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Chapter 12

Temporal Expression of Isozymes, Alozymes and Metabolic Markers at the Early Ontogeny of *Prochilodus argenteus* (Characidae – Prochilodontidae) from São Francisco Basin, Três Marias, Minas Gerais, Brazil

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Additional information is available at the end of the chapter

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1. Introduction

It is well-known phenomenon that all changes in a population depend on reproduction, growth and mortality. Analysis of differential expression of genes which encode enzymes has made it possible to relate developmental changes at the molecular level to the general physiological changes whit accompany differentiation. According to [1], the specific protein expression during the different stages of development indicates the gene activity that can be started or turned off during embryogenesis. These properties make the multiple forms particularly interesting for the beginning ontogenetic gene regulation. Many enzymes exist as isozymes, and these isozymes are often differentially expressed during embryogenesis [2]. Thus, the enzymatic studies, including isozymes and allozymes must be informative about gene's activity and regulation during the early development. The electrophoretic and kinetics studies can be employed to investigate when genes are started during development, and how these enzymes are increased or reduced during ontogeny.

The Três Marias hydroelectric station was built in 1960s in the main canal of the São Francisco River in Minas Gerais state. According to [3] it has been observed that several migratory fish collected at downstream region closet to the dam, are smaller in size and have immature gonads during the spawning season. A distinct condition is observed 30 Km downstream from the dam, where these animals generally are normal-sized and have

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developed gonads. These facts reveal that conditions on this region are less favorable to they reproduction. There are two possible factors, among others caused by hydroelectric station, as a lower water temperature and oxygenation.

The Prochilodontidade family is composed by iliophagous-migratory fish, that swimming upstream to deposit their eggs every year in the end of dry season. *Prochilodus argenteus,* is an endemic fish specie, that migrate to the water spring for the spawning. It is called curimbatá, and today is endangered specie of fish from São Francisco basin.

2. Objective

The general objectives are to verify the correspondence among enzymatic levels, early ontogeny and physiological activities, as well as the correspondence between spatial and temporal enzymatic activities and their metabolic role, through electrophoretic and spectrophotometric studies, verifying the moment of gene activation, monomorphism or polymorphism of the enzymatic systems, synchrony or asynchrony of paternal and maternal genes, what kind of metabolism (glycolytic or aerobic) is predominant and changes in enzymes activities that occurring during the early ontogeny of *P. argenteus*.

3. Material and methods

- Five adults and mature couples of *P. argenteus* were induced to reproduce through a hypophysation process. After the gametes extrusion, fertilized eggs or larvae were collected at 0, 4, 8, 12, 16, 20, 36, 60, 87, and, 135 hours post fertilization (h.p.f.) and immediately iced. Temperature, solved oxygen and pH of water measured in each incubation tank, whit a Horiba U10 instrument. After the reproduction, the adults were sacrificed and, muscle, heart, and liver were collected and iced.
- Were performed electrophoretic analysis for alcoholic dehydrogenase (ADH, EC 1.1.1.1), glucose phosphate dehydrogenase (GPI, EC 5.3.1.9), glucose 6 phosphate dehydrogenase (G6PDH, EC 1.1.1.49), isocitrate dehydrogenase NADP-dependent (IDHP, EC 1.1.1.41), lactate dehydrogenase (LDH, EC 1.1.1.27); malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme NADP-dependent (ME, EC 1.1.1.38), 6-phosphogluconate dehydrogenase (6PGDH, EC. 1.1.1.44), and enzymes activities analysis for lactate dehydrogenase (LDH, EC1.1.1.27), malate dehydrogenase (MDH, EC1.1.1.37), piruvate kinase (PK, EC 2.7.40), and citrate synthase (CS, EC 4.1.3.7).
- A small piece of each tissue for adults or a few eggs or larvae was/were homogenized (w/v) in 50mM phosphate potassium buffer (pH 7.0), using a manual homogenizer and centrifuged at 27000g for 30 min at 4^{o}C in a Sorvall RC5B centrifuge. The resulting crude extracts were used for electrophoretic analysis. Electrophoreses were carried out employing a horizontal gels containing 13% (pH 8.7) (for GPI, G6PDH, LDH and, 6PGDH) or 14% (pH 6.9)(for ADH, IDHP, sMDH, mMDH, ME) corn starch prepared according to [4]. A voltage gradient of 5V/cm was applied for 12-14 h at 4^{o}C. After electrophoreses, the gels were sliced lengthwise and the lower halves incubated in a specific staining solution. The histochemical solutions used were described by [5] and modified by us, for G6PDH,

6PGDH, IDHP, LDH and ME. For ADH and MDH, we used the solution described by [6] with modifications and, