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Colossoma macropomum (Characiformes: Serrasalmidae) adapted to new climate regime: differential gene expression from farmed tambaqui juveniles raised in subtropical and tropical regions

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Abstract
The Brazilian native fish species with the highest potential in fish farm is *Colossoma macropomum*. This work investigated whether two populations of farmed *C. macropomum* respond equally to their captive habitats. 20 tambaqui juveniles from the Brazilian fish farms Balbina and Brumado were selected. We examined genes of specific metabolic pathways: *hsp70*, *hif-1α*, *per-1*, *cry-1*, *acly*, *mstn*, *ube3a*, *ras* and *ogt*. Expression of *ras* (p = 0.041), *cry-1* (p = 0.001), *per-1* (p = 0.001), *ogt* (p = 0.001) and *acly* (p = 0.025) were significantly lower in Brumado fish. Heatmaps showed extreme relative expression differences for some genes. Integrated biomarker response values for Balbina and Brumado populations were 42.7 and 6.79, respectively. In general, the Brumado population presented lower gene expression than Balbina fish. The differential genetic structure of the populations is likely the most impacting factor, suggesting local adaptation.

Keywords: Acclimatization, aquaculture, metabolic comparison, local adaptation

1. Introduction
In Brazil, the native species with the highest potential and excellent performance in fish farming systems is *Colossoma macropomum* (Cuvier, 1818) a fish from Characiformes, popularly known as tambaqui (Midande, 2010) [25]. Although it is a tropical species commonly found in Northern Brazil, it is cultivated in almost all Brazilian States (Brabo *et al*., 2016) [7].
Tambaqui has many adaptive mechanisms: high tolerance to hypoxic conditions (Almeira-Val *et al*., 1995; Robertson *et al*., 2015) [2, 31], high temperature (Dragan, 2014) [11] and pH variations (Val *et al*., 1998; Wood *et al*., 2018) [37, 39]. Consider a model specie among tropical fish for studies in aquaculture (VAL; OLIVEIRA, 2021) [36]. Genetic isolation from natural populations, aided by geographic isolation in fish farm tanks, directly affects gene flow and induces changes in gene frequencies and mutation accumulation (Bijilma & Loeschcke, 2012) [5]. Also individuals from genetically distinct populations can present specific gene expression in response to stressful conditions (Picard & Schulte, 2004) [28].
Knowledge about population genetic variability enables improvements (Bohling *et al*., 2016) [6]. Recently, Gonçalves and colleagues (2018) [15] reported the loss of genetic variability and high co-ancestry in all populations cultures in six tambaqui farms. The founder effect, bottleneck effect and absence of gene flow may potentialise these phenomena (Knibb *et al*., 2014) [21]. Besides, little is known about how each population responds to their captive habitat. Farmed animals are under the influence of both environmental and anthropogenic factors. Latitude is another critical factor for aquaculture production (Conover *et al*., 1997) [10].
Genes involved in metabolic processes can indicate adaptive. Fish energy metabolism is affected by diet (Overturf *et al*., 2016) [27], temperature (Wen *et al*., 2017) [38], contaminants (Li *et al*., 2016) [22] and other factors. Metabolic rhythm can be altered by latitude (Hut *et al*., 2013) [19] and seasonal variations (Herrero & Lepesant, 2014) [17].
Protein metabolism can fluctuate especially with temperature changes (Balchin *et al*., 2016) [4].

Since tambaqui is a species of high economic value and relevancy for human consumption, it is necessary to improve studies on its metabolism. Only by understanding the mechanisms of its metabolism will it be possible to develop strategies to increment its production. Considering all the above, this work aims to investigate whether there is a difference in gene expression of tambaqui populations from two fish farms located in distinct latitudes.

2. Material and Methods

2.1 Ethical statement

All experimental protocols employed in the present study were performed in accordance with Brazilian Guidelines from the National Board of Control and Care for Ethics in the use of Experimental Animals (CONCEA, 2013) and approved by the Committee of Ethics on Animal Care (CEUA) at the Brazilian National Institute for Research of the Amazon (INPA) with protocol number 032/2016.

2.2 Population sampling

We selected 10 tambaqui juveniles (42.5±4.7 g; 11.2±0.4 cm long) from each Brazilian fish farms, Balbina (Amazonas; 1°55'54.4"S 59°24'39.1"W) and Brumado (São Paulo; 22°31'16.00"S 46°53'5.71"W). Both sampling occur during summer season ate each region and water temperature in Balbina's rearing tank recorded 29, 5 ºC and in Brumado's rearing tank recorded 21 ºC. The dissolved oxygen concentration in both tanks ranged from 5 to 7 $mg.L^{-1}$. At each farm, fish liver was collected in early morning, before fish were feed and promptly placed in RNA later™ Stabilization Solution (Invitrogen™, Thermo Fisher Scientific), according to manufacturer's instructions. Samples were stored at -20 ºC.

2.3 Total RNA isolation, electrophoresis, and complementary DNA (cDNA) synthesis

Liver RNA extraction was performed using the RNeasy® Mini Kit (Qiagen, Hilden, DE) on the Qiacube (Qiagen) automated workstation. Microfluidic electrophoresis confirmed the sample integrity with Bioanalyzer RIN analysis (Agilent Technologies, Santa Clara, CA). We used the DNase I kit (Thermo Fisher Scientific, Massachusetts, USA) to remove genomic DNA. CDNA was synthesised with the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Vilnius, Lithuania). The integrity of the RNA was verified by 1% agarose gel electrophoresis, showing intact 28S and 18S rRNA bands. We quantified RNA concentration with a Nano Drop 2000 Spectrophotometer (Thermo Fisher Scientific).

2.4 Relative gene expression analysis with quantitative real-time polymerase chain reaction (qRT-PCR)

We choose genes involved in several crucial metabolic pathways: hsp70 – heat shock protein 70 (protein folding); hif-1α – hypoxia-inducible factor-1alpha (oxygen-dependent genes); per-1 – period circadian regulator-1 (circadian cycle); cry-1 – cryptochrome circadian regulator (circadian cycle); acly – ATP citrate lyase (lipid metabolism); mstn – myostatin (skeletal muscle production); ube3a – ubiquitin protein ligase E3A (ubiquitin metabolism); ras (oncogene and cell growth); ogt – O- linked N- acetyl glucosamine transferases (multiple biological pathways) (Table 1).

Out of the nine analysed genes, primers for six were previously validated for tambaqui: *ras*, *hsp-70* and *hif-1α* (Silva *et al*., 2017) [34], *mstn* and *β-tubulin* (Lima, 2018) [23] and *β-actin* (Ferreira *et al*., in prep.). For the other genes, specific primer sequences were designed from gene sequences obtained from the tambaqui transcriptome (Fé-Gonçalves *et al*., 2020) [12] (Access number: PRJNA547332). For primer design, we used Oligo Explorer 1.2 (Gene Link); primers were 18 to 22 base pairs (bp) long, generated a 137-150-bp fragment, and had a ≥ 50% GC content, a melting temperature (Tm) from 58-62 º C and no primer annealing. For primer validation, we generated a standard curve with eight points obtained from a dilution (1:2) of basal samples (Balbina fish), and we considered an R^2 value of 0.98-0.99 and an efficiency (%) of 100.7 to 104.1 (Table 1).

Table 1: Details of target genes (*hif-1α*, *hsp70*, *ras*, *mstn*, *acly*, *per-1*, *cry-1*, *ube3a* and *ogt*) and reference genes (*β-tubulin* and *β-actin*) primers.

Gene	Length (bp)	R^2	Efficiency (%)	Primers sequence (5′-3′) forward/reverse
tubulin-F	20	0.99	109.5	GACGTGGTGCCCAAAGATGT
tubulin-R	18			TGGATGGTGCGCTTGGT
β-actin-F	21	0.99	100.5	GCTGTTTTCCCCTCCATTGTT
β-actin-R	19			TCCCATGCCAACCATCACT
hif-1α-F	20	0.99	105.2	CTTCTGAGCTCTGATGAGGC
hif-1α-R	20			GAAAGCACCATCAGGAAGCC
hsp-70-F	20	0.99	100.9	GCAAGGAGAACAAGATCACC
hsp-70-R	19			CACTCCGTTGCACTTGTCC
mstn-F	20	0.98	100.5	AATCCAAGCGAGGGAAAAGC
mstn-R	22			CCTCCATCACCTGAAAGGTCTT
ras-F	20	0.97	99.31	CCAGTACATGAGGACAGGAG
ras-R	20			CAAGCACCATTGGCACATCG
acly-F	19	0.99	100.7	ATCATCTCCCGCACTACAG
acly-R	19			TACCTCCAATCTCTCCCAG
ube3a-F	21	0.98	103.3	GCCATAAGCAAGCAGCACAAC
ube3a-R	19			CCAGTCAGTCCGCACATCG
per-1-F	20	0.98	104.1	TGTTGAAGTTTGTGCCCCAG
per-1-R	18			CAGTCCAGATGCTCCTCC
cry-1-F	19	0.99	103.6	GTCCAACAGCCCTCAAACT
cry-1-R	18			TACGCCAAGCACTCCAGA
ogt-F	19	0.99	104.1	CCTCCCTTTGCTGTGTTCC
ogt-R	20			TGTCTGCTTTCCGCTTTCGC

Relative gene expression was quantified on a Vii ATM 7 Real-Time PCR system (Thermo Fisher Scientific) platform. Two reference genes were used for normalization calculations (*β-tubulin* and *β-actin*), and relative gene expression was calculated according to Livak & Schmittgen (2001) [24]. PCR reactions were as follows: 5 µL Fast SYBR® Green Master Mix (Applied Biosystems), 1 µL forward primer (2 pmol), 1 µl reverse primer (2 pmol), 2 µL nuclease-free water – not DEPC treated (Ambion) and 1 µL cDNA (~ 0.140 mg/µL). Each assay was performed in triplicate. The qRT-PCR thermal cycling protocol was: Hold (one cycle) - 95 ºC for 20 s; PCR (40 cycles) - 95 ºC for 1 s and 60 ºC for 20 s; melting curve stage (one cycle) - 95 ºC for 15 s and 60 ºC for 1 min.

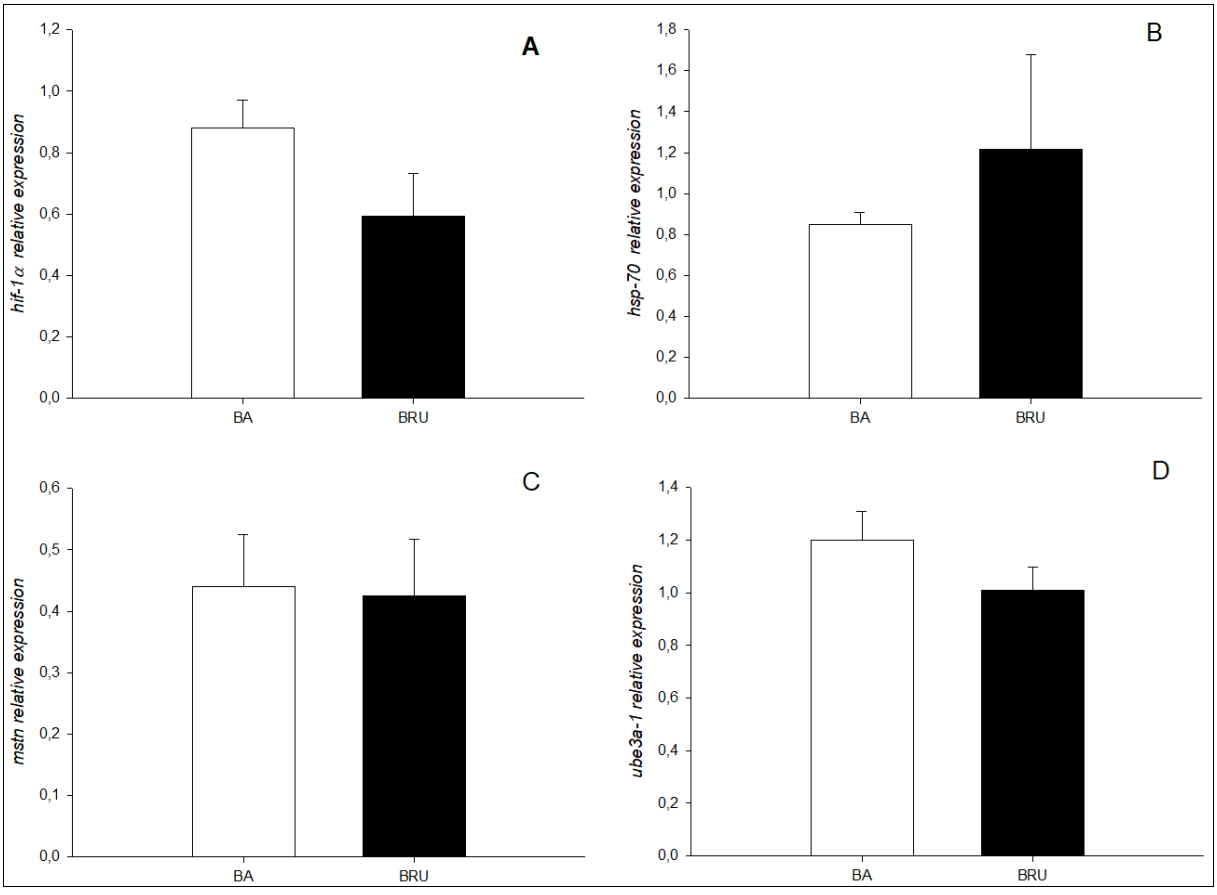
2.5 Statistical analysis

Sigma Plot 11.0 software was used to analyse relative gene expression with the t-test, presented as mean ± standard error of the mean (SEM; n = 5). We also built cluster analyses and heatmaps in Metabo Analyst 4.0 software using log-transformed and auto-scaled data. Cluster analyses followed the criteria: i) cluster rows, ii) similarity metrics by Euclidean and iii) clustering method by Ward. The heatmap color scale ranged from blue (low transcript levels) to red (high transcript levels). The integrated biomarker response (IBR) was calculated according to Beliaeff & Burdeot (2002) using Excel (Microsoft, USA).

3. Results

The *hif-1α* (p = 0.137), *hsp-70* (p = 0.465), *mstn* (p = 0.907) and *ube3a* (p = 0.205) relative gene expressions were not statistically significantly different between Balbina and Brumado populations (Fig. 1A-D). The oncogene *ras* from the Brumado population exhibited lower relative expression (p = 0.041) compared to the Balbina population (Fig. 1E). The Balbina population *cry-1* (p = 0.001) relative expression was 4-fold higher than in the Brumado population (Fig. 1F). *Per-1* was even more differentially expressed; it was 13-fold higher in the Balbina compared to Brumado fish (Fig. 1G; p = 0.001). *Ogt* relative expression was 6-fold higher in the Balbina population (Fig. 1H; p = 0.001). Finally, *acly* was 3-fold higher in Balbina fish (Fig. 1I; p = 0.001).

The heatmap also reflected these marked differences in some relative gene expressions (Fig. 2). Based on cluster analyses, the Balbina and Brumado individuals were grouped separately (Fig. 2). The heatmap also showed that the analysed genes were generally more expressed in the Balbina compared to the Brumado population. The IBR values for Balbina.



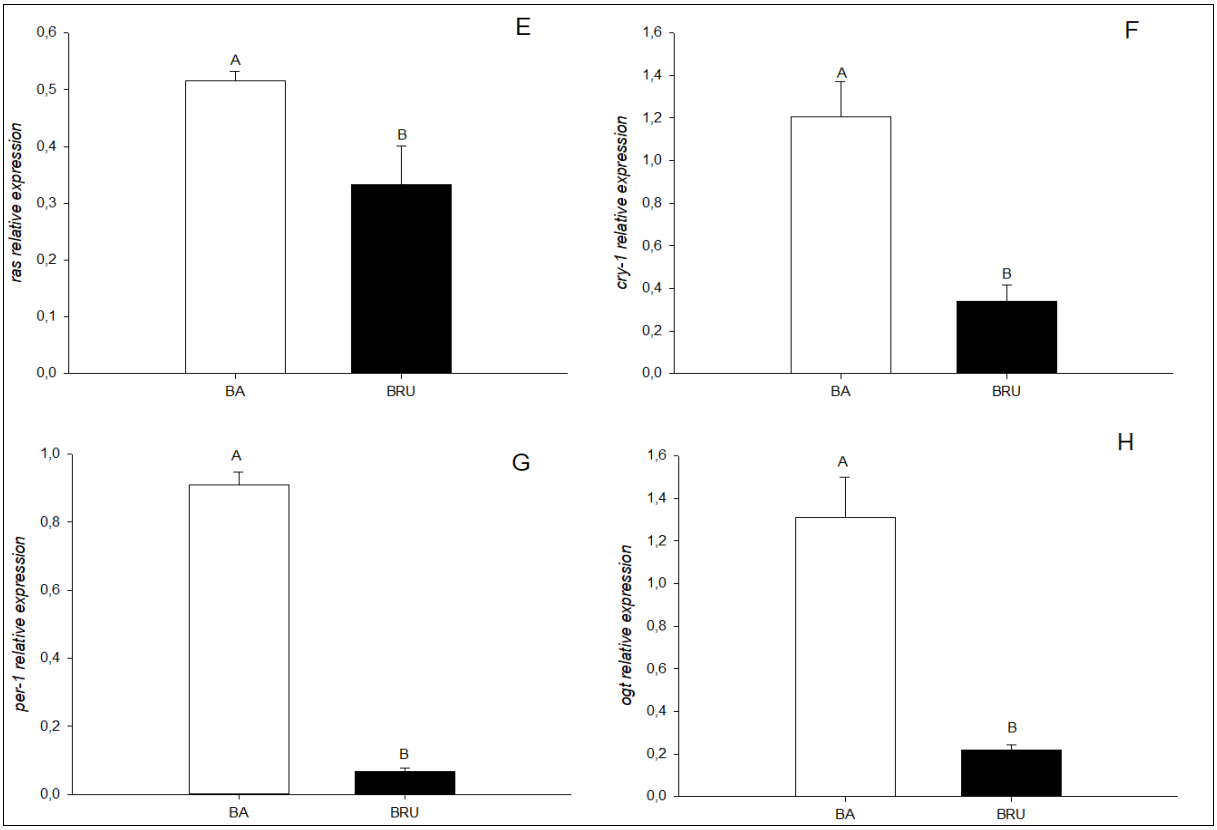


Fig 1: Relative gene expression in tambaqui juveniles farmed in two Brazilian regions: Northern (Balbina; BA) and Southeast (Brumado; BRU). Different letters represent statistical differences between populations. The graphs show expression of A) *hif-1α* (p = 0.137), B) *hsp-70* (p = 0.465), C) *mstn* (p = 0.907), D) *ube3a* (p = 0.205), E) *ras* (p = 0.041), F) *cry-1* (p = 0.001), G) *per-1* (p = 0.001), H) *ogt* (p = 0.001) and I) *acly* (p = 0.025).

