Mitochondrial Remodeling During Physiological Cardiac Hypertrophy in the Burmese Python

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Abstract

I. Background: *Cardiac hypertrophy occurs in response to both pathological and physiological stimuli, with the latter providing a beneficial effect on cardiac function. In pathological hypertrophy, mitochondrial dysfunction occurs due to oxidative stress, however, in physiological hypertrophy, sustained energy production is supported by either mitochondrial biogenesis or via enlargement of existing mitochondria. To date, there are no approved drugs to address mitochondrial dysfunction in heart failure. Thorough characterization of the signaling and genetic profiles involved in metabolic remodeling during physiological hypertrophy could shed light on novel drug targets that support energy production and potentially reverse metabolic dysfunction in the failing heart. II. Methods and Results: Electron microscopy revealed increased area occupied and redistribution of mitochondria in the Burmese python ventricle, a model of physiological hypertrophy, after feeding. Measurement of phosphorylation states of 46 signaling molecules was performed and demonstrated activation of signaling pathways that mediate both cardiac hypertrophy and mitochondrial biogenesis. To validate that mitochondrial biogenesis is occurring, expression of 15 genes involved in mitochondrial biogenesis, mitochondrial function, and termination of mitochondrial biogenesis was measured. Dynamic expression was observed during hypertrophy and regression, consistent with mitochondrial biogenesis. III. Conclusions: The results suggest that physiologic cardiac hypertrophy is supported by activation of signaling pathways that mediate mitochondrial biogenesis in the Burmese python heart. The patterns of gene expression are consistent with this observation. Data presented here represent a broad view of physiological metabolic remodeling in the heart as well as possible novel targets to support contractility of the failing heart.*

Keywords: cardiac hypertrophy, mitochondrial biogenesis, Burmese python, gene expression, intracellular signaling, electron microscopy.

Introduction

Cardiovascular disease (CVD) kills 600,000 people annually in the United States and as many as 16.7 million worldwide [1]. Risk factors including hypertension, hyperlipidemia, and tobacco use are present in nearly half the American population [2]. Estimates indicate that CVD costs the United States over $108.9 billion annually [3]. Due to the serious consequences of CVD, a need exists to identify novel drug targets that decrease the pathology associated with this disease.

Pathological cardiac hypertrophy, which is characterized by an abnormal increase in cardiomyocyte size, myofibrillar disarray, fibrosis, and hemodynamic dysfunction [4], is often observed in CVD. This type of growth, after a certain point, is irreversible and can lead to cardiac dilation and heart failure. Interestingly, not all cardiac growth is unfavorable and irreversible. Pregnant women and endurance athletes exhibit *physiological* cardiac hypertrophy, a reversible state of cardiac enlargement that lacks the detrimental changes in cardiac tissue, organization, fibrosis, and function that are typical of pathological hypertrophy. Instead, *physiological* hypertrophy produces a sustainable increase in cardiac function [5,1]. Indeed, exercise in the setting of CVD is beneficial. However, many people with CVD are not capable of the aerobic exercise required to obtain this benefit [6]. Pharmacologially replicating the pathways in the heart that are altered during exercise could be beneficial in the setting of CVD. In CVD, mitochondria are often both the target and the origin of pathways that lead to the progression of cardiac dysfunction. Dysfunctional mitochondria can promote accumulation of toxic intermediates such as long chain acyl-CoA, as well as reactive oxygen species (ROS) and reactive nitrogen species, which in turn affect mitochondrial function by reducing ATP synthesis [7], especially in the later stages of disease. Reduced energy negatively affects cardiomyocyte contractility in the heart and begins the progression to heart failure. Conversely, mitochondrial function during physiological hypertrophy is sufficient to support increased cardiomyocyte size and contractility emphasizing the importance of understanding the pathways that mediate this increase in mitochondrial function [8].

Mitochondrial dysfunction in pathological hypertrophy is accompanied by unchanged mitochondrial enzyme and oxidative phosphorylation gene expression despite the increased need for ATP [9,10]. In physiological cardiac hypertrophy, the mechanisms mediating the increase in mitochondrial function remain unclear. After exercise conditioning, canine ventricles exhibited increases in mitochondrial enzyme expression suggesting an increase in the number of mitochondria in the ventricular tissue [11]. Similarly, in a rat model of myocardial infarction, exercise training reduced metabolic deficits by increasing expression of mitochondrial enzymes in a pattern consistent with mitochondrial biogenesis [12]. By contrast, aerobic training in rats has also been shown to promote physiological cardiac hypertrophy, but without alterations in the density or distribution of mitochondria [13]. Although mitochondrial biogenesis is a logical target to increase metabolic function in heart failure, to date, there are no approved drugs to promote such an effect [14]. Therefore, further investigation is required to fully characterize the signaling and genetic profiles that regulate mitochondrial function during physiological cardiac hypertrophy. Data obtained from a thorough characterization could provide a means to identify strategies for maintaining metabolic function in heart failure.

A promising model for evaluating the molecular basis of metabolic remodeling in the heart is the Burmese python (*Python molurus*) [5]. The Burmese python regularly undergoes cardiac hypertrophy within 48-72 hours after feeding, characterized by a rapid 40% increase in mass and concomitant increase in cardiac output, followed by regression within 7-10 days [5]. The rapid hypertrophy of the python heart facilitates the digestion of large, infrequent, and energetically-expensive meals that require a large increase in systemic perfusion [15]. The molecular mechanisms mediating cardiomyocyte growth are similar to mammalian physiological hypertrophy but are more robust and occur in a shorter time frame, making the python an excellent model to efficiently study cardiac growth [16,5]. The mechanisms mediating production of the ATP required for increased cardiac output and a 44-fold increase in metabolism associated with the observed hypertrophy, have not yet been described [17].

The roles of ROS damage to mitochondria and insufficient energy production in CVD have been clearly demonstrated. However, the precise mechanisms mediating the adaptive changes during physiological hypertrophy have not been fully elucidated [18]. We aimed to examine the pathways that regulate mitochondrial function to determine whether mitochondrial biogenesis is responsible for maintenance of sufficient energy during physiological hypertrophy. We further aimed to provide the basis for identification of novel drug targets for the treatment of mitochondrial insufficiency in CVD.

We hypothesized that following feeding, activity of signaling molecules and subsequent downstream expression of the genes required for mitochondrial biogenesis would be increased. To test this hypothesis, we first measured activation of signaling molecules in the Burmese python ventricle to isolate pathways that may be involved in mitochondrial remodeling after feeding. Using real time PCR, we focused on nuclear-derived transcripts to allow conclusions about intracellular signaling pathways and the nuclear transcripts that regulate genes expressed in mitochondrial DNA to be drawn. We also examined, using electron microscopy, the area occupied by mitochondria in the python ventricle. This area increases after feeding, and mitochondria are delocalized such that they are aligned between myofibers when cardiac hypertrophy is observed. This study addresses inconsistencies in the literature about mitochondrial remodeling during physiological hypertrophy and links intracellular signaling pathways to genetic changes consistent with mitochondrial biogenesis rather than individual mitochondrial enlargement. Additionally, the data presented here provide the basis for further study of mitochondrial remodeling to validate potential targets that up regulate mitochondrial biogenesis in heart failure.

Methods

Tissue isolation. All animal experiments were performed with the approval of the University of Colorado at Boulder Institutional Animal Care and Use Committee (Protocol #1102.02). Two to three commercially raised juvenile Burmese pythons of unknown gender (Strictly Reptiles, Hollywood, FL) were used per time point in this study, totaling 22 pythons. Pythons were housed in light- and temperature-controlled conditions (12 hours light/12 hours dark and 28-32°C), and fed rats equal to approximately 25% of individual python body weights bi-monthly. Water was provided *ad libitum*. Before rapid decapitation and dissection, pythons were deeply anesthetized using isoflurane; depth of anesthesia was tested by firm tail pinch. Cardiac ventricular tissue was quickly dissected, flash frozen in liquid nitrogen, and stored at -80°C until later use.

Electron microscopy. Electron microscopy was performed as described [19]. Briefly, hearts were retrogradely perfused with 2% gluteraldehyde in 0.1 M cacodylate buffer. The ventricle was then post-fixed in 2% osmium tetroxide and 1% uranyl acetate for one hour each. Dehydration was achieved via increasing concentration of ethanol washes. Tissue was then rinsed in propylene oxide followed by a 1:1 mix of propylene oxide and Epon-Araldite epoxy resin overnight. The block was then polymerized at 60 °C for 48 hours. Sections were cut with a Diatome Ultra diamond knife and post-stained in 2% uranyl acetate followed by Reynold's lead citrate. Images with greater than 50% longitudinally cut contractile fibers were evaluated using a Philips CM100 electron microscope.

