



Variant expression signatures of microRNAs and protein related to growth in a crossbreed between two strains of Nile tilapia (*Oreochromis niloticus*)

Marcos Edgar Herkenhoff^{a,*}, Luiz A. Bovolenta^b, Oliver Broedel^c, Lucilene D. dos Santos^d, Arthur C. de Oliveira^a, Luiz G.A. Chuffa^e, Amanda de O. Ribeiro^a, Luiz A. Lupi Jr^e, Marco A. D. Dias^{f,g}, Alexandre W.S. Hilsdorf^{f,g}, Marcus Frohme^c, Danilo Pinhal^{a,*}

^a Department of Chemical and Biological Sciences, Institute of Biosciences of Botucatu, São Paulo State University (UNESP), Botucatu, SP, Brazil

^b Department of Biophysics and Pharmacology, Institute of Biosciences of Botucatu, São Paulo State University (UNESP), Botucatu, SP, Brazil

^c Division Molecular Biotechnology and Functional Genomics, Technical University of Applied Sciences Wildau, Wildau, Germany

^d Center for the Study of Venoms and Venomous Animals (CEVAP), São Paulo State University (UNESP), Botucatu, SP, Brazil

^e Department of Structural and Functional Biology, Institute of Biosciences of Botucatu, São Paulo State University (UNESP), Botucatu, SP, Brazil

^f Unit of Biotechnology, University of Mogi das Cruzes, Mogi das Cruzes, SP, Brazil

^g Department of Animal Sciences, Federal University of Lavras, Lavras, MG, Brazil

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ABSTRACT

Nile tilapia (*Oreochromis niloticus*) is a species of worldwide importance for aquaculture. A crossbred lineage was developed through introgressive backcross breeding techniques and combines the high growth performance of the Chitralada (CHIT) lwith attractive reddish color of the Red Stirling (REDS) strains. Since the crossbreed has an unknown genetically improved background, the objective of this work was to characterize expression signatures that portray the advantageous phenotype of the crossbreeds. We characterized the microRNA transcriptome by high throughput sequencing (RNA-seq) and the proteome through mass spectrometry (ESI-Q-TOF-MS) and applied bioinformatics for the comparative analysis of such molecular data on the three strains. Crossbreed expressed a distinct set of miRNAs and proteins compared to the parents. They comprised several microRNAs regulate traits of economic interest. Proteomic profiles revealed differences between parental and crossbreed in expression of proteins associated with glycolysis. Distinctive miRNA and protein signatures contribute to the phenotype of crossbreed.

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is currently a major aquaculture species worldwide. This is evidenced by the FAO [31] data, which rank Nile tilapia as the third aquaculture freshwater finfish in production behind Grass carp, *Ctenopharyngodon idellus*, and Silver carp, *Hypophthalmichthys molitrix*. Therefore, Nile tilapia is regarded as one of the most important tropical and subtropical species as a source of animal protein and income in many countries [103].

Genetic enhancement programs such as GIFT [28], GIANT-G9 [53], and those to developed red tilapia strains [123] have been implemented since the 1980s to boost tilapia productivity globally. Crossbreeding

between Chitralada (CHIT) and Red Stirling (REDS) strains led to the production of animals with high heterosis [87]. REDS strain has slower growth than CHIT; however, its reddish appearance is attractive to the consumer [69]. Currently, a crossbreed (CB) between these strains (e.g., genotype 7/8 Chitralada: 1/8 Red Stirling) has been produced by successive crosses and has shown high-quality heterosis [68]. However, the molecular basis of such economically valuable phenotype remains largely elusive.

In this sense, the sharp control of protein production by non-coding RNAs, notably microRNAs (miRNAs), has been regarded as a major mechanism of metazoan genome to regulate gene expression. MiRNAs are a large class of non-coding RNA of roughly 22 nucleotides highly

* Corresponding author at: Department of Chemical and Biological Sciences, Institute of Biosciences of Botucatu, São Paulo State University (UNESP), Botucatu, SP, Brazil.

E-mail addresses: herkenhoff@usp.br (M.E. Herkenhoff), oliver.broedel@th-wildau.de (O. Broedel), arthur.c.oliveira@unesp.br (A.C. de Oliveira), luiz-gustavo.chuffa@unesp.br (L.G.A. Chuffa), lupijr@ibb.unesp.br (L.A. Lupi), wagner@umc.br (A.W.S. Hilsdorf), mfrohme@th-wildau.de (M. Frohme), danillo.pinhall@unesp.br (D. Pinhal).

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abundant in the genome of animals and plants [7,62,71,140]. MiRNAs as managers of gene regulation and protein output have soon become targets in animal breeding research. So far, several studies have been carried out in bovines ([23]; Gu et al., 2007; [118]), pigs [111,136], poultries [24,25,39,48,49], and in Nile tilapia [45,52,88,142–146] seeking to identify miRNAs potentially linked to traits of relevance for aquaculture productivity improvement.

An immediate consequence of the pervasive and activity of miRNAs at the post-transcriptional level is the differential modulation of protein expression in distinct cells, organs and organisms. As the final product of the mRNA processing, proteins contribute qualitative and quantitatively to determine multiple phenotypic traits in various body tissues. The study of whole proteins expressed in a given tissue (proteome) has enormous potential for animal production. Proteomics has been carried out using biological models of economic interest, such as cattle, to assess proteome alterations in semitendinosus muscles during postmortem storage [148]. One of the most critical studies has shown that feed restriction in cattle leads to dissimilar production indices and changes the protein composition of meat [1]. Proteomics in fishes has been used to evaluate resistance to bacterial infection in Nile tilapia [17,73], cryopreservation of rainbow trout semen (*O. mykiss*) [90], quality and texture of salmon meat (*S. salar*) [94], and hepatic toxicology too in salmon (*S. salar*) [44].

The combination of different parental miRNA sequences may cause heterosis in animals [120], which implies better performance (more vigor or greater production) of the offspring relative to the parents. Alterations in miRNA expression may promote either greater or lower expression of targets or lead to the acquaintance of novel targets thereby largely influencing the protein expression profiles output.

Against this background, we characterized the miRNA transcriptome and the proteome of a Nile tilapia crossbred and its parental strains to get insights into the emergence of superior phenotype traits linked to heterosis. By using RNA-seq, bioinformatics, and mass spectrometry, we

identified distinctive miRNAs and protein signatures among strains and point out targets that can be modulated to aquaculture improvement.

2. Results

2.1. miRNA expression profiles overview

Six miRNA libraries were sequenced from animals aged at 5th and 6th months from the three strains investigated (i.e, CHIT, REDS and CB), corresponding to the period of marked growth of these animals [46]. Libraries from 5th and 6th months generated 2,084,755 and 2,568,552 reads for CHIT, respectively; 5th and 6th of REDS had 2,036,241 and 3,182,923 reads, and 5th and 6th of CB had 1.949.529 and 3.453.399 reads.

Regarding the expression of miRNAs, some of them should be highlighted. There was no significant difference in miRNAs expression between CHIT and CB. Both miR-192-5p and miR-122-5p were lower expressed in CHIT and CB in comparison to REDS in the 5th month (Fig. 1). Conversely, miR-144-5p and miR-144-3p were not differentially expressed in CB, but were higher expressed in CHIT comparing to REDS in 5th month. Equally, miR-9b-1-3p was also not differentially expressed in CB, whereas it was lower expressed in CHIT in relation to REDS in 6th month.

There were also miRNAs that showed differences in expression between the 5th and 6th month. The expression of miR-192-5p, miR-122-5p, 196a-5p and miR-10d-5p was lower at 5th month, showing an increase at 6th month in the three studied groups (Fig. 1). In the cross-breed miR-204-5p, miR-204a-5p and miR-153a-1-3p were higher expressed in the 5th month compared to 6th month. MiR-196a-2-5p, miR-429-3p and miR216b-5p were lower expressed in the 5th month compared to 6th month in CHIT, while miR-144-5p and miR-2188-5p were higher expressed in this same comparison. MiR-196-5p, miR7-5p, miR-7a-5p and miR-9b-3p were down-regulated in the 5th month

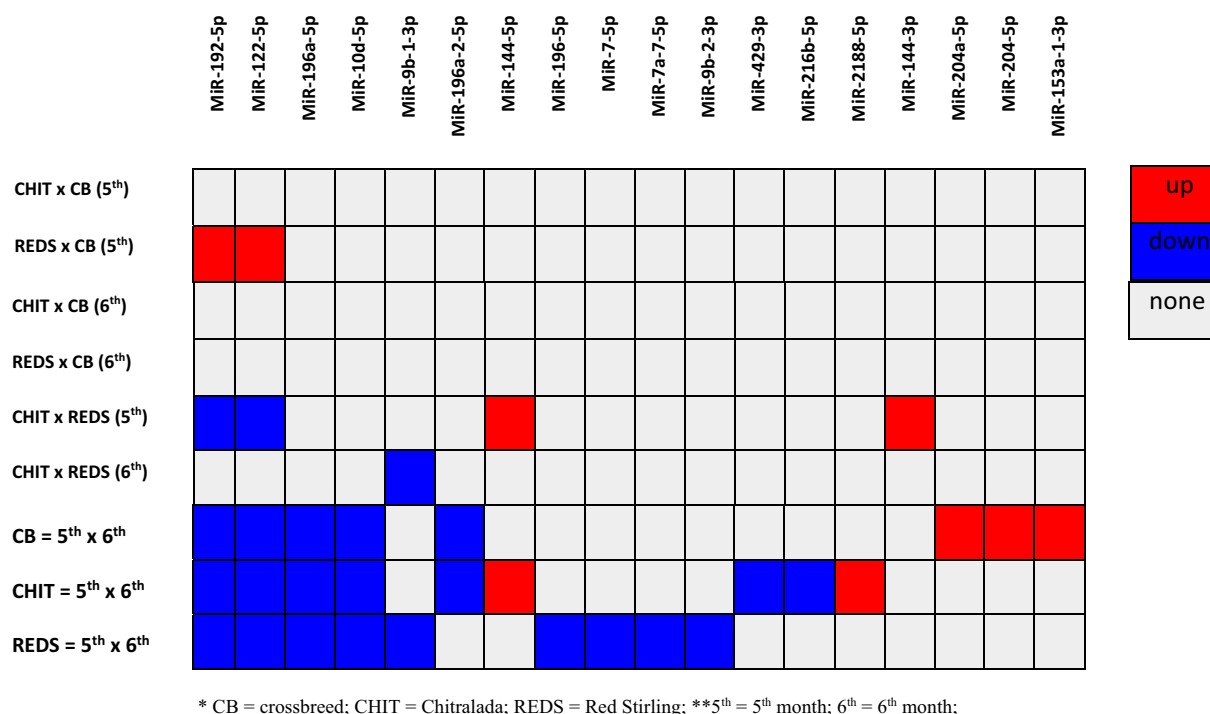


Fig. 1. Relationship of miRNAs with differential expression. The miRNAs were compared between the strains and their crossbreed, as well as between the 5th and the 6th month in the same lineage. The blue color indicates that it is down-regulated, the red color indicates that it is up-regulated when compared, and the gray color indicates that there is no difference in expression ($p < 0.05$). The second strain is compared with the first, for example, the first line [CHIT x CB (5th)] indicated the expression of CB compared to CHIT. (COLOR SHOULD NOT BE USED IN PRINT VERSION). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compared to 6th month in REDS.