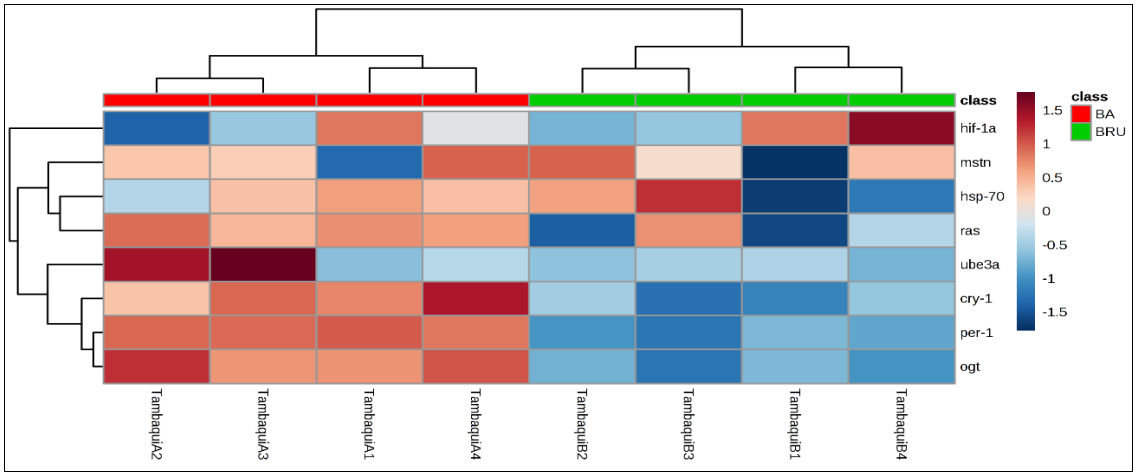


Fig 2: Heatmap of relative expression in Balbina (BA) and Brumado (BRU) populations. The colour scale ranges from blue (low transcript levels) to red (high transcript levels).

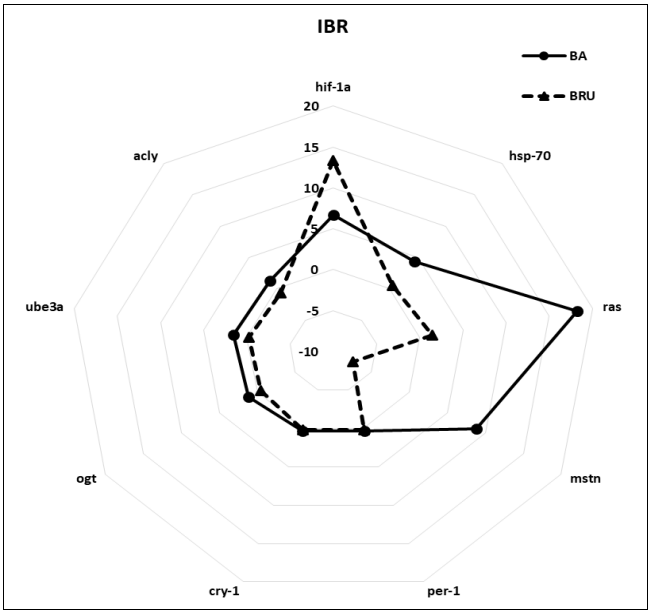


Fig 3: IBR analyses of relative gene expression in Balbina (BA) and Brumado (BRU) populations. The IBR values are 42.7 (Balbina) and 6.79 (Brumado).

And Brumado populations were 42.7 and 6.79, respectively (Fig. 3). Besides the different IBR values, the populations also had different area conformations.

4. Discussion

Each farm fish is located in a complete different climate region. Not only with a distingue temperature range, also with a different photoperiod dynamic during the year. Considering Köppen's classification, Brumado is on a humid subtropical zone with hot summer (Cwa) and Balbina is on a tropical zone without dry season (Af) (Alvares *et al*., 2013) [3]. In each climate zones all abiotic factors changes creating a complete different environment that will affect the entire metabolism of every organism.

Metabolic pathways are required to maintain homeostasis, which allows an organism to fully function. Metabolic functions are directly related to circadian rhythms, and its processes are divided into anabolism and catabolism (Challet, 2013) [9]. The Balbina fish population showed high anabolic activity, with active metabolic routes for lipid production and cell growth. Low relative expression of *ogt*, *acly* and *ras* in the Brumado population indicates that probably this population has a higher metabolic demand to preserve homeostasis. Thus, the Brumado fish would not have disposable energy for alternative stock energy routes, and this profile may slow growth. Still, *mstn* relative expression was not different between populations. *Mstn* act as a growth and differentiation factor by negatively regulating skeletal muscle mass. Although its role as a negative regulator of skeletal muscle growth is well established, it also affects other metabolic routes (Huang *et al*., 2011) [18] in liver and adipose tissue (Allen *et al*., 2011) [1]. Lima (2018) [23] reported higher *mstn* relative expression in control tambaqui compared to a group exposed to a climate change scenario. The absence of an *mstn* difference between the populations can indicates local adaptation after 50 years of acclimatization under different weather cycles.