Signaling molecule antibody array. Human Phospho-Kinase Antibody Arrays (R & D Systems, Minneapolis, MN) were used to measure changes in activation of 46 different signaling molecules, according to the manufacturer's protocol. 150µg isolated protein from three Burmese python ventricles at each of three time points [fasted, 3 days post fed (dpf), and 6dpf] was incubated with individual arrays spotted in duplicate with antibodies specific for the activated states of the signaling molecules. Pan-phospho-specific antibodies conjugated to HRP were incubated with the arrays and levels of phosphorylation were detected and visualized using chemiluminescence and X-ray film. Analysis was performed using ImageJ according to instructions provided by R&D Systems. Phosphorylation levels at two postprandial time points were normalized to activity of individual signaling molecules in the fasted state. Heat map was produced using Matrix2PNG (http://www.chibi.ubc.ca/matrix2png/).

Primer design and *in silico* validation of assembled transcript. At the time of this study, the Burmese python genome was not fully annotated and transcript sequences were not available. However, a database of contiguous DNA sequences (contigs) was available as a Whole Genome Shotgun (WGS) [20]. Primer design began by obtaining cDNA transcripts of closely related species from Ensembl Genome Browser (www.ensembl.org). Transcripts from either *Gallus gallus* (chicken) or *Anolis carolinesis* (anole lizard), species evolutionarily related to Burmese python, were aligned with the WGS of *Python molurus* using Basic Local Alignment Search Tool, Nucleotide (BLASTn) (blast.ncbi.nlm.nih.gov). Contigs with highly similar nucleotide sequences were recorded and assembled into a putative python transcript using the reference species transcript as a model to preserve exon-exon boundaries and prevent repeats and frameshifts. Using ExPASy Translation Tool (www.expasy.org/translate/) and NCBI protein BLAST (BLASTp), the assembled python transcript was validated to ensure that the translated protein was complete, free of introduced stop codons, and predicted to encode the expected protein in a closely related species.

Primers were designed from the validated python transcript using Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) to span an exon-exon boundary, thus preventing amplification of genomic DNA, and produce a single product between 75 and 200 nucleotides long. Primers were validated using NCBI's primer BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) to demonstrate that the primers would anneal efficiently to the target sequence of the reference species *in silico*. (**Table 1**).

RNA isolation and determination of purity. Total RNA was isolated from two pooled and homogenized python ventricles at each time point using TRIzol reagent (Molecular Research Center, Cincinnati, OH), according to manufacturer protocol. RNA concentration and purity were measured on a Nano Drop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). A260/280 values above 1.8 indicated pure RNA.

cDNA synthesis. Complementary DNA (cDNA) was synthesized from RNA using a reverse transcriptase reaction mediated by Super Script® III Reverse Transcriptase (SSIII, Life Technologies, Grand Island, NY), according to the manufacturer's protocol. Briefly, 2µg of RNA (volume based on spectrophotometer readings) was combined with 1µl of 10mM deoxyribonucleotides (dNTPs), 1µl of 300pM random primers, and RNase-free H_2O to bring the final volume to 13µl. The samples were heated to 65°C for 5 minutes and placed on ice. A master mix was prepared, containing 4µL 5X first strand buffer (FSB), 1µL 0.1M dithiothreitol (DTT), 1µL RNase OUT™ (Life Technologies, Grand Island, NY), and 1µL SSIII. No template control (NTC) samples were also made, omitting SSIII. Master mix was added to the samples on ice, and PCR was run with a 60 minute elongation step at 55°C, and a 15 minute inactivation step at 70°C.

***In vitro* primer validation.** Primers were validated by performing conventional polymerase chain reaction (PCR) using an Applied Biosystems Thermal Cycler (Life Technologies, Grand Island, NY) (40 reaction cycles: 94°C denaturation, 60°C annealing, and 72°C elongation). The PCR products were run on a 2.2% agarose gel in tris ethylenediaminete traacetic acid (TE) for 2 hours at 40 volts with 6X Orange G loading buffer. Hypoxanthine-guanine phosphoribosyl transferase (HPRT), tubulin 2A (TUBA2) or 18S ribosomal RNA was used as a positive control. This process aimed to validate production of a single product and product size. A quantitative (qPCR) melt curve was also performed to confirm that one unique product was amplified from each primer pair.

Specificity of qPCR primers was validated *in silico* using several online databases. The assembled nucleotide sequence for each gene produced an amino acid sequence free of stop codons, and protein BLAST returned the correct protein for each gene in species closely related to the Burmese python with 98-100% alignment. The assembled sequence was used to design primer pairs for each gene. The primer pairs obtained were each successfully validated using primer BLAST by returning a product in the predicted mRNA sequence in a closely related species. *In silico*, the primers reliably produced the expected size PCR product, which was between 75-200 bps in length. *In vitro* validation of primer specificity was confirmed using cDNA synthesized from python RNA isolated from ventricular tissue through comparison of the predicted product sizes using agarose gel electrophoresis. Products were 75-200 bps in length for all genes examined, as expected. These data confirmed that primers were appropriately designed to measure the expression of each gene of interest using qPCR.

The efficiencies of the primer sets were determined by a standard curve produced using the Bio-Rad CFX96 qPCR software. Slopes were between -2.3 and -3.6, indicating efficiencies between 89.6% and 110%. qPCR melt curves produced by increasing the temperature after amplification by 2°C every 10 seconds confirmed the presence of a single PCR product with a single melting temperature for each gene. qPCR amplification curves also revealed distinct and repeatable cycle thresholds for each gene at each time point.

Quantitative PCR. qPCR was used to quantify gene expression at different time points after feeding. Expression was measured using a Bio-Rad CFX 9600 Real Time PCR System (Hercules, CA), as previously described.[21] Cycling parameters were as follows: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of denaturation and elongation (95°C for 15 seconds followed by 60°C for one minute). The melt temperature (T_m) was determined at the end of the protocol by measuring SYBR Green® (Life Technologies, Grand Island, NY) fluorescence every five seconds as temperature increased by 2°C. This technique provided a second validation of the production of a single product for each primer set. TUBA2 or 18s rRNA was used as a reference gene to normalize the expression of each gene of interest in each sample**.** Each reaction contained 4-8 ng of cDNA. All samples for qPCR were loaded in triplicate to ensure reproducibility.

qPCR data analysis. Amplicon concentration for each sample was determined using Bio-Rad CFX96 qPCR software and the standard curve method.

Values for mRNA expression at each time point were generated by normalizing the relative fluorescence units (RFUs) of each of three triplicate wells to the reference gene RFUs and adjusting for primer efficiency, a method that is integrated in the Bio-Rad CFX96 algorithm. Values were averaged for each time point and normalized to the fasted condition to determine the fold-change value. The fold-change at each time point was analyzed for statistical significance using a Student's unpaired t-test, comparing fasted to each time point; p values less than 0.05 were considered statistically significant. All qPCR plates were repeated a minimum of two times to ensure reproducibility. Statistical outliers were identified and removed using a quartile test. Error bars represent the standard error of the mean (S.E.M.). Analyses were performed and graphs were generated using GraphPad Prism software.

Results

Electron microscopy analysis of the Burmese python ventricle after feeding reveals an increase in mitochondrial area and distinct alignment between myofibers. Previous studies demonstrated increased heart size and a dramatic increase in oxidative capacity to support hypertrophy and increased contractility in the Burmese python heart after feeding [5]. However, evaluation of the mitochondria in the Burmese python ventricle has not been reported.

To examine the morphology and area of mitochondria in the python heart, we examined the ventricles of one fasted and one 3dpf python using electron microscopy. This analysis revealed distinct differences in mitochondrial localization and area occupied between the fasted and postprandial states (**Figure 1**). Images revealed myofibers that were dramatically disorganized at all time points, rather than the parallel arrangement observed in mammals [22] (**Figure 1a**). Mitochondria were clustered in the fasted state and became markedly aligned between myofibers at 3dpf (**Figure 1b**). At 3dpf, contractile tissue contained 9% more area occupied by mitochondria when compared to similar area in the fasted state ($p = 0.158$)(**Figure 1c**).

Pathways involved in growth factor signaling are increased in the Burmese python ventricle after feeding. In light of previously published data that report increased oxidative metabolism and our electron microscopy data indicated an increase in area of the ventricle occupied by mitochondria, we were interested in examining the signaling pathways they are activated or inhibited after feeding that may be responsible for this increase. To examine the role of intracellular signaling in mitochondrial remodeling after feeding in the Burmese python ventricle, we measured phosphorylation of a panel of 46 signaling molecules important for cardiac function and metabolic remodeling in protein lysates isolated from three pooled ventricles from fasted, 3 dpf, and 6dpf snakes. 3dpf represents the time point when the greatest increase in metabolism and cardiac growth is observed, while at 6dpf, the heart is returning to its resting size [5]. Interestingly, most signaling molecules in both the 3dpf and 6dpf ventricles remained unchanged (+/- 5% of fasted levels) (**Supplemental Table 1**). Among those signaling molecules with notable increases in activation at 3dpf were AMP-activated protein kinase α2 (AMPKa2) (1.26-fold), extracellular-signaling regulated kinase 1/2 (Erk1/2) (1.43-fold), c-Jun N-terminal kinase (JNK) (1.24-fold), p38α (1.26-fold), and target of rapamycin (TOR) (1.23-fold) (**Figure 2**), each of which is required for growth factor signaling and mitochondrial biogenesis and several of which were previously reported as increased using Western blots, which validates this assay [23,5]. The molecules with the greatest decrease in activation at 3dpf were fibroblast growth factor (FGF) (-1.45-fold), heat shock protein 27 (Hsp27) (-1.29-fold), and p70 S6 kinase T389 (-1.42-fold) (**Figure 2)**.

Expression of genes involved in mitochondrial biogenesis in Burmese python after feeding. In light of activation of signaling molecules that mediate growth factor signaling in the heart and mitochondrial biogenesis [23], expression of genes involved in biogenesis, function, and termination of biogenesis was measured at 1, 3, and 10dpf. We hypothesized that the increase in oxidative capacity previously reported is due to mitochondrial biogenesis rather than enlargement of existing mitochondria. Therefore, we chose genes that represent several components of the process of biogenesis. The 1dpf time point was added to capture changes that precede maximum cardiac hypertrophy. Levels of nuclear-encoded transcription factor mRNAs varied postprandially with two trends: either an initial increase or decrease in expression followed by return to baseline expression levels (**Figure 3**).

Expression of estrogen-related receptor α (ESRRα) peaked at 1dpf with a 2.89-fold increase compared to fasted levels and began returning to baseline by 3dpf. Expression of nuclear factor erythroid 2-related factor (NRF2) also increased to 2.08-fold at 1dpf, then decreased to below fasted levels (0.73-fold and 0.58-fold at 3 and 10dpf, respectively). In contrast to ESRRα and NRF2, myocyte-specific enhancer factor 2A (MEF2A) and peroxisome proliferator-activated receptor γ co activator 1 α (PCG1α) decreased at 1dpf.

MEF2A expression decreased to 0.24 relative to fasted then increased to 1.65-fold at 3dpf, while expression of PGC1α decreased to 0.67 at 1dpf, decreased further to 0.29 at 3dpf, and then increased expression at 10dpf, returning to baseline. (**Figure 3a**).

Expression of nuclear-encoded mitochondrial transcription factors that are transported into mitochondria and activate mitochondrial genes all increased dramatically after feeding. Mitochondrial transcription termination factor D2 (MTERFD2) mRNA increased at 3dpf to 37.5-fold and remained elevated at 10dpf. Transcription factor B1, mitochondrial, (TFB1M) mRNA also increased 6.8-fold at 1dpf and 8.76-fold at 3dpf. Similarly, transcription factor B2, mitochondrial (TFB2M) mRNA peaked at 4.33-fold at 1dpf, while at subsequent postprandial days, expression decreased. DNA-directed RNA polymerase, mitochondrial, (POLRMT) mRNA peaked at 1dpf with a 45.5-fold increase, then progressively decreased over time (**Figure 3b**).

Expression of structural mitochondrial components revealed increases that peaked at 1dpf and returned to baseline by 10dpf (**Figure 4a**). Expression of translocase of outer mitochondrial membrane 22 (TOMM22) and voltage-dependent anion channel 1 (VDAC1) peaked at 1dpf with 1.7-fold and 2.3-fold expression relative to fasted, respectively.