2.2. Proteome analysis

Shotgun proteomics have evidenced 64 differently expressed proteins or unique proteins comparing CHIT with REDS, 64 proteins comparing CHIT with crossbreed, and 40 proteins comparing REDS with the crossbreed. After functional enrichment, the performance of CHIT-exclusive proteins compared to REDS was described to be involved in oxidoreductase activity and nucleoside monophosphate and nucleoside triphosphate-related metabolic processes (Table S2). Comparing REDS with CHIT, two REDS exclusive proteins appeared to be related with nucleoside monophosphate and metabolic processes; otherwise, when compared to crossbreed, a number of important proteins were mostly associated with catalytic reactions. Comparing CHIT with the crossbreed, CHIT-exclusive proteins were mainly involved in the catalytic

activity of the muscle fibers.

Using the STRING software, we constructed three functional protein-binding networks (Fig. 2A-C). The first network shows the interactions of proteins that were up-regulated or exclusively found in CHIT compared to crossbreed (Fig. 2A). The second network shows interactions of proteins that were up-regulated or unique to CHIT compared to REDS (Fig. 2B). And the third shows up-regulated or exclusive interactions of REDS proteins compared to crossbreed (Fig. 2C). Among the networks, the proteins up-regulated in CHIT compared to REDS (Fig. 2B), and REDS in relation to crossbreed, showed a larger network (Fig. 2C).

2.3. miRNA and protein interaction

Proteins and their putative regulatory miRNAs (Table S1) were found to be largely involved in glycolysis. Several of the glycolysis proteins are

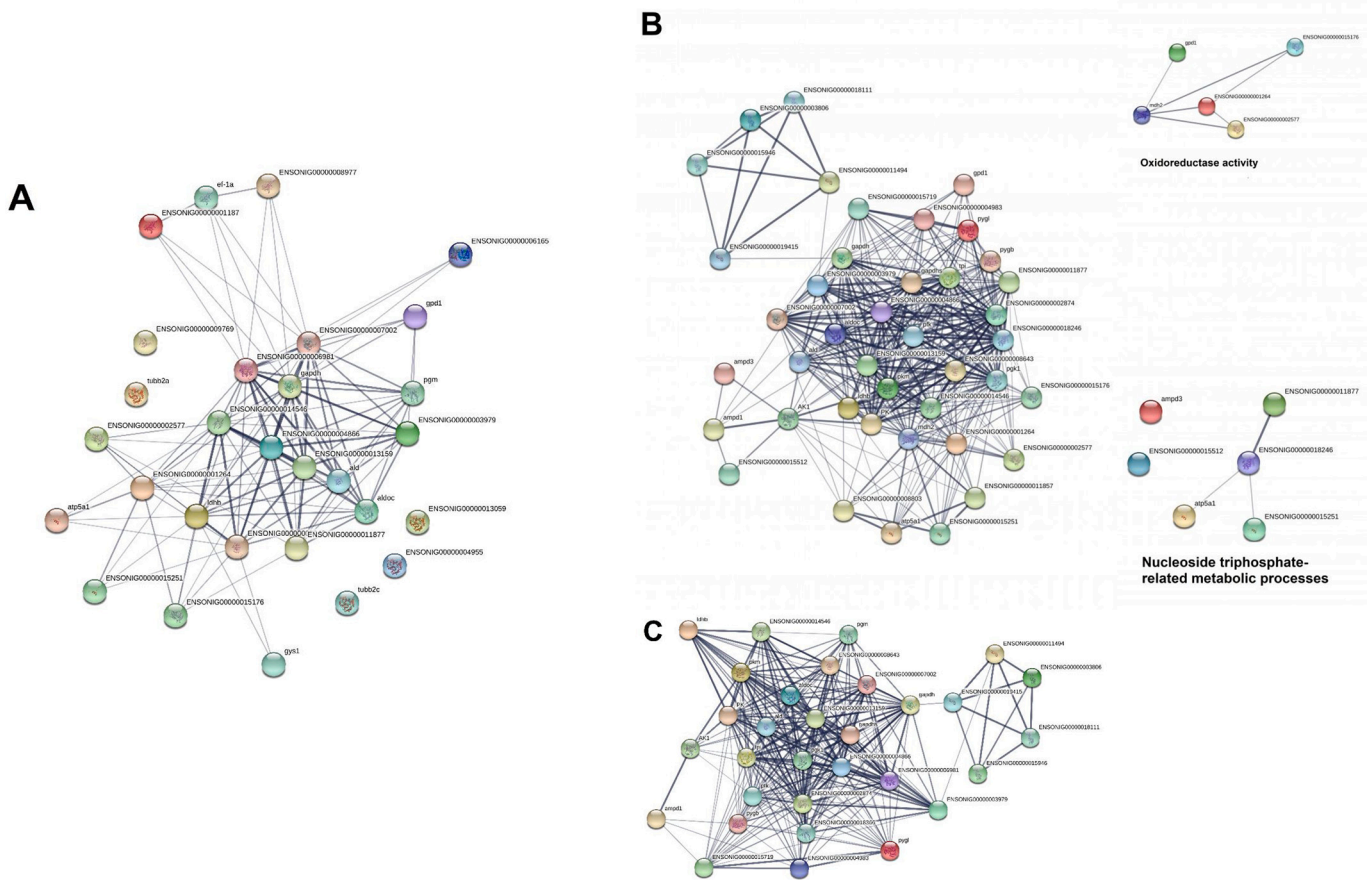


Fig. 2. Functional protein association networks. Protein-protein interaction are based on different cellular and molecular processes. The dark features represent high interaction, while the lighter features represent weaker interactions. AK1: Adenylate kinase isoenzyme 1; ALD: Aldolase A; ALDOC: Aldolase C; AMPD: AMP deaminase; ATP5A1: ATP synthase subunit alpha; EF-1a: Elongation factor 1-alpha; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GAPDHS: *Oreochromis niloticus* glyceraldehyde 3-phosphate dehydrogenase; GPD1: Glycerol-3-phosphate dehydrogenase 1b; GYS1: Glycogen synthase; LDHB: Lactate dehydrogenase Ba; MDH2: Malate dehydrogenase 2; PFK: ATP-dependent 6-phosphofructokinase; PGK1: Phosphoglycerate kinase 1; PGM: Phosphoglycerate mutase 1b; PK: Pyruvate kinase; PKM: Pyruvate kinase; PYGB: Alpha-1,4 glucan phosphorylase; PYGL: Alpha-1,4 glucan phosphorylase; TPI: Triosephosphate isomerase 1b; TUBB: Tubulin beta chain. Other proteins are represented not by symbols, but by registration number, such as: Aldolase b (ENSONIG00000004866); Aldolase c (ENSONIG000000013159); Alpha-1,4 glucan phosphorylase (ENSONIG00000004983, ENSONIG000000015251, ENSONIG000000015719); AMP deaminase (ENSONIG000000015512); ATP-dependent 6-phosphofructokinase (ENSONIG000000011877); ATP synthase subunit beta (ENSONIG000000008803, ENSONIG000000011857); Calcium-transporting ATPase (ENSONIG000000003806, ENSONIG000000011494, ENSONIG000000015946, ENSONIG000000018111, ENSONIG000000019415); Elongation factor 1-alpha (ENSONIP00000001501, ENSONIG00000006165, ENSONIG00000008977); Glucose-6-phosphate isomerase (ENSONIG000000018246); Glucose phosphate isomerase b (ENSONIG00000002874); Glyceraldehyde-3-phosphate dehydrogenase (ENSONIG00000007002); Histone-lysine N-methyltransferase (ENSONIG00000009769); Isocitrate dehydrogenase 2 (NADP+) (ENSONIG00000002577, ENSONIG000000015176); Lactate dehydrogenase A4 (ENSONIG000000014546); Lactate dehydrogenase Bb (ENSONIG00000008643); (Malate dehydrogenase 1 (ENSONIG00000001264); Phosphoglycerate mutase 1a (ENSONIG000000018366); Phosphoglycerate mutase 2 (ENSONIG00000003979); Triosephosphate isomerase 1a (ENSONIG00000006981); and Tubulin beta chain (ENSONIG00000004955, ENSONIG000000013059). A) Associated proteins of CHIT x crossbreed groups; B) Associated proteins of CHIT x REDS groups; C) Associated proteins of REDS x crossbreed groups. (COLOR SHOULD NOT BE USED IN PRINT VERSION).

known to be active in other cellular processes, such as gluconeogenesis and fatty acid synthesis. However, these processes cannot be operating simultaneously [36,119].

3. Discussion

Orchestration of the protein repertoire production by miRNAs is a central mechanism of metazoan cells. In fish, numerous miRNAs have been continuously identified, including the miRNAs discovery in Nile tilapia. Studies focused on fish miRNAs have provided sufficient evidence for linking miRNA activity to economic interest aspects, as observed for muscle development and hypertrophy control [45].

In the current study, we found that miRNAs were higher expressed in the 6th month to all strains studied. This was reflected by a greater activity in number and diversity of miRNAs expressed in this period (Fig. 1), which matches to the month of greatest growth of Nile tilapia [46,93].

In the context of heterosis, we found a distinctive set of miRNAs and proteins expressed in the crossbreed compared to their parents. The comparison among the studied strains revealed a significant relationship between the expression profiles of miRNAs and their putatively regulated proteins. We observed four situations: (i) sub-expression of miRNAs accompanied by the super-expression of its predicted targets, (ii) super-expression of miRNAs followed by the sub-expression of its targets, (iii) super-expression of miRNAs followed by the super-expression of its targets, and (iv) super-expression of predicted targets with no changes on corresponding miRNAs. The first two cases stand for the usual inversely correlated profiles of the miRNAs with its target genes, mainly obtained by the canonical regulatory mechanism. Such mechanism occurs through the interaction of the miRNA-RISC complex with binding sites of the target mRNA, thus inhibiting its expression (Lee and Dutta, 2009) and protein output by either translation repression or RNA degradation.

However, in addition to these standard reverse correlations, other expression patterns between miRNAs and their targets are also conceivable, such as the simultaneous down or up regulation of both miRNAs and its targets, as verified in our dataset. One possible explanation for the first scenario may be a decrease on mRNA transcription levels, which would lead to an attenuation of its miRNA regulator production, while in the second instance an increase on mRNA production may be followed by an increase on miRNA expression in an attempt to maintain cell homeostasis [128]. Additionally, miRNAs were also reported to be able to promote direct mRNA up regulation in specific circumstances ([126,127]). Such non-canonical miRNA roles, like the interplay with transcription factors ([156]; [157]), leading to the indirect regulation of hundreds of genes – may promote a myriad of distinct modulatory effects on gene and protein expression. Definitely, indirect non-canonical regulation is one of the multiple facets that make up the miRNA modus operandi ([158]).

3.1. miRNAs with economic potential are differentially expressed in the crossbreed

Exhibiting noticeable differential expression in Nile tilapia crossbreed and simultaneously in just one of the parental strains, we bellow call attention to miRNAs already associated with traits of economic interest in fish and other species.

