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eIF2α 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. eIF2α is the target of phosphorylation by a family of kinases that respond to a range of physiological stressors. Phosphorylation of eIF2α inhibits overall protein synthesis, but also facilitates the reprogramming of gene expression to adapt to, and recover from, stress. The deduced coding sequence of cobia eIF2α has 94% identity to both zebrafish (*Danio rerio*) and human eIF2α sequences with identical phosphorylation and kinase docking sites. Here we use cobia larvae and a cobia cell line derived from muscle (Cm cells) to investigate the response of cobia eIF2α to various stressors. In Cm cells, phosphorylation of eIF2α is increased by nutrient deficiency and endoplasmic reticulum stress (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 eIF2α. We conclude that evaluation of eIF2α phosphorylation could function as an early marker to evaluate diet, environmental stressors, and disease in cobia and may be of particular use in optimizing conditions for rearing cobia larvae and juveniles.

Keywords: eIF2α; eIF2α-kinases; diets; stressors; *Rachycentron canadum*; cobia cells

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1. Introduction

Cobia, *R. canadum*, is a marine perciform found in the Atlantic Ocean, the Caribbean, and the Indo-Pacific Ocean off Australia, Japan, and India. This species is eurythermal and euryhaline, tolerating temperatures from 1.6 to 32.2 °C and salinities from 5 to 44.5 ppt. Cobia commands a relatively high price commercially. However, because it is a solitary species, there is no designated wild fishery. It is currently farmed in aquaculture [1–3] and exhibits rapid growth in both off-shore cage and marine recirculating systems [4]. Difficulties persist in large-scale commercial aquaculture production despite continuing improvements in aquaculture conditions. Production is dependent on seed stock from hatcheries, but high mortalities of larvae persist, reflecting stress and disease [5]. Furthermore, there is a continuing reliance on unsustainable fish meal diets in rearing fingerlings. The expansion of cobia farming requires the development of sensitive assays to monitor stress during larval culture and optimizing plant-based diets to increase production and sustainability [6–8]. Diet studies are both time-consuming and expensive and could be facilitated by developing faster indicators of diet quality. Similarly, a sensitive indicator of stress has the potential to assist in the optimization of larviculture conditions.

In eukaryotes, from protists to vertebrates, phosphorylation of Ser51 in the α-subunit of the translational initiation factor eIF2 is a critical cellular response to many stressors, including nutrient deficiency, hypoxia, endoplasmic reticulum stress, and viral infection [9–11].

Phosphorylation of eIF2α on Ser51 or its equivalent reduces the rate of protein synthesis overall. However, increased eIF2α phosphorylation stimulates the recruitment of a subset of mRNAs allowing the transient activation of an alternate gene expression program that enables the cell or organism to respond to stressors [10]. There are five eIF2α-specific kinases in vertebrates [12], five in teleost fish and amphibians, and four in tetrapods [13–15] that can phosphorylate eIF2α. Different stressors activate each of these. HRI (EIF2AK1), which is stimulated by heme depletion in erythroid cells, also responds to oxidative stress, arsenite, and osmotic shock, and is the major eIF2α kinase responsive to heat shock [16–19]. PKR (EIF2AK2) is stimulated by viral infection [20,21] but also plays a more general role in transferring metabolic information [22]. It is activated in response to disparate signals ranging from oxidative and ER stress [23–25], as well as cytokine and growth factor signaling [23,26], and is implicated in the pathological effects of obesity [27,28]. PERK (EIF2AK3) is an endoplasmic reticulum (ER) transmembrane protein activated by misfolded proteins in the ER; a phenomenon termed the unfolded protein (UPR) or ER stress response [29]. Changes in Ca^{2+} levels within the ER negatively affect the ability of a 78 kDa glucose-regulated protein (GRP-78), also known as the binding immunoglobulin protein (BiP), to maintain PERK in its inactive state [30]. Many signals such as inhibition of the SERCA calcium pump, glucose deprivation, and high levels of fatty acids reduce calcium concentration in the lumen of the ER and activate PERK [31,32]. GCN2 is the only eIF2α-kinase conserved among plants and metazoans and is the principal responder to nutritional deficiency. Falling amino acid levels in yeast and mammalian cells activate GCN2 through uncharged tRNAs and the ribosomal P-stalk [33–36]. GCN2 is also activated by glucose deprivation [37] and is involved in various organismal functions in vertebrates, such as feeding behavior and long-term memory formation [38]. These four members of the eIF2α kinase family share overall homology in their catalytic domains and phosphorylate eIF2α at the same residue, equivalent to Ser51 in human/mice [12]. Teleost fish and amphibians also have a PKR-like kinase, PKZ, more closely related to PKR than the kinase domains of the other three eIF2α kinases [13,15]. Like PKR, PKZ is involved in the innate immune response [39].

With the known relationship between eIF2α phosphorylation, nutritional status, and food choices in mammals [40], it was of interest to determine whether eIFα phosphorylation could be an early indicator for evaluating experimental diets and aquaculture conditions in fish. Studies were initiated in zebrafish, a tractable model system, to provide a foundation for looking at fish of interest to aquaculture. Zebrafish ZFL cells respond to various stressors that activate a range of eIF2α–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. Several cell lines have been described from economically important marine fish species. These include cell lines from groupers [41,42], tilapia [43], seabass [44], red sea bream [45], turbot [46], flounder [47,48], sea perch [49], and gilthead seabream [50,51]. The isolation of two cobia cell lines, Cb and Cf, from brain and fin, respectively, was previously reported and characterized but subsequently lost [52]. Here we report establishing and using a new cell line from cobia muscle, referred to as Cm cells. To facilitate studies on Cm cells and cobia, we have successfully cloned cobia eIF2α cDNA using degenerate primers based on eIF2α sequences from other fish species and completed the coding sequence using 5′ & 3′ RACE.

We set out to examine whether the eIF2α phosphorylation state in Cm cells responds to known stress-activated eIF2α-kinases by increasing eIF2α phosphorylation. Furthermore, additional studies in cobia juveniles show that changes in eIF2α phosphorylation can arise from nutritional deficiencies and temperature changes. These results suggest that eIF2α phosphorylation could provide a valuable indicator to monitor cobia performance and response to diets and disease.

2. Materials and Methods

2.1. Establishment and Culture of Cobia Cell Line

The cobia Cm cell line was established and characterized as described previously for cell lines from cobia brain and fin [52] from 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. Dissociated muscle cells were cultured in Leibovitz-15 medium (Gibco) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 lU/mL streptomycin, lacking sodium bicarbonate and CO_2 at 28 °C. Optimal growth was found at 28 °C in 10% FBS. One-third of the medium was replaced every 10–14 days until the attached cells reached 90% confluence and sub-cultured at a ratio of 1:2. The Cm cells grow as a monolayer and are fibroblast-like in appearance (Figure 1). Cells were cryopreserved in liquid nitrogen after the 50th passage.

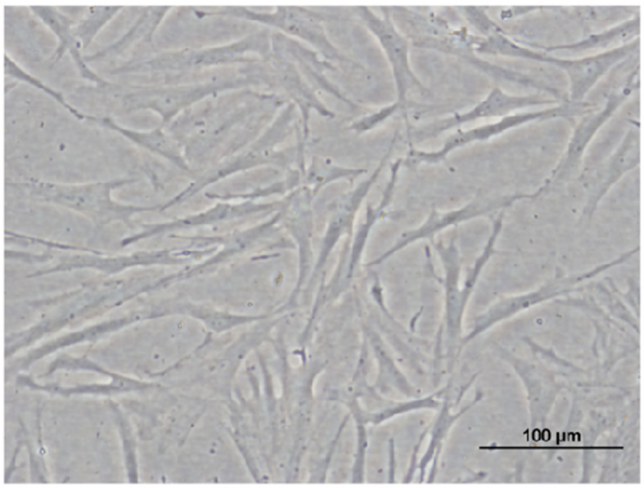

Figure 1. Cm cells in culture resemble fibroblast morphology.

2.2. Identification of Cm Cell Origin

The Cm mitochondrial cytochrome oxidase subunit I gene (cox I) sequence was used to verify whether the cell lines were derived from cobia, essentially as described [52]. RNA was isolated from Cm cells and cobia muscle using the PureLink® RNA Mini Kit (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Revertaid™ M-MULV reverse transcriptase and random hexamer primers were used for cDNA synthesis. Using the cox I primers described [53], endpoint PCR showed amplification of cDNA fragments of the predicted size for cobia cox I. The 650 bp cDNA products were recovered after separation using 1% agarose in Tris-acetate-EDTA gel electrophoresis using a QIAquick PCR purification Kit (Qiagen, Hilden, Germany) followed by sequencing. CLC and NCBI Blast were used to compare sequence identity.

2.3. Primer Design for Analysis of Cobia eIF2α

Degenerate primers were designed from five closely related fish species, zebrafish, catfish, pufferfish, Atlantic salmon, and rainbow trout, and used to amplify cDNA encoding eIF2α for cloning into pGEM-T. DNA sequence analysis was carried out using an Applied Biosystems Automated Sequencer (Thermofisher, Waltham, MA, USA). 5′ and 3′ rapid amplification of cDNA ends (RACE)-PCR was used to assemble the entire cDNA sequence. Primers used to amplify the cobia full-length eIF2α cDNAs were designed using Primer 3 software. For a list of primers, see Supplemental Table S1.

2.4. Preparation of RNA from Cm Cells and Cobia Muscle and cDNA Synthesis

Following the manufacturer's protocol, total RNA was extracted from the cobia cell line, liver, and muscle using the PureLink® RNA Mini Kit (Ambion, Thermofisher, Waltham,

MA, USA). The recovered RNA was analyzed spectrally for concentration at 260 nm and purity using both 260/280 and 260/230 ratios by Nanodrop ND-1000 spectrophotometry and automated electrophoresis using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA). DNA with 260/280 ratios and 260/230 ratios of >2 was considered sufficiently pure.

2.5. Cobia eIF2α Cloning and Generation of eIF2αS51A Constructs

Degenerate primers were designed for RT-PCR from the closely related fish species, zebrafish, catfish, pufferfish, Atlantic salmon, and rainbow trout. The resulting 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. All primers sets were designed to provide Nde I and BamHI recognition sites at the 5′- and 3′-ends (Supplemental Table S1). 5′ and 3′ rapid amplification of cDNA ends (RACE)-PCR was used to construct the entire length of the cDNA sequence. For cloning of full-length eIF2α, cDNA was prepared using random hexamers. The open reading frame of eIF2α was excised from pGEMT/eIF2α using the NdeI and BamHI restriction sites and transferred into pCITE4a. eIF2α sequence was verified from the sequencing of five primary transformants. Radiolabelled eIF2α was synthesized from the pCITE4a constructs using the rabbit reticulocyte TNTQuick system (Promega, Madison, WI, USA). Generation of eIF2α-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.

2.6. Preparation of Cell Extracts for Analysis of eIF2α Phosphorylation

Extracts from ~10^7 Cm cells were essentially prepared as described to prepare ZFL cell extracts with precautions to inhibit protease and phosphatase activities [54]. Clarified lysates were snap-frozen and stored in liquid N2. Extracts contained approximately 2–3 mg/mL of protein.

2.7. Immunoblot Analysis to detect eIF2α Phosphorylation

Cobia Cm cell extracts were fractionated by 15% SDS-PAGE, electrotransferred to PVDF membranes, and subjected to immunoblot analysis using a polyclonal antibody against the Ser51 phosphorylated form of mammalian eIF2α (3597, 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. The stripped blot was used to determine total eIF2α levels by reprobing with a rabbit antibody that equally recognizes both phosphorylated and unphosphorylated forms of eIF2α (9722, CellSignaling, Danvers, MA, USA). Values obtained from phosphorylated eIF2α were normalized for the total level of eIF2α present in the sample and expressed as the relative density of phosphorylated to total eIF2α.

2.8. In Vitro Phosphorylation of Cobia eIF2α

35S-radiolabeled eIF2α 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 purified mammalian recombinant HRI (EIF2AK1), PKR (EIF2AK2), PERK (EIF2AK3) or GCN2 (EIF2AK4) in in the presence of microcystin (0.5 μM) to inhibit phosphatase activity, essentially as described [54]. Samples were diluted in 10 volumes of 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. Proteins were separated by vertical slab isoelectric focusing (VSIEF) essentially as described, using a narrow pH range, in the presence of 8.8 M urea, 3% acrylamide, 1% CHAPS, 5% Pharmalyte (pH 4.5–6), and 50 mM DTT [55]. Electrophoresis was conducted 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 imaged using a Typhoon imaging system (Amersham Biosciences, Piscataway, NJ, USA).

2.9. Experimental Fish and Systems

This study was conducted per the Institutional Animal Care and Use Committee of the University of Maryland Medical School (IACUC protocol # 0610015). Juvenile (~2 g) cobia, *R. canadum*, were purchased from the Virginia Agricultural Experiment Station, Virginia Tech, Hampton, VA. Juveniles were maintained at 27 °C in the marine recirculating system at the Aquaculture Research Center, Institute of Marine and Environmental Technology, Baltimore, MD. Three replicates for each dietary treatment were performed. The full details are described in Watson et al. [8]. Approximately 500 juvenile cobia (~2 g each) were obtained from the University of Miami, Miami, FL. Fish were housed at the Institute of Marine and Environmental Technology's Aquaculture Research Center, Baltimore, MD. Cobias were "cold-banked" [7]; maintained at 20 °C, and fed a maintenance ration five days a week at 1.5% of body weight (BW)/day for six weeks prior to acclimation, with cold-banking parameters based on Holt et al. [5]. Acclimation to study conditions at 27 °C and 5% BW feeding daily occurred over the course of two weeks, increasing 1 °C/day and 0.5% BW/day for one week, followed by maintenance at 27 °C for one week prior to grading and stocking. Fish were maintained on the reference diet until they reached an average weight of ~18 ± 0.9 g. At that point, 12 fish were stocked into each of 21 identical tanks and randomly assigned one of the seven experimental diets with three replicate tanks per dietary treatment. The 21 tanks were within a group containing four independent sets of six 340-L tanks connected to bubble-bead and biological filtration that constituted the recirculating systems used simultaneously during the growth trial with photoperiod maintained at 14L:10D throughout. Water quality parameters were maintained at 26.8 ± 1.8 ppt, pH 7.8 ± 0.2; total ammonia nitrogen, 0.2 ± 0.2 mg/L; nitrite 0.4 ± 0.2 mg/L and alkalinity, 139.8 ± 48.1 mEq/L, with no significant differences (P N 0.05) in water quality parameters among systems utilized over time. Oxygen levels were routinely monitored and maintained at ≥6 mg L^{-1}. The trial was conducted over twelve weeks, with the collective weights of fish in each tank recorded and feeding rates adjusted weekly to 5% BW/day for the first six weeks, reduced to 3.5% from weeks 6–10, and 3% for the final two weeks of the trial. Fish were fed by hand four times daily to maintain apparent satiation and avoid overfeeding.

Fish diet formulations are provided in Supplemental Table S2 and prepared as described [8]. The performance characteristics, including specific growth rate (SGR), protein efficiency ratio (PER), and condition factor (CF), were calculated as described by Watson et al. [8].

2.10. Preparation of Cobia Liver Samples

Liver samples 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, USA) (3 lysis buffer:1 tissue ratio). Tissue was disrupted using FastPrep-24 5G (MP Biomedicals, Solon, OH, USA) for 40 s, kept on ice for 5 min, and microcentrifuged at 12,000× *g* for 15 min at 4 °C. Supernatants were snap-frozen in liquid N_2 immediately and stored at −80 °C for later analyses. Phosphorylation of eIF2α was determined by immunoblot analysis of SDS-PAGE fractionated proteins as described [54].

3. Results

3.1. Identification of Cm Cell Origin

The identification of Cm cells as derived from cobia was confirmed by sequence comparison of cox 1 cDNA from Cm cells and a muscle sample. The PCR-amplified cox 1 cDNA amplicons from Cm cells and a muscle sample, obtained using two different primer sets, were of the predicted sizes; each ~650 bp as determined by agarose gel electrophoresis.

DNA sequencing demonstrated that cox 1 cDNAs from cobia muscle tissue and 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).

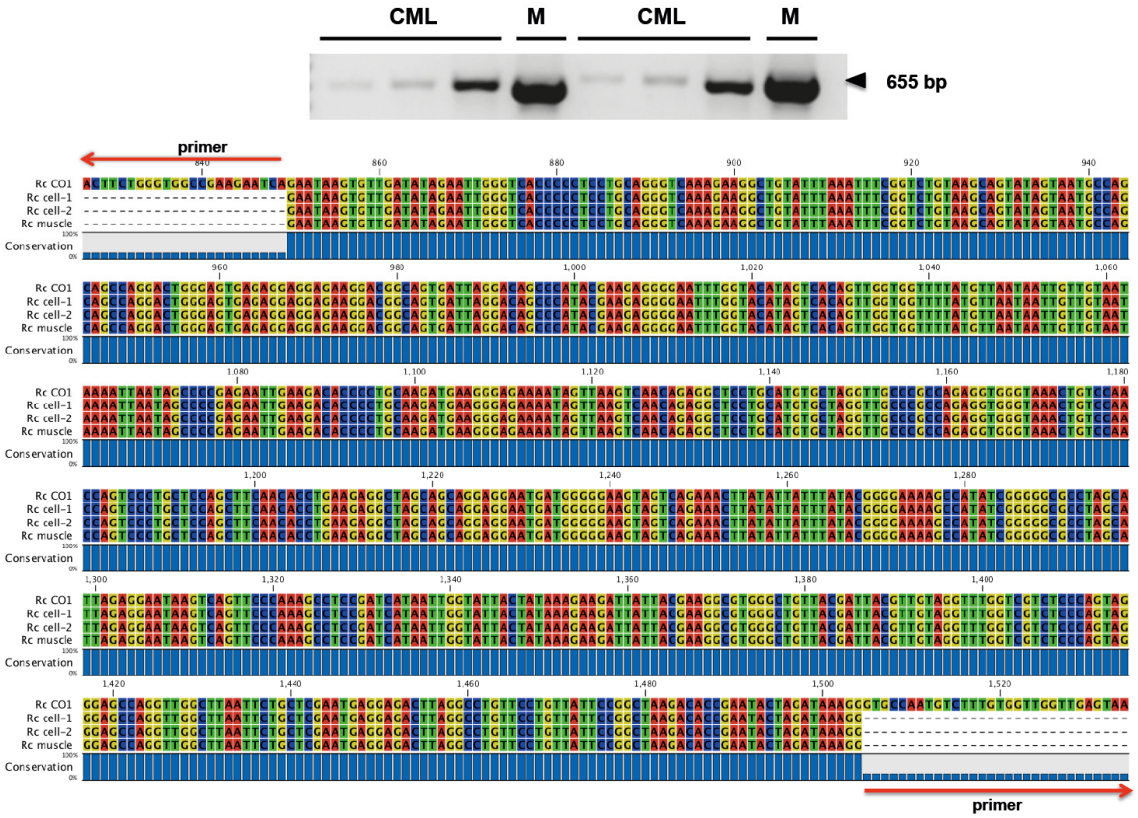

Figure 2. Identification of cobia cell origin: The upper panel shows PCR-amplified DNA from cobia muscle cell line (Cm) and cobia muscle (M) using primer pairs specific for cobia cytochrome oxidase subunit 1 (COI) gene and primers specified in Supplemental Table S1. Amplicons were fractionated by 1% agarose gel electrophoresis. The lower panel shows a multiple alignment of amplified COI DNA sequences from Cm cells and a cobia muscle sample compared with the cobia cytochrome oxidase subunit 1 (COI) reference sequence (GenBank accession # KJ202194.1) using the ClustalW program. The red arrows indicate the primer position.

3.2. Cobia Have Two eIF2α Transcripts but Identical Coding Sequences

Two transcripts of eIF2α are found in cobia, although the coding sequence for each is identical (accession # KJ513464). Compared to human and zebrafish eIF2α, the cobia coding sequence is 94% identical at the amino acid level.

Table 1 shows the amino acid composition percentages, predicted molecular weights, and isoelectric points of cobia eIF2α. The predicted isoelectric points and molecular weights of the eIF2αs are in excellent conformity with those of human and zebrafish eIF2α. The cobia eIF2α is predicted to be 35.97 kDa compared with a molecular weight of 36.1 kDa for human eIF2α. The deduced isoelectric points are also very similar, 4.84 for cobia eIF2α compared to 5.02 for human eIF2α. Figure 3 shows the multiple-alignment of the cobia eIF2α compared to human and zebrafish eIF2αs. Residues in the phosphorylation loop region are identical to those found in humans. Similarly, residues in the PKR binding site (residues 79–83) of cobia eIF2α are identical to those in the human and zebrafish sequences, suggesting it is a suitable substrate for eIF2α-specific protein kinases. In addition, residues critical for interaction with the α-, β-, and δ-subunits of the guanine nucleotide exchange factor, eIF2B [56], are present in cobia eIF2α signifying that the interactions between cobia eIF2α and eIF2B are also conserved [57].

Table 1. Characteristics of cobia eIF2α.

eIF2α	Gene	ID#	#aas	%ID	MW(kDa)	pI	Chrom	Location	Accession #
R. canadum	eif2s1		315	94	35.97	5.02			KJ513464
***D. rerio* eIF2α-a**	eif2s1a	321807	315	94	36.13	4.97	17	NC_007128.6	NM_199569.1
***D. rerio* eIF2α-b**	eif2s1b	321564	315	94	36.17	4.98	20	NC_007131.6	NM_131800.2
H. sapiens	EIF2S1	1969	315	100	36.1	5.01	14	14q23.3	NM_004094

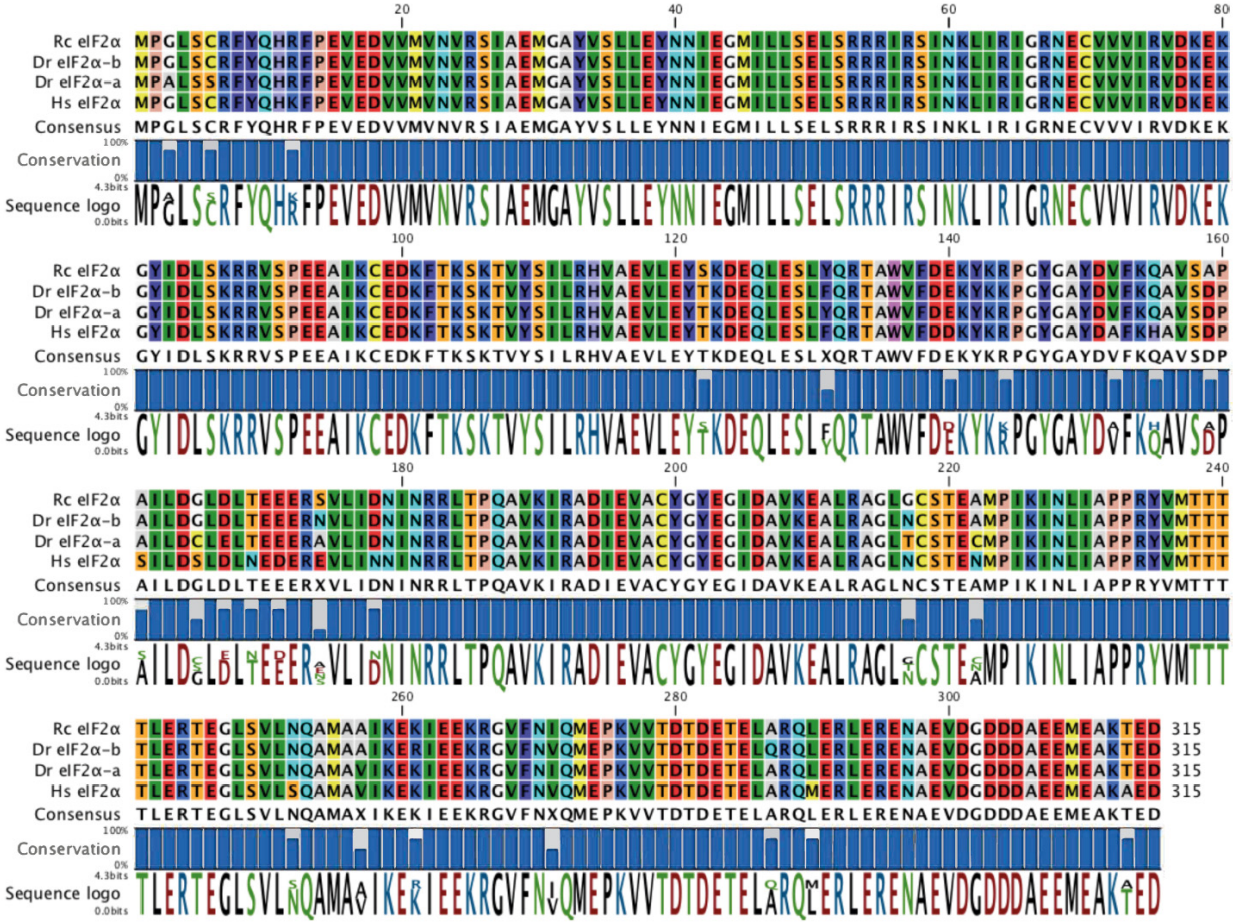

Figure 3. Multiple alignment of eIF2α from cobia, zebrafish, and human eIF2α from cobia, *Rachycentrum canadum*, compared with zebrafish eIF2αs and human.

Each of the four eIF2α-kinases, HRI, PKR, PERK, and GCN2, can be activated in mammalian cells by various stressors [11]. Thapsigargin is an inhibitor of Ca^{2+} ATPase in the endoplasmic reticulum, blocking calcium entry into the sarcoplasmic and endoplasmic reticulum [58]. The resulting depletion of ER calcium stores initiates endoplasmic reticulum (ER) stress, triggering the unfolded protein response, including PERK activation [59,60]. GCN2 is activated by amino acid and glucose starvation [37,61]. Leucinol is the alcohol formed by the total reduction of the carboxylic acid group of leucine. It is an inhibitor of leucyl-tRNA synthetase and mimics leucine deficiency in cells [62], activating GCN2 [37,61]. *N*-methylprotoporphyrin (NMPP) is a powerful inhibitor of ferrochelatase (Ki is approximately 10 nM), the terminal enzyme of the heme biosynthetic pathway [63], and activates the heme-sensitive kinase HRI [64]. Polyinosinic polycytidylic acid (poly I:C) is a synthetic dsRNA used to mimic viral infection in vivo, reviewed in [65], and activate PKR in vivo and in vitro.

The response of Cm cells to these known activators of eIF2α-kinases was examined, as shown in Figure 4. Cm cells treated with known activators of eIF2α-kinases; incubation in nutrient limiting medium or in the presence of leucinol (4 μM) for activation of GCN2; thapsigargin (1 μM) for activation of PERK; polyI:C (50 μg/mL with 200 μg/mL DEAE dextran) for activation of PKR [54] and NMPP (2 μM) for activation of HRI. Cells were harvested at various time points, as determined by preliminary experiments, and analyzed by immunoblotting.

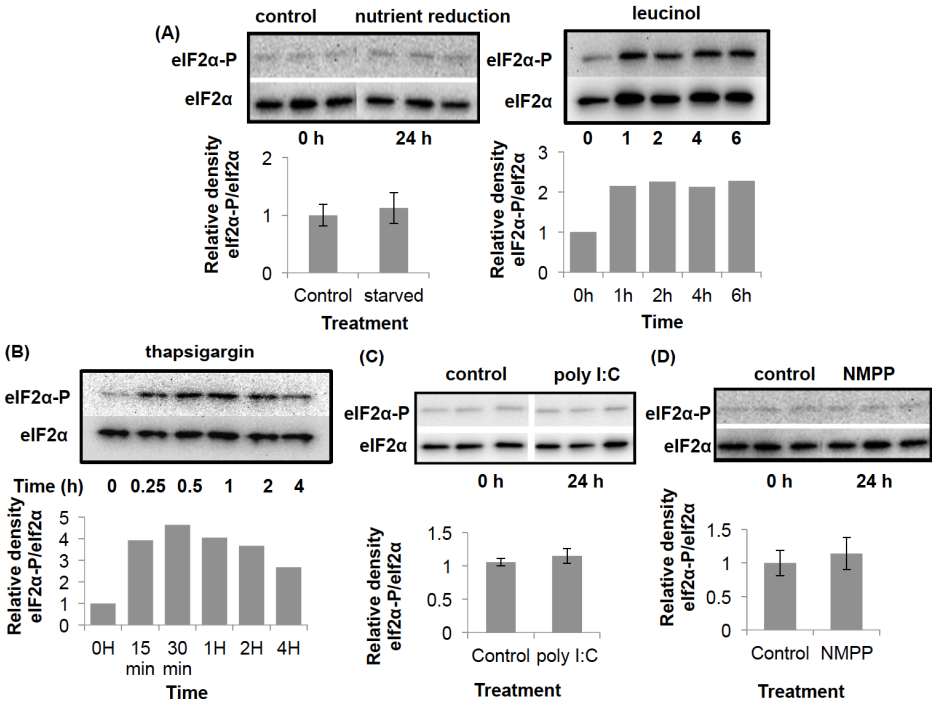

Figure 4. Response of cobia muscle cells to activators of eIF2-kinases Cobia muscle cells treated with known activators of eIF2α-kinases: (**A**) nutrient reduction cells were maintained in 30% L-15 cell culture medium in PBS for 24 h; (**A**) leucinol (4 mM), for up to 24 h, for activation of GCN2; (**B**) thapsigargin (1 μM), for up to 2 h, for activation of PERK; (**C**) poly I:C (50 μg/mL), for 24 h, for activation of PKR; (**D**) NMPP (2 μM), for up to 24 h, for activation of HRI. Proteins in cell extracts were fractionated by SDS-PAGE, and eIF2α was visualized by chemiluminescence following electro-transfer to PVDF. The blots were probed first with antibodies to phosphorylated eIF2α and, after stripping, antibodies to total eIF2α. Chemiluminescence was detected and an image generated (upper panels) using the ProteinSimple Fluorochem E, with quantification using ImageJ. The bar diagrams in the lower panels represent the quantification of the blots corresponding to the respective lanes in the upper panels and reflect the relative density of eIF2α to phosphorylated eIF2α.

3.3. 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, eIF2α phosphorylation was increased (Figure 4A(left)), consistent with the activation of GCN2. When leucinol (4 mM) was included in the complete culture medium, an increase in eIF2α phosphorylation could be seen within 1 h and continued to increase over 24 h (Figure 4A(right)). This also strongly suggests activation of GCN2 and is consistent with the response of mouse ES cells to leucinol [66]. Mouse ES cells also respond very rapidly to leucinol treatment. However, during the first 3 h of leucine deprivation, eIF2α phosphorylation in ES cells from mice lacking GCN2 (gcn2-/-) does not increase, demonstrating that the effect of leucine deprivation on eIF2α phosphorylation can be attributed to GCN2.

3.4. Effects of Thapsigargin on Cm Cells

Thapsigargin, a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase, SERCA, leads to PERK activation [67]. In Cm cells, eIF2α phosphorylation increases over the first hour of incubation with thapsigargin (1 μM) (Figure 4B). These results indicate that PERK can be activated in cobia cells.

3.5. Effects of Poly I:C on Cm Cells

Cm cells were treated with 50 μg/mL poly I:C and 200 μg/mL DEAE dextran. Figure 4C shows that treatment for 24 h showed almost no increase in eIF2α phosphorylation. This result differs from the effect of poly I:C in rainbow trout RTG2 cells and zebrafish ZFL cells [54]. These results could indicate that PKR is not expressed in Cm cells, is not activated, or that cobia eIF2α is not a good substrate for PKR.