Functional mitochondrial components (**Figure 4b**) displayed varying trends in expression, with citrate synthase (CS), mitofusin 1 (MFN1), and mitochondrial fission 1 protein (FIS1) all increasing in expression at 1dpf. Similar to many of the other genes in this study, CS expression peaked at 1dpf, with a 27-fold increase then decreased toward baseline by 10dpf. MFN1 mRNA expression increased at 1dpf, and maintained elevated expression at 3dpf and 10dpf. FIS1 expression increased at 1dpf to 3.4-fold increase was reduced to 1.7-fold at 3dpf, and returned to 3.9-fold at 10dpf. By contrast, NAD-dependent deacetylase 3 (SIRT3) and presenilin-associated, rhomboid-like (PARL) expression decreased at 1dpf to 0.2-fold and 0.4-fold, respectively.

Discussion

The postprandial metabolic rate of the Burmese python increases a remarkable 44-fold compared to fasting [17]. The mechanisms mediating the rapid production of ATP required for increased cardiac output have not been fully described and in fact, are contradictory in the literature [12,9,11,10]. Increased oxidative capacity was previously observed in the ventricle of the Burmese python, as evidence by a lack of lipid accumulation, increased cytochrome oxidase 2 expression, and increased expression of superoxide dismutase 2, which mitigates the effects of reactive oxygen species production by mitochondria [5]. This dramatic increase in cardiac metabolism likely exceeds the capacity of existing mitochondria and requires an increase in either mitochondrial size or number.

We used electron microscopy to examine mitochondrial morphology and number in the python ventricle of the fasted and postprandial state. Disorganization of the myofibers in the ventricle precluded evaluation of membrane morphology or counting individual mitochondria, however, we did observe an increase in the area occupied by mitochondria in the contractile tissue. Additionally, localization of the mitochondria changed at 3dpf such that alignment of mitochondria between myofibers was observed, possibly to promote efficient delivery of ATP to the contractile apparatus of the sarcomeres. This would be particularly important in the postprandial state when heart rate increases 3-4-fold [17]. Signaling molecules that respond to growth factor signaling in the heart exhibited increased phosphorylation at 3dpf. Growth factors are released in cardiac tissue in response to physiological hypertrophic stimuli and bind to receptor tyrosine kinases [23]. This binding promotes the activity of mTOR, glycogen synthase kinase 3β (GSK3β), ERK, JNK, and p38α. This signaling pattern is typically examined in the context of expression of genes that regulate or are involved in production of contractile force in the heart but, importantly, these molecules also regulate mitochondrial biogenesis in many cell types [24,23]. Because activation of signaling molecules consistent with both cardiac hypertrophy and mitochondrial biogenesis was observed, we were interested in examining the specific effects of these pathways on expression of genes that regulate or are involved in mitochondrial biogenesis.

In human hearts, when AMP accumulates from high levels of metabolic activity, AMPK is activated [25]. Indeed, we and others report an increase in AMPK signaling in the postprandial python ventricle [5]. Interestingly, we also observed decreased p70 S6 kinase phosphorylation, which further increases AMPK signaling in other tissues including muscle by increasing AMP:ATP ratios [26]. AMPK phosphorylates PGC1α, which promotes expression of genes that regulate mitochondrial biogenesis such as ESRRα.

Co activation of ESRRα causes Err to auto dimerism and initiate transcription of genes that regulate processes ranging from energy homeostasis to ATP generation [27-29]. Increases in both ESRRα and NRF2 expression at 1dpf and 3dpf are consistent with increased energy usage of the ventricle and suggest that the heart is responding in a physiological manner to increased energy demand. Unexpectedly, expression of PGC1α was inversely proportional to heart growth, with its lowest expression at peak hypertrophy. Although PGC1α is a known activator of ESRRα and NRFs [30-33], it is possible that the true peak expression of PGC1α was not captured during the time points used in this study.

Peak expression of PGC1α may have occurred within hours of feeding [34]. An early increase could have been followed by a decrease to negatively regulate mitochondrial biogenesis; indeed, mitochondrial mutations induced by PGC1α constitutive activity lead to cardiomyopathy [30]. Additionally, previous studies and our antibody array data revealed increased activation of AMPK that activates PGC1α beginning at 1dpf [5]. Therefore, we hypothesize that a rapid increase in PGC1α activity is required for activation of ESRRα and other genes responsible for mitochondrial remodeling (**Figure 4**).