The cyclic action of circadian genes induces metabolic changes so that the organism can deal with daily environmental changes. Alterations during the circadian cycle promote modifications in hepatic lipid metabolism and energy homeostasis (Yang *et al*., 2017) [40]. *per-1* and *cry-1* are part of the especially elaborated circadian clock and can be downregulated by the CLOCK/BMAL heterodimer, usually hin a 24-hour cycle (Foulkes *et al*., 2016) [14]. Photoperiod changes throughout the year affect organisms differently depending on latitude (Hut *et al*., 2013) [19] what could explain the difference in expression of *per-1* and *cry-1* between populations. Herrero and Lepesant (2014) [17] considered that photoperiod influences daily oscillations of these genes.

Our results also showed that protein metabolism appears to work at the same intensity in both populations. The relative expression of *ube3a* and *hsp70* were not different between populations. These genes are essential for protein formation and ubiquitination. *Ube3a* is responsible for the ubiquitination ligase step (Pickart & Eddins, 2004) [29]. Ubiquitination involves three steps, and the ligase step is the last one when the protein is the target of the ubiquitin protein for degradation (Gustin *et al*., 2010) [16]. This process affects many pathways including lipid metabolism (Qi *et al*., 2006) [30]. *Hsp70* is a heat shock protein that ensures proteins in non-native conformations will be fixed or targeted for degraded (Feder & Hofmann, 1999) [13]. *C. macropomum* commonly express *hsp70* (Souza-Bastos *et al*., 2016) [35]; it is usually overexpressed under thermal stress conditions (Kelly *et al*., 2018) [20]. Local adaptations must be the explanation for the similar *hsp-70* values, since temperatures were different at the time of sampling.

Another gene that is regulated by temperature is *hif-1*□. Both *hif-1* and *hsp-70* can serve as thermal shift biomarkers (Mladineo & Block, 2009) [26]. Shen and colleagues (2010) [33], when studying *Megalobrama amblycephala*, showed that *hif-1* and *hif-2* are involved in different physiological functions in hypoxic situations. The absence of gene expression differences between the populations may indicate that both artificial environments promote similar thermal pressure as well as oxygen availability.

Gonçalves and colleagues (2018) [15] analysed six Brazillian tambaqui fish farms to provide genetic basis of their broodstock. Two of those six fish farms are the same two studied in the present research. Authors analysed 15 microsatellite loci and stablished an increased inbreeding and a loss in genetic variability in the tambaqui fish farms. A previous work analysed fish farms from North and North East already showed genetic loss probably influenced by the founder effect (Santos *et al*., 2016) [31]. Moreover, they confirmed that all populations are different and genetically structured. These facts corroborate our data and explain why we obtained different gene expression between the two fish farm populations.

When relative expression of all genes was plotted together, we noticed a separation between fish farm populations with both heatmap and IBR analyses. Differences in IBR area and shape reflect distinct metabolic strategies that indicate local adaptation. In general, the Brumado population presented lower gene expression than Balbina fish, a finding that indicates a latitudinal influence. Campos *et al*. (2021) [8] already discusses phenotypic shifting in a species after a long time of exposure to environmental change. Besides, the long period (50 years) during which the populations were raised in isolation, when location adaptation and epigenetic episodes occurred, likely caused the different genetic structure between populations.

5. Conclusion

Our results suggest that these two populations present local adaptation to each environment. This statement is reinforced by the absence of differences in gene expression of *hsp-70*, *hif-1*, and *mstn* between populations. Data corroborates with previous works that propose a different genetically structured based on heatmap and IBR results. Considering the heatmap, IBR, and gene expression of *ras, ogt, acly, per-1,* and *cry-1*, also the difference in latitude, may reflect distinct metabolic strategies between populations. An investigation should be done into this hypothesis because it can affect fish production. We suggest future experiments using tambaqui from different latitudes to explore the metabolic strategy.

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