We found miR-192 was low expressed in the white muscle of CHIT and crossbreed, lineages of higher body growth than REDS. Crossbreed was expected to have some growth characteristics that make them closer to CHIT than REDS, since CHIT and crossbreed are the fastest growing genetic groups and crossbreed has more CHIT in its genetic material than REDS. By contrast, in Atlantic cod miR-192 was higher expressed in larvae exhibiting better growth and developmental performance after fed with natural zooplankton as a start-feed [10]. In blunt snout bream (*Megalobrama amblycephala*), miR-192 was associated with normal

metabolism, with no interference from the diet [150] and as a regulator of genes of starch-associated (glucose) metabolism in the liver [85]. Some changes, mainly associated with the hypomethylation of DNA in the liver and muscle, stimulated with initial doses of glucose, have an effect on positive metabolic programming plus trade in life, improving the performance of these animals [64] and early feeding programs with a high carbohydrate content proved to be effective for a nutritional program in Nile tilapia with improved growth performance, linked to a better ability to sweat glucose, induce lipogenesis and suppress amino acid catabolism [65]. The liver plays an important role in the growth of fish, as it is the center of intermediate carbohydrate metabolism [100], with muscle being the largest part of the weight of the fish and is considered a stronger user of glucose as an energy source than other tissues [86,137].

Using NGS, miR-192 was associated with glucose metabolism and identified in the liver of blunt Snout Bream (*M. amblycephala*) as a regulator of genes of starch-associated metabolism [85]. Nutritional regulation of glucose metabolism in Nile tilapia revealed that intake of high dietary carbohydrates (up to 40%) was associated with better growth performance and down-regulation of gluconeogenesis and an increase in glycogen and lipid deposits in the liver and muscle [11], compared with carnivorous fish species [60].

The present study aimed to investigate nutritional programming of carbohydrate metabolism in Nile tilapia. Early nutritional intervention stimulus was achieved by feeding fry with high-protein/low-carbohydrate (HP/LC) or low-protein/high-carbohydrate (LP/HC) diet since first feeding for 4 weeks, and the effect of nutritional stimulus on carbohydrate and its related metabolism was evaluated through the adult stage.

MiR-122, a highly expressed miRNA in liver, has been proved to regulate cholesterol and free fatty acid [30,63] and with liver metabolism in Rainbow trout (*O. mykiss*) [83]. Overexpression or inhibition of miR-122 leads to changes of cholesterol and fatty acid synthesis [151]. In skeletal muscle, Sun et al. [121] showed that miR-122 expression declined in skeletal muscle fibrosis, which was a consequence of transforming growth factor- β and then overexpression retarded fibrotic process. Muscular fibrosis is a physiological process that impairs muscle regeneration, thus, the high expression miR-122 can play a fundamental role in myogenesis.

MiR-144-5p and miR-144-3p were up-regulated in the 5th month of CHIT compared to REDS. MiR-144 is a regulator of the growth factor receptor-bound protein 2 in Atlantic salmon (*S. salar*) [6]. MiR-144-3p was highly up-regulated in type 2 diabetes (T2D) and could impair insulin signaling [59] and this phenotype of insulin resistance, which is strongly associated with T2D, adipocyte differentiation and obesity [35,57]. MiR-144-3p high expression is positive related with adipocyte volume in both lean and obese pigs [72]. Shen et al. [112] observed that miR-144-3p could promote adipogenesis in mice injected with miR-144-3p agomir through tail-vein injection. In fish, especially in Nile tilapia, a better ability to use glucose induce adipogenesis, and suppress amino acid catabolism [64]. Thus, an increase in possible adipogenesis, possibly indicated by the up-regulated miR-144, indicates a best use of glucose, which leads to muscle growth, as well as a low protein catabolism in CHIT, indicating one of the possible molecular mechanisms that make CHIT perform better than REDS.

In this study, miR-153a-1-5p, miR-204-5p and miR-204a-5p showed a decrease in the 6th month in the crossbreed. Previously, miR-204-5p expression gradually decreased when myoblasts were induced to differentiate suggesting this miRNA blunted myoblast differentiation in C2C12 myoblasts cells [20]. The miR-204-5p crossbreed down-regulation can evidence an early maturity in crossbreed. MiR-204 was significantly more highly expressed in Min pig race skeletal muscle comparing with Large White race pig characterized by high lean meat percentage, fast-growing muscle, and high body weight [113]. The authors concluded that miR-204 might exert an impact on postnatal hypertrophy of skeletal muscle by regulating myoblast proliferation. Thus,

the down-regulated miR-204 in the 6th month in the crossbreed may be one of the factors that contribute to the greater growth of this lineage, due the low expression of this miRNA with fast-growing.

Using deep sequencing and in situ localization, it was showed that miR-153 when up-regulated stimulates neuronal development [122,133,134,138]. Besides, Song et al. [115] showed that miR-153 stretch-stimulated proliferation of venous smooth muscle cells via activation of the insulin-like growth factor-1 receptor. Like as angiogenesis and neural development are evidence of tumor formation, here they may be indicating greater tissue development, in particular, muscle development. In this way, miR-153 can be an indirect indicator of growth through individual development.

3.2. Key proteins are upregulated in the crossbreed

The most of differentially expressed proteins found in this study are involved with glycolysis. Glycolysis is one of the major routes for ATP generation in cells and is present in all tissue types. Glycolysis proteins are involved in other processes, such as gluconeogenesis and fatty acid synthesis; however, the processes can therefore not be simultaneously active. Cells with high-energy demand have a more pronounced glycolytic activity [36,119]. Thus, high glycolytic activity may indicate low fatty acid synthesis and gluconeogenesis, which are responsible for muscle growth. In general, these proteins being upregulated in CHIT and REDS compared to crossbreed may indicate a high glycolytic activity and low muscle growth in the parents.

Furthermore, some considerations are necessary regarding the main proteins found in this study. L-lactate is a glucose precursor in liver and kidney and being recognized as an energy substrate in most cells via its oxidation to pyruvate, which is catalyzed by L-lactate dehydrogenase (LDH) with pyruvate in cytosol subsequently catabolized in the mitochondria [95]. Deletions L-lactate dehydrogenase gene in microorganisms hinders the growth and hampered infection in hosts [91]. LDH is a cytoplasmic enzyme, which is released from cells following plasma membrane damage and its activity in culture supernatant therefore correlates to degree of cell damage in an explant culture system [108]. Regarding animal production, comparison of two lines of chicken, revealed several proteins in semen, including LDH, which were more expressed in White Leghorns than in Red Junglefowl, with White Leghorn being more productive and more fertile [4]. These data highlight the importance of LDH for growth and cell maintenance.