3.6. Effects of N-Methylprotoporphyrin on Cm Cells

N-methylprotoporphyrin (NMPP), a transition-state analog, inhibits ferrochelatase. It has been used experimentally to generate heme deficiency in rat hepatocytes and has been shown to increase eIF2α phosphorylation [64]. Cobia Cm cells were treated with NMPP (2 μM) for up to 24 h. Unlike rat hepatocytes, which respond rapidly to NMPP, Cm cells show no increases in eIF2α phosphorylation after 24 h (Figure 4D). These results indicate that HRI may not be expressed in Cm cells, is not activated, or that cobia eIF2α is not a good substrate for HRI.

3.7. All Four eIF2α-Kinases Can Phosphorylate Cobia eIF2α In Vitro

Although Cm cells responded to activators of GCN2 and PERK with increased eIF2α phosphorylation, they did not respond to activators of PKR or HRI. We took the alternative approach of looking at the capacity of recombinant cobia eIF2α to be phosphorylated by the kinases in vitro, as we have previously shown for recombinant zebrafish and rainbow trout eIF2α [54]. 35S-labeled cobia eIF2α was synthesized in a reticulocyte translation system to which we later added purified mammalian eIF2α-kinases. Phosphorylated and non-phosphorylated forms were separated by vertical slab gel isoelectric focusing (VSIEF) (Figure 5). This analysis showed that cobia eIF2α can be phosphorylated by eIF2α-kinases and that PKR and HRI and is consistent with our interpretation that these kinases are either not well expressed or not activated by poly I.C or NMPP in Cm cells.

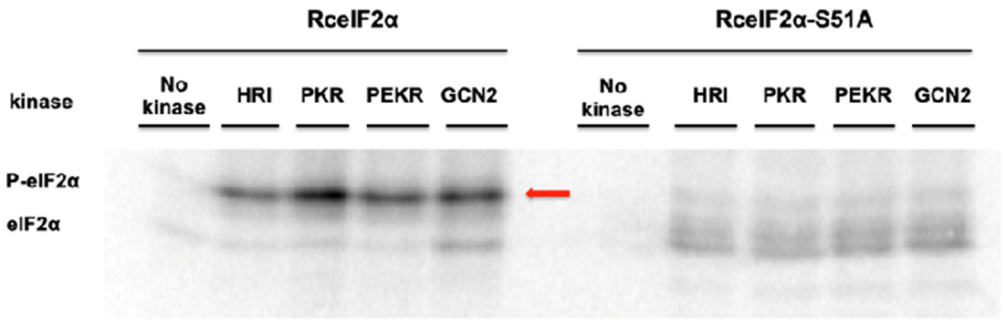

Figure 5. In vitro phosphorylation of cobia eIF2α by purified recombinant mammalian eIF2α-kinases ^{35}S-radiolabeled cobia eIF2α, eIF2α-S51A were synthesized in the reticulocyte TnT cell-free transcription/translation system by incubation at 30 °C for 30 min after which they were supplemented with x 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 μM) was included to inhibit phosphatase activity. Each sample was diluted in an 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 eIF2α.

3.8. eIF2α Phosphorylation in the Livers of Cobia Juveniles

3.8.1. Comparison of eIF2α Phosphorylation in Fish Fed a Fish Meal versus an All Plant Protein Diet

To apply to aquaculture, it was essential to demonstrate that eIF2α 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 formulating plant protein diets [7]. We took advantage of this by sampling fish from a few diet trials. Our investigation was not intended to be an exhaustive analysis of eIF2α phosphorylation under all dietary regimes, but fish samples were taken to ask if changes in eIF2α 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 eIF2α in the livers of these fish at the conclusion of the feeding trial is shown in Figure 6. Higher eIF2α phosphorylation levels can be seen in the fish fed the plant protein diet without taurine supplementation. In fact, the level of eIF2α phosphorylation is as high as that observed in juveniles maintained without feeding for 7 days.

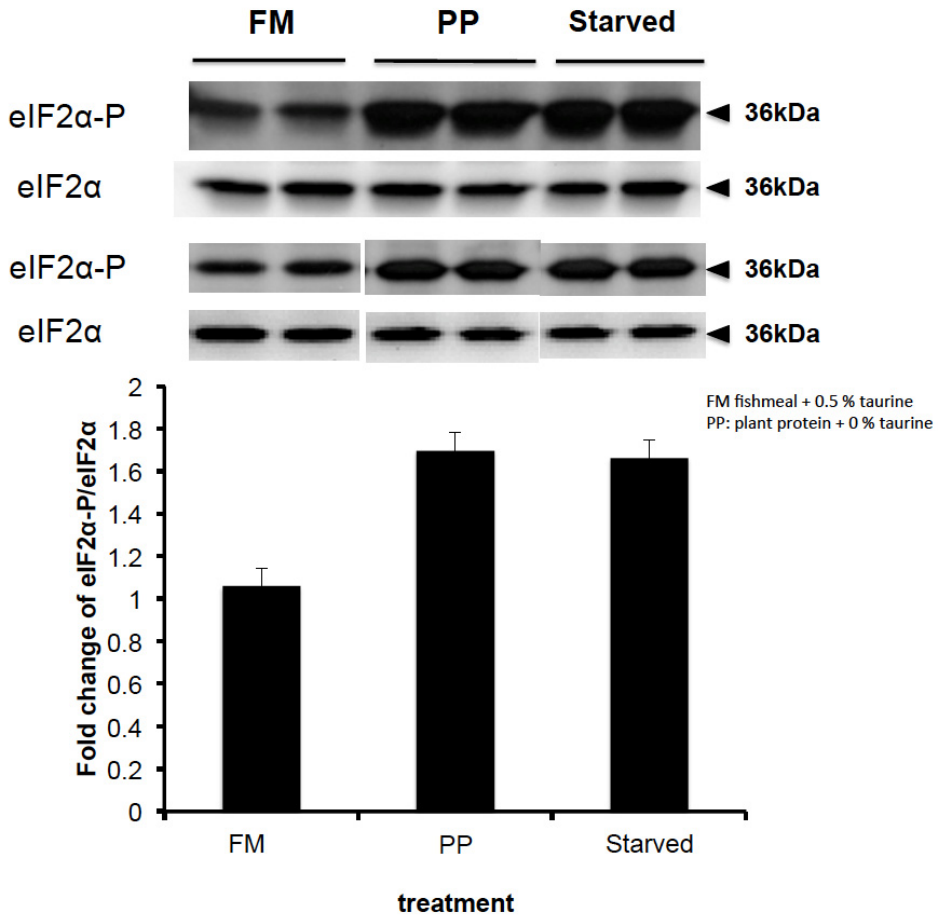

Figure 6. Comparison of eIF2α phosphorylation in livers of cobia fed a fish meal versus a plant protein diet. Cobia juveniles maintained for 12 weeks on an all plant protein diet, PP, were compared with animals fed a mixed fish meal/plant protein diet, FM [6] and unfed fish. The complete formulations are given in Supplemental Table S2. Proteins in liver extracts were fractionated by SDS-PAGE and