Transcription factor MEF2A regulates genes that specify cardiac muscle cell differentiation, manage the buildup of ROS, and are crucial to mitochondrial localization [35,36]. The early 4-fold decrease in expression at 1dpf was unexpected, although MEF2A activity may be regulated by histone deacetylases at the onset of hypertrophic stimuli [37]. The 2-fold increase in expression of MEF2A at 3dpf corresponds with an observed 1.26-fold increase in p38α activity. p38α is part of the MAP kinase signaling pathway, which is known to phosphorylate MEF2A to promote activation during cardiac muscle growth and differentiation [38,36]. Additionally, EM imaging shows mitochondrial alignment at this time, indicative of activated mitochondrial localization genes that lie downstream of MEF2A. These data support the conclusion that growth factor signaling may cause activated MAP kinase signaling cascades and eventually downstream gene expression through post-translational modifications. NRF2 mediates repair of the damaging effects of ROS and regulates metabolic responses by changing mitochondrial bioenergetics [39]. The 2.08-fold increase in expression of NRF2 would cause up regulation of genes involved in mitochondrial gene transcription such as mitochondrial transcription factor A (TFAM) and TFB2M [40,41] to maintain the stoichiometry of structural and functional elements of existing and/or new mitochondria in the energetically demanding ventricle. These factors, along with POLRMT and TFB1M, are responsible for initiating transcription of mammalian mitochondrial DNA [42]. Indeed, expression of POLRMT, TFB1M, and TFB2M increased at 1dpf. The observed expression patterns of each of these mitochondrial transcription factors should result in increased mitochondrial DNA transcription and thus increased concentration of the 13 necessary subunits of the respiratory pathway associated with the inner mitochondrial membrane [43].

Structural and functional proteins associated with the maintenance of the mitochondria would also require upregulation. Indeed, increased expression of CS, essential for the first step of the citric acid cycle [44], TOMM22, an organizer of the TOM complex [45], and VDAC1, an important aquaporin [46], was observed. In fact, increased CS expression has been observed with mitochondrial biogenesis following exercise training in mammals [47] and is considered predictive of mitochondrial number [12]. The up regulation of FIS1 further supports increased mitochondrial biogenesis because its expression has been shown to correlate with the number of mitochondria in human cells [48]. Its role in recruiting mitochondrial fission proteins to the outer membrane of the mitochondria [49] and its measured increase in expression signifies that the mitochondria should be increasing in number if all required proteins of the fission complex are functioning within the cell.

Unexpectedly, MTERFD2, an important regulator of mitochondrial ribosomal synthesis [50], continued to increase through all time points. There are two potential interpretations for this pattern of gene expression. The first is that MTERFD2 expression is relatively low in the fasted python heart and must increase dramatically in order to meet the increased demands of energy production during cardiac hypertrophy. The sustained increase in expression of this gene may represent the role of MTERFD2 in the translation of regulatory components important to mitochondrial function or number. The second involves the largely unexplored function of MTERFD2 as a mitochondrial transcription terminator. MTERFD2 is structurally similar to other MTERF isoforms; it contains the same nucleic acid-binding protein domain [51]. Thus, it is possible that MTERFD2 begins to regulate the transcription of mitochondrial genes involved in biogenesis at 3dpf as regression begins and continues to down-regulate these genes at 10dpf.

Although outside the scope of this study, our results from the late postprandial time point (10dpf) raise interesting questions about the mechanism by which the function or number of mitochondria may be reduced to fasted levels postprandial. The expression patterns of FIS1 and its counterpart MFN1, which is responsible for fusing mitochondria, are not consistent with the hypothesis of a fusion-fission mechanism for mitochondrial duplication and reduction during hypertrophy. FIS1 mRNA levels were significantly increased at 10dpf compared to fasted, and were the highest of all time points measured contrary to the hypothesized return to fasted conditions. In contrast expression of FIS1, MFN1 expression failed to increase significantly and remained relatively stable throughout all time points examined. The increase in FIS1 and unchanged MFN1 expression indicates that fusion, which is expected to be necessary in consolidating mitochondria, may not be the mechanism for reducing active mitochondria during cardiac regression.