Fructose-bisphosphate aldolase (FBA) or aldolase occupies a central position in glycolysis and gluconeogenesis pathways [155]. FBA is a key metabolic enzyme and essential for cell growth [13,15,79,80,114]. This enzyme can catalyze the reversible cleavage of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate [21,78,124] and a single amino acid mutation can decrease aldolase activity and inhibit growth performance of vertebrates and plants [43,51]. Wang et al. [131] showed that allele frequencies of three SNPs in FBA and the genotype frequency were significantly different between the two populations with different growth performance.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a conserved enzyme of central metabolism present in bacteria and eukaryotes [33]. This enzyme is involved in glycolysis and gluconeogenesis by catalyzing the reversible oxidation and phosphorylation of D-glyceraldehyde-3-phosphate (GAP) to 1,3-bisphosphoglycerate (1,3-BPG) [109].

Pyruvate kinase (PK) showed high expression in blunt snout bream (*M. amblycephala*) [74] and juvenile largemouth bass (*Micropterus salmoides*) [76] both treated with a high carbohydrate diet. PK is an important enzyme that catalyses the irreversible transfer of the high-energy phosphate group from phosphoenolpyruvate (PEP) to ADP to synthesize pyruvate and ATP [2,125]. The product pyruvate can generate acetyl-CoA, which can be used as the initial substrate to participate in the tricarboxylic acid (TCA) cycle to generate ATP. Besides, acetyl-CoA can be involved in fatty acid [42].

FBA, PK and GAPDH are proteins related to glycolysis and gluconeogenesis. Glycolysis, which is the breakdown of the six-carbon glucose into two molecules of the pyruvate with three carbon. In this metabolism process, FBA cleavage of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate [21,78,124], GAPDH catalyze D-glyceraldehyde-3-phosphate (GAP) to 1,3-bisphosphoglycerate (1,3-BPG) [109] and finally the energy is then released as the two molecules of 1,3-bisphosphoglycerate are converted to two molecules of pyruvate by PK [42].

The proteins up-regulated in crossbreed may indicate that this lineage had a high availability of glucose, which increases insulin secretion. However, one of the evidences of its marked presence is the high expression of calcium-transporting ATPases, which has the function of intracellular transport of the Ca ion.

Intracellular Ca²⁺ is important for endocrine function, particularly insulin secretion. Ca²⁺ signaling evoked by glucose stimulation shows the unique feature of oscillatory increases regulated by interplay between Ca²⁺ influx through voltage-gated L-type Ca²⁺ channels and Ca²⁺ mobilization from intracellular stores [27,107]. Evidences suggest that both the amplitude and the frequency of cytosolic Ca²⁺ are altered in subjects with type 2 diabetes mellitus [22,37].

It is known that insulin in mammals modify growth hormone (GH)-induced IGF-I expression at the hepatic level. In carp liver, there are data that suggest that insulin and GH can act in a synergistic manner to up-regulate IGF-I and -II expression through protein:protein interaction at the receptor level followed by potentiation in post-receptor signaling [55]. In tilapia, insulin can exert opposite effects on the two IGFs, with inhibition on IGF-I but stimulation on IGF-II mRNA expression. In salmon, insulin can also enhance GH-induced IGF-II expression but with no effect on the corresponding response for IGF-I [97]. IGFs play a key role in fish growth, and in that way, they may be responsible for the best crossbreed growth.

3.3. Expression signatures reveal direct and indirect miRNA/protein interactions

Overexpression of both, miRNAs and protein, in the same period, may be due a biological response, which shows that miRNA expression may followed the expression of its target protein (Table S2 and S3). On the other hand, miRNA up-regulation and protein down-regulation (Table S3) shows that miRNA alters its expression so as to bring the expression of the protein back to normal (back to a basal pattern). The sub-expression of certain miRNAs in comparison to REDS resulted in the increase of the proteins that have their genes as targets of the same miRNAs.

The following miRNAs were found up-regulated in crossbreed with their respective targets down-regulated compared to CHIT: miR-145 and miR-203 with fructose bisphosphate aldolase; miR-122 and miR-204 with L-lactate dehydrogenase; And the following miRNAs and theirs targets were found overexpressed in crossbreed compared to Chitralada: miR-182, miR-338, miR-722 with fructose bisphosphate aldolase; miR-122, miR-204, miR-221, miR-222 and miR-462 with L-lactate dehydrogenase (Table S2 and S3).

Comparing with Red Stirling, the following miRNAs and theirs targets were found overexpressed in crossbreeds: miR-15, miR-19a, miR-122, miR-130, miR-193a and miR-203 with pyruvate kinase; miR-19a, miR-122 and miR-204 with L-lactate dehydrogenase; miR-18-1, miR-18-2, miR-19a, miR-101, miR-101a, miR-101b, miR-122, miR-139, miR-144, miR-217 and miR-429 with glyceraldehyde-3-phosphate dehydrogenase; miR-217 with alpha-1,4 glucan phosphorylase; miR-30a, miR-30c, miR-126 and miR-143 with calcium-transporting ATPase; miR-19a, miR-145, miR-199a, miR-199c and miR-203 with fructose-bisphosphate aldolase; and miR-217 with α -1,4-Glucan phosphorylases (Table S2 and S3).

