour	237.7	175.5
Soybean meal, solvent extracted	90	90
Wheat Gluten meal	0	22
Blood meal, spray	39	0
Menhaden fish oil	90	120
Vitamin pre-mix	20	20
Mono-Dical Phosphate	0	42.5
Lecithin	0	20
L-Lysine	0	19.9
Choline CL	6	6
Potassium Chloride	0	5.6
DL-methionine	0	5
Threonine	0	2.8
Sodium Chloride	0	2.8
Stay-C	2	2
Trace mineral pre-mix	1	1
Magnesium Oxid	0	0.5
Mycozorb	2	2
Taurine	5	0
Performance characteristics (extrapolated from two different experiments)		
SGR	4.72+/-0.02	0.57+/-0.12

Table S3. Diet formulations and proximate compositions of the experimental diets

Ingredient (g 100g ⁻¹)	Reference	3010-50
Menhaden meal	45.5	22.9
Poultry meal	7.5	3.8
Wheat Flour	16	15.0
Soy protein concentrate	7.5	3.8
NPFI-3010	--	35.1*
Corn	17	9.4
Menhaden oil	3.9	6.4*
Vitamin pre-mix	1.0	1.0
Trace mineral pre-mix	0.1	0.1
Taurine	1.5	1.5
Lysine HCL	--	0.1*
DL-Methionine	--	0.8*
Proximate Composition (g 100g⁻¹)^a		
Moisture (g 100g ⁻¹)	7.2	11.1
Protein (g 100g ⁻¹ dm)	46.3	42.6
Protein on dry matter basis	49.9	47.9
Fat (g 100g ⁻¹ dm)	10.8	11.4
Fiber (g 100g ⁻¹ dm)	1.2	1.0
Ash (g 100g ⁻¹ dm)	9.1	8.8
Carbohydrate ^b (g 100g ⁻¹ dm)	28.72	29.01
Energy (MJ kg ⁻¹)	18.56	18.97
Performance characteristics		
SGR	3.29+/-0.08	3.45+/-0.08
PER	1.74+/-0.04	1.74+/-0.04
CF	0.637+/-0.04	0.715+/-0.04

^a New Jersey Feeds Labs analysis, Trenton, NJ.^b Calculated by difference (100-Moisture-Protein-Ash-Fat-Fiber).

Author Contributions: Conceptualization, A.R.P., R.J.; cell culture, C.L.L.; bioinformatic searches, C.L.L.; cloning protein extraction, in vitro translation, western blots, C.L.L., E.D.; fish culture and dietary studies, A.M.W., A.R.P.; writing—original draft preparation, C.L.L., R.J.; writing—review and editing, C.L.L., A.R.P. and R.J.; supervision, A.R.P., R.J.; project administration, A.R.P., R.J.; funding acquisition, A.R.P. and R.J. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All experiments involving fish were carried out in accordance with the guidelines of the International Animal Care and Use Committee of the University of Maryland Medical School: IACUC protocol #0610015 for cobia. Fish used for tissue sampling were anesthetized with Tricaine methanesulfonate (MS- 222, 70 mg L⁻¹) for blood sampling and then euthanized with MS-222 (150 mg L⁻¹).

Data Availability Statement: The cDNA sequence for the *Rachycentron canadum* eIF2 α has been deposited in GenBank (accession # KJ513464).

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Conflicts of Interest: The authors declare no conflict of interest.

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