One hypothesis is that mitochondrial damage occurs during the highly metabolic digestion period, and the damaged areas are selectively removed through mitophagy [52]. The second hypothesis is that MFN2, a homologue of MFN1, could be utilized simultaneously to decrease mitochondrial numbers via fusion [53]. Both of these mechanisms could explain the lack of expression of MFN1 and thus the lack of fusion during cardiac regression [5], although the selective elimination of damaged mitochondria would prove especially useful to cardiomyocytes. This pathway requires further examination and could be important in further characterizing cardiac metabolism during hypertrophy in the python. As with all qPCR data, the amount of mRNA may not directly correlate with the amount of protein or its activity within the cell without further testing.

Therefore, expanding the study to quantify the protein products of each gene would be beneficial to validating the results, but would require reagents not currently available for the Burmese python such as antibodies for use in western blotting. Further examination of the number of mitochondria in the python ventricle over course of the hypertrophy could also yield significant additional findings. This may prove difficult, however, as cardiomyocytes in the python heart are extremely disorganized, unlike the mammalian heart. Despite a need to understand and address mitochondrial dysfunction that occurs with CVD, there exist no approved drugs to increase energy production in the failing heart [14]. One approach to treating this issue is to improve energy production, however, the mechanisms by which energy production is sustained in physiological cardiac hypertrophy are not well understood due to conflicting reports and incomplete studies in various mammalian models [12,9,11,10]. This study is the first to our knowledge to comprehensively examine the patterns of intracellular signaling and gene expression regulating mitochondrial function in the Burmese python during physiological cardiac hypertrophy. Our data provide a greater understanding of the significance of mitochondrial biogenesis in physiologic hypertrophy, which could lead to the development of novel therapeutics capable of reverting pathological hypertrophy to physiological hypertrophy and potentially reducing the progression of CVD.

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Figure Legends

Table 1 List of primers designed using the Burmese python whole genome shotgun and expected qPCR product sizes.

Figure 1 Electron microscopy of fasted and 3 days post-fed (3dpf) Burmese python ventricles. Representative images of fasted (**a**) and 3dpf (**b**) ventricles. Bar indicates 500 nm. **c**. Quantification of mitochondrial area in fasted (white bars) and 3dpf (gray bars) ventricles. M, mitochondria. Error bars indicate standard error from the mean (SEM); n = 1 ventricle per time point.

Figure 2 Activation of signaling molecules in the Burmese python ventricle. Heat map describing activation states of 46 signaling molecules involved in cardiac function at 3 days and 6 days after feeding (3dpf and 6dpf, respectively), relative to fasted. Amount of phosphorylation reported is based on average pixel intensity of duplicate antibody spots for each molecule. Green indicates decreased and red indicates increased phosphorylation relative to fasted. n = 3 pooled ventricles per time point.

Figure 3 Expression of genes encoded in the nucleus that regulate transcription of nuclear genes (**a**) and mitochondrial genes (**b**). Expression was measured 28 days after feeding (fasted), 1 day after feeding (1dpf), 3 days after feeding (3dpf), and 10 days after feeding (10dpf). Values are reported relative to expression in the fasted state. Error bars represent standard error of the mean (SEM); n = 2 pooled python ventricles and three technical replicates (reference and gene of interest) per time point.

Figure 4 Expression of genes encoded in the nucleus that contribute to the structure (**a**) and function (**b**) of mitochondria. Expression was measured 28 days after feeding (fasted), 1 day after feeding (1dpf), 3 days after feeding (3dpf), and 10 days after feeding (10dpf). Values are reported relative to expression in the fasted state. Error bars represent standard error of the mean (SEM); n = 2 pooled python ventricles and three technical replicates per gene (reference and gene of interest) per time point.

Figure 5 Proposed pathway stimulated by feeding in the Burmese python. Feeding provides the stimulus for increased energy demand. Increased intracellular AMP stimulates transcription of nuclear transcription factors that promote transcription of both structural mitochondrial protein and other transcription factors that act on mitochondrial DNA. Transcription of mRNAs responsible for mitochondrial biogenesis is increased, as are transcripts that are translated into components of the machinery responsible for mitochondrial fission. As the stimulus decreases, the active mitochondrial pool is decreased by reduced transcription of the mRNAs that contribute to mitochondrial biogenesis and fusion is promoted to decrease the number of mitochondria. Image adapted from [54].