transferred to PVDF. Phosphorylated and total eIF2α were visualized by enhanced chemiluminescence, using an antibody to phosphorylated eIF2α and (after stripping) antibodies to total eIF2α, as described in the legend in Figure 4. Values obtained for the level of phosphorylated eIF2α were normalized for the total level of eIF2α present in the sample and expressed as the relative density of phosphorylated to total eIF2α with the bar diagrams in lower panels corresponding to the respective lanes in the upper panel.

3.8.2. Comparison of eIF2α Phosphorylation in Livers of Fish Fed a Reference Diet versus a Diet with Soy Protein Using the Non-GMO Soybean Cultivar 3010

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 soybean cultivars 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, and 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 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]. The performance characteristics are consistent with the level of eIF2α phosphorylation, which is lower in fish fed the 3010-50 diet (Figure 7). Note that levels of phosphorylated eIF2α are low in each condition; the relative density of phosphorylated eIF2α to total eIF2α in the reference diet is equivalent to that observed in the FM diet (Diet 6) in Figure 6.

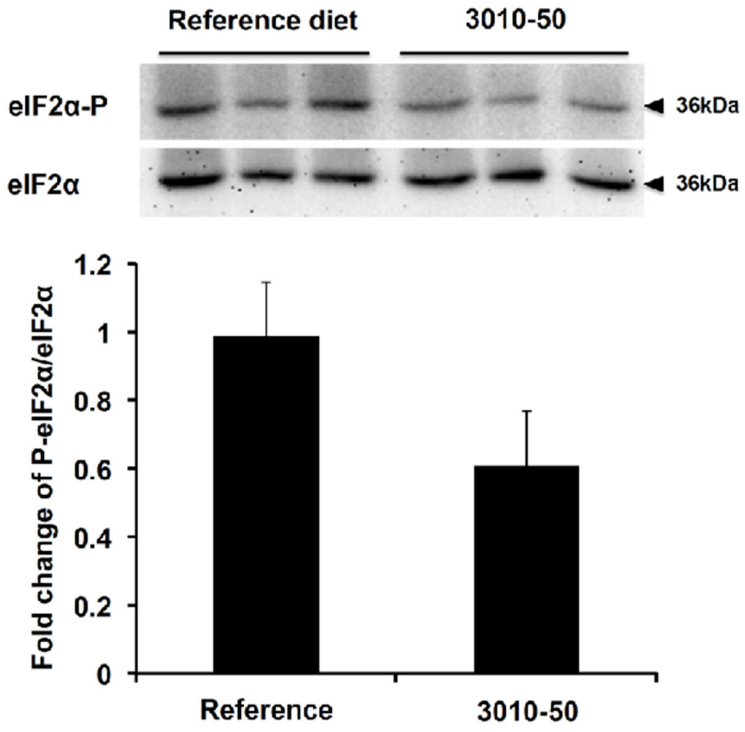

Figure 7. Comparison of eIF2α phosphorylation in liver extracts from cobia juveniles fed a reference diet versus a diet with soy protein concentrate using the non-GMO soybean cultivar 3010. Juvenile

cobia fed with either a reference diet (formula given in Table S3) or a diet with partial replacement of fish meal, poultry by-product (reference diet), and soy protein concentrate with a non-GMO soybean cultivar (3010-50). Proteins in tissue extracts were fractionated by SDS-PAGE and transferred to PVDF. Phosphorylated and total eIF2α were visualized by enhanced chemiluminescence, using an antibody to phosphorylated eIF2α and (after stripping) antibodies to total eIF2α, as described in the legend in Figure 4. Values obtained for phosphorylated eIF2α were normalized for the total level of eIF2α present in the sample and expressed as the relative density of phosphorylated to total eIF2α, with the bar diagrams in lower panels corresponding to the respective lanes in the upper panel.

3.8.3. eIF2α Phosphorylation during "Cold Banking" and Acclimation

"Cold banking" is a carefully employed technique to slow down the fish growth rate. It is especially effective with fingerlings when an investigator tries to stagger fish production. A comparison was made of "cold banked" juveniles, maintained at 20 °C and fed a maintenance ration equivalent to 1.5% body weight (bw) daily, five days a week, for six weeks prior to acclimation with cold-banking parameters based on Holt et al. [5] Acclimation to 27 °C and 5% bw daily feeding was established over two weeks, with temperature increasing 1 °C per day and 0.5% bw per day for one week, followed by maintenance at 27 °C and 5% bw daily feeding for one week. eIF2α phosphorylation was compared in the cold-banked and acclimated fish in Figure 8. eIF2α phosphorylation was higher in the fish acclimated to 27 °C. This surprised us since cold shock activates PERK in mammals [68]. However, a similar response can be seen in human livers kept at cold temperatures for transportation [69]. Cold transportation temperatures activate the IRE-1 pathway component of ER stress early. However, at colder temperatures, ATP levels are low, so PERK is not activated (requires autophosphorylation), and levels of eIF2α phosphorylation are low. However, PERK activation happens as a second phase once the liver is re-perfused and the temperature increases.