Fructose bisphosphate aldolase and pyruvate kinase, which occupies a central position in glycolysis and gluconeogenesis pathways, showed

to be targeted by miR-122 in goose [151]. Glyceraldehyde-3-phosphate dehydrogenase, another metabolic protein, but does not have any studies involving interactions with miRNAs and animal breeding. Calcium-transporting ATPase is a pump that transports calcium ions from the cytoplasm into the sarcoplasmic reticulum [96]. The transportation of calcium from the cytoplasm after muscle contraction is required for the relaxation of muscle cells [77]. Wei et al. [135] showed that miR-151-3p likely regulates myosin expression and muscle fiber types indirectly through Calcium-transporting ATPase. L-lactate dehydrogenase are responsible for the biosynthesis of phenyllactic acid through a phenylalanine mechanism [54,104,153], however, miRNAs studies with l-lactate dehydrogenase focused only in cancer and tumor.

We conclude that distinctive miRNA and protein expression signatures contribute to the unique phenotype of the advantageous crossbreed red-tilapia lineage. Several miRNAs, including myomiRs miR-1, miR-133a and miR-206, and miRNAs miR-30c, miR-33, miR-122, miR-148a, miR-204 and miR-429, all related previously with animal breeding, were differentially expressed in the genetically improved crossbreed. In addition, proteins involved in glycolysis such as Fructose-bisphosphate aldolase (FBA), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Pyruvate kinase (PK), and other in the glycolysis cycle, as L-lactate dehydrogenase (LDH) catalyze L-lactate and calcium-transporting ATPases were highly expressed and seems to contribute to the genetic gain in crossbreeds.

4. Material and methods

4.1. Experimental animals

The two strains used in the present study were Thai Chitralada strain (CHIT) from the Asian Institute of Technology and the Red-Stirling strain (REDS) from the University of Stirling. Both broodstock strains has been kept by the Brazilian fish farming company (Indústria Brasileira do Peixe Ltd.) as part of a breeding program to produce better lines of red strains. Contemporary fingerling of REDS and CHIT as well as their crossbred (CB; 7/8 CHIT: 1/8 REDS) were produced in reproduction “hapas” where they were kept to the minimum size to be marked with electronic microchips (Passive Integrated Transponder (PIT) tags). Tagged fingerlings were transferred to net cages according to the study carried out by Lago et al. (2016). Twelve fishes were randomly collected during the 5th and 6th months of age from each genetic group (twenty four in total). The growth curve of the animals presented a sigmoidal shape, the first stage of life being slow followed by a marked growth. This inflection of the line varies from 121.2 to 156.2 days for Nile tilapia females, and 134.1 to 166.6 days for males, which corresponds to about 4 to 5 months [93]. In a previous study, these strains of tilapia and their crossbreed showed this marked growth from the 5th to the 6th month [46], which justifies the collection in these months of development. The animals were sacrificed using tricaine methanesulfonate (MS-222, Sigma Chemical, St. Louis, MO). After euthanasia, samples of white muscle were collected and stored at -80°C . The use of experimental animals in this study was approved by the Ethical Committee of the Institute of Bioscience/UNESP (CEUA, protocol 792/2015), and the guide for the care and use of these animals was strictly followed in all experiments.

4.2. RNA isolation and small RNA sequencing

Total RNA was extracted using TRIzol (Life Technologies, EUA), according to the manufacturer’s instruction. RNA integrity was assessed by electrophoresis on 1.0% agarose gel stained with GelRed (Uniscience, USA) and RIN (RNA Integrity Number) by using the 2100 Bioanalyzer system (Agilent, USA) (Fleige and Pfaffl, 2006; Becker et al., 2010). For subsequent analysis, we only used samples with RIN > 7.0 . The RNA samples were also quantified by using a NanoDrop 1000 (Thermo Scientific, USA). RNA purity was measured by the ratio of A260/280 and

A260/230, and only samples with ratio > 1.8 were used.

Small RNA cloning was performed using the TrueSeq Small RNA Sample Prep kit (Illumina, EUA), according to the manufacturer’s instruction. The previously isolated RNA was fractionated into fragments of different sizes using polyacrylamide gel electrophoresis (SDS-PAGE). The fragments ranging from 15 to 30 nucleotides, were excised from the gel and recovered by cooling overnight in saline solution, followed by extraction, precipitation and filtration by micropore. The adapters were bounded to the 3’ and 5’ of the isolated small RNA fragments. Primers (provided by the kit) were then bound to the sequence of the adapters and were used for reverse transcription and selective amplification of small fragments of RNA by RT-PCR. The cDNA fragments were purified and used to build the libraries to generate clusters and subsequent sequencing. The libraries were sequenced using Illumina/Solexa RNA-Seq by LC Sciences enterprise (USA).

4.3. miRNA bioinformatics analysis

The raw reads were checked for quality inspection with FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) being the reads with Phred Score < 30 removed from the analysis. The adapters were trimmed by using FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). The sequences among 18 and 25 nt were selected for miRNAs identification using UEA sRNA workbench v3.2 [117]. We also discard reads from tRNA and rRNA fragmentation or low complexity sequence. The reads were mapped to genome by using Bowtie2.0. Conserved known and novel miRNAs were identified using miRDeep2 [34]. Expression values of these miRNAs were obtained using the weight, which is the number of total reads divided by the number of the loci that matched with that read. Weight count data was normalized by TMM from egdeR package [106]. For statistical tests, the quasi-likelihood F-test (qltr) was used, a test indicated for small quantities of replicates. We also adjusted data with dispersion estimates using data from samples collected at different times as replicates, based on the pipeline for comparisons without replicates from the General-Linear Model (GLM) indicated in TMM manual. We consider as comparisons crossbreed vs CHIT, REDS vs crossbreed, REDS vs CHIT, REDS vs CHIT where CHIT differs from crossbreed, crossbreed vs CHIT where CHIT differs from REDS. FOLD CHANGE also was used to compare the pseudocounts among the libraries (\log_2 of the ration of two samples - one sample divided by the other sample).

In addition, miRNA nomenclature compared with [98] and miRBase v. 22 data and comparison are available in Supplementary File S1. Target prediction was performed using TargetScan (v 6.2) with a cutoff of Context+Score ≤ -0.2 . The 3’UTR sequences of Nile tilapia (v 1.0) were downloaded at BioMart in Ensembl database. For genes with no annotated 3’UTR sequence, we used the 500 first nucleotides after the stop codon.