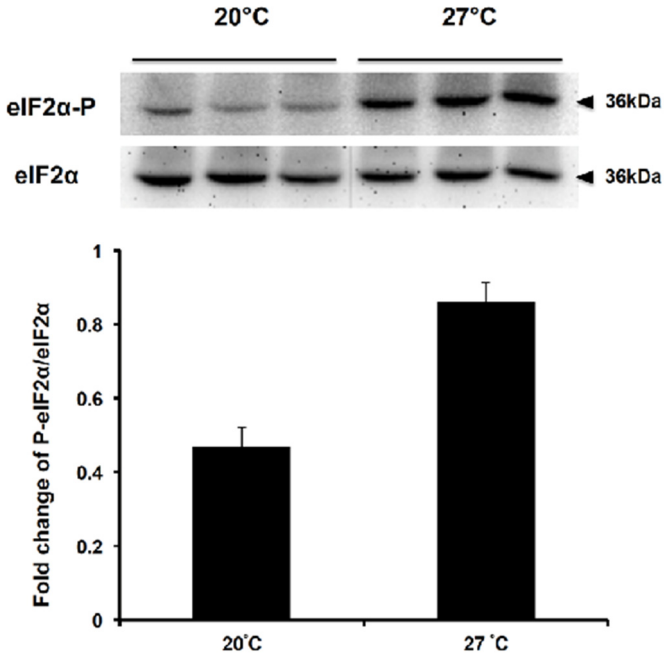

Figure 8. Effects of "cold banking" on eIF2α phosphorylation level in liver extracts.

Juvenile cobia were held in tanks at 20 °C and 27 °C. Proteins in cell extracts were fractionated by SDS-PAGE and transferred to PVDF. Phosphorylated and total eIF2α were visualized by enhanced chemiluminescence, using an antibody to phosphorylated eIF2α and (after stripping) antibodies to total eIF2α, as described in the legend in Figure 4. Values obtained for phosphorylated eIF2α were normalized for the total level of eIF2α present in

the sample and expressed as the relative density of phosphorylated to total eIF2α, with the bar diagrams in lower panels corresponding to the respective lanes in the upper panel.

4. Discussion

The present study investigated the response of the marine fish cobia, *R. 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 alpha-subunit of eIF2 is the target of a family of stress-activated protein kinases. The high conservation of eIF2α throughout vertebrates allowed us to use commercial antibodies to eIF2α and phosphorylated eIF2α 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 mimics that observed in mammalian and ZFL cells. The lack of response to poly I:C and NMPP most likely reflects the low-level expression of these kinases in the Cm cell line. However, investigation of this will depend on uncovering the sequences of cobia eIF2α 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 investigating fatty acyl elongase, an essential 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 and at the fry and fingerling stages of cobia [52,70].

Beyond investigations in Cm cells, we asked if eIF2α phosphorylation could be used as a rapid indicator of physical fish condition. There is an accumulation of reports of the eIF2α kinases in fish. The investigations have focused on the dsRNA-activated eIF2α 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*, pufferfish, *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* [15,71–74]. All are interferon-stimulated genes and play roles in interferon-mediated antiviral responses. In addition to its antiviral role, PKR may also be involved in the adaptive response to forestall the accumulation of energy in the overfed state. It has been proposed that PKR may also form part of the adaptive response to forestall further accumulation of energy in the overfed state. It is of interest to note that the RNA binding domain of PKR is required for its activation in response to lipids [28]. PERK has been identified in the Chinese rare minnow, *Rhynchocypris oxycephalus,* and medaka, *Oryzias latipes*, which is involved in ER stress [75,76], and in zebrafish in which it is involved in response to tributyltin and other metal poisonings [77,78]. PERK is also known to respond 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 in 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 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]. Our study opens up the whole area of fish diet and eIF2α phosphorylation since GCN2 is the principal responder to nutritional deprivation. PERK is also known to respond 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 in zebrafish larvae [78]. GCN2 is also engaged in various organismal functions in vertebrates, such as feeding behavior [38,81]. Omnivores choose among available food sources to ensure an adequate supply of nutrients. This process can be seen in the innate distaste of omnivores

to foods with imbalanced amino acid content. This aversive response is impaired by brain-specific inactivation of GCN2 [38]. In mice, rapid activation of GCN2 can be observed after consuming a leucine-deficient diet in the mediobasal hypothalamus, a response reduced after a knockdown of GCN2 in this region of the brain [81].

5. Conclusions

Our preliminary studies have shown that in cobia juveniles, responses at the level of eIF2α phosphorylation can be observed in response to diet and water temperature. This highlights the usefulness of monitoring eIF2α phosphorylation in establishing optimum diets and aquaculture conditions and provides a means to investigate the reasons underlying poorly accepted diets. This proof of principle opens the way to a more systematic investigation of responses at the level of eIF2α phosphorylation in cobia and other species of aquaculture interest. We conclude that eIF2α phosphorylation may be useful to indicate fish condition and health in aquaculture situations. The development of eIF2α phosphorylation as a routine assay for fish health is unlikely because of the level of technical skill needed to use the method usefully. Nevertheless, the monitoring of eIF2α phosphorylation can provide a useful tool for analysis in determining 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: https://www.mdpi.com/article/10.3390/jmse10050709/s1. Table S1: Primer pairs used for *eIF2a*-RACE, cloning of cobia eIF2α and cobia COX1 gene recognition: Primers used to amplify the cobia eIF2α 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]. Table S2: Dietary formulations for the fish meal and plant protein diets: PP corresponds to PP1 in Table 4.4.1 in Watson, A.M., Ph.D. thesis, 2013: Taurine: An Indispensable Ingredient in the Development of Sustainable Aquafeeds, except for inclusion of 0.02% taurine. Table S3: Diet formulations and proximate compositions of reference and experimental diets: [a] New Jersey Feeds Labs analysis, Trenton, NJ. [b] Calculated by difference (100-Moisture-Protein-Ash-Fat-Fiber). 3010-50, as in Watson, A.M., Ph.D. thesis, 2013: Taurine: An Indispensable Ingredient in the Development of Sustainable Aquafeeds, formulated to replace 50% protein from fish meal with NPF1-3010. Diets ~identical in levels of protein, fat, fiber, carbohydrate, taurine. Diets differ significantly in menhaden and poultry meal, soy protein concentrate, NPF1-3010, menhaden oil. * higher in 3010-50.

Author Contributions: Conceptualization, A.R.P. and R.J.; cell culture, C.L.L.; bioinformatic searches, C.L.L.; cloning protein extraction, in vitro translation, western blots, C.L.L. and E.A.D.; fish culture and dietary studies, A.M.W. and A.R.P.; writing—original draft preparation, C.L.L. and R.J.; writing—review and editing, C.L.L., A.R.P. and R.J.; supervision, A.R.P. and R.J.; project administration, A.R.P. and 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 fish experiments were carried out following the Institutional 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 methanosulfonate (MS-222, 70 mg/L) for blood sampling and then euthanized with MS-222 (150 mg/L).

Informed Consent Statement: Not applicable.

Data Availability Statement: The cDNA sequence for the *Rachycentrum canadum* eIF2α 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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