4.4. Protein extraction and quantification

In this study, we used the shotgun proteomics approach to evaluate the whole set proteins in skeletal muscle and detect those with differential expression between experimental groups. Samples were prepared according to the protocol described by Bouley et al. (2004). White muscle tissue pools were prepared from five individuals with six months aged per group (CHIT, REDS and crossbreed). For protein extraction, frozen white muscle samples (50–100 mg) were mechanically homogenized in a lysis solution containing 8.3 M urea, 2 M thiourea, 1% (m/v) DTT, 2% (m/v) CHAPS and 2% (v/v) IPG pH 3–10 buffer with a rotor stator homogenizer (Polytron). Then the samples were centrifuged at 10,000g for 30 min and the supernatants were collected. Protein concentration was determined by the Bradford method with bovine serum albumin (BSA) as the standard protein [12].

4.5. In solution protein digestion

CHIT, REDS and crossbreed white muscle samples (50 µg protein) were submitted to in solution protein digestion. In the reduction procedure, protein extracts were processed in a solution containing 10 mM dithiothreitol (DTT) and 50 mM ammonium bicarbonate and alkylation was performed in a solution of 45 mM iodoacetamide (IAA) and 50 mM ammonium bicarbonate, centrifuged (14,000 g, 45 min), and the flow through was discarded. The samples were hydrolyzed by trypsin at a ratio of 1:50 (enzyme: substrate) in 50 mM ammonium bicarbonate buffer (pH 7.8) for 16 h at 37 °C. The hydrolysis was terminated by addition of 1% (v/v) formic acid to the sample volume. Then, the samples were desalted using Sep-Pak Vac C18 cartridges, reduced in SpeedVac vacuum concentrator and maintained at 4 °C until the time of mass spectrometry analysis [130].

4.6. ESI-Q-TOF mass spectrometry analysis of tryptic digests from complex mixture samples

The tryptic peptides of CHIT, REDS and crossbreed white muscle samples were run in a nano ACQUITY UPLC system (Waters, Milliford, USA) coupled to the Xevo Q-TOF G2 mass spectrometer (Waters, Milliford, USA). The nano ACQUITY UPLC system was equipped with a HSS T3 (Acquity UPLC HSS T3 column 75 mm × 150 mm, 1.8 µm, Waters) column, previously equilibrated with 7% of mobile phase B (100% (v/v) ACN + 0.1% (v/v) formic acid). The peptides were separated by a linear gradient of 7–85% mobile phase B for 20 min at a flow rate of 0.35 µL/min and the column temperature maintained at 45 °C. The MS was operated in positive ion mode, with the data acquisition time of 20 min. The acquisition of spectra was Lock mass: [Glu1]-Fibrinopeptide B human 1 pmol/µL (*m/z* 785.8426), scan time: 0.3 s, acquisition mode: resolution +. ProteinLynx GlobalServer (PLGS) version 3.0 software (Waters, Milliford, USA) was used for data processing. Ion accounting algorithm of PLGS searched against *O. niloticus* database downloaded from NCBI. Search parameters were as follows: automatic tolerance for precursor ions and for product ions, minimum 2 ions fragments matched per peptide, minimum 5 ions fragment matched per protein, minimum 2 peptide matched per protein, 1 missed cleavage, carbamidomethylation of cysteines and oxidation of methionines as fixed and variable modifications, false positive rate (FPR) of the identification algorithm under 1% and 100 fmol of the ADH internal standard set as calibration protein concentration.

Mass spectrometry analysis of CHIT, REDS and crossbreed complex mixture were performed in triplicate. Spectral count values were normalized for each identified protein and differential protein expression were obtained using ANOVA with *t*-test, and a *p*-value of less than 0.05 was considered to indicate a statistically significant difference. Moreover, the differential protein expression was also estimated by the normalized fold changes.

4.7. Analysis of miRNAs and protein interactions

The functional enrichment analysis was done using the g:Profiler tool (<http://biit.cs.ut.ee/gprofiler/>) using the Nile tilapia database and g:SCS threshold as a statistical parameter. Network interactions between proteins were obtained using STRING software (<http://string-db.org>).

To establish the possible involvement of the proteome profiling and miRnoma data, genes correlated with those altered proteins were accessed in the databases and their access numbers retrieved. Using this criterion, we identified by TargetScan, the regulatory miRNAs of these target genes. In the next step, miRNAs with differential expression were set apart to find matches with the proteins of interest.

Nile tilapia (*O. niloticus*) is currently a major aquaculture species worldwide. This is evidenced by the FAO [31] data, which rank Nile tilapia as the third aquaculture freshwater finfish in production behind Grass carp, *Ctenopharyngodon idellus*, and Silver carp, *H. molitrix*.

Therefore, Nile tilapia is regarded as one of the most important tropical and subtropical species as a source of animal protein and income in many countries [103].

Authors contribution statement

Experimental design: MEH. Animal experimental design: MEH, MADD, AWSH. Animal experimental execution: MEH, MADD. miRNA design and experimental: MEH, LAB. miRNA bioinformatic analysis: LAB. Proteomic experimental design: MEH, OB, LDS, MF. Proteomic experimental execution: MEH, OB, LDS, LGAC, LAL Jr. Proteomic data analysis: MEH, OB, LDS. Data discussion: MEH, LAB, OB, LDS, ACO, MF, AMRO, AWSH, MF, DP. Writing of the first draft of the manuscript: MEH, LAB, DP. Critically revision and final manuscript writing: MEH, LAB, OB, LDS, ACO, MF, AWSH, MF, DP.

All authors read and approved the final manuscript.

Declaration of competing interest

Authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jgeno.2021.11.008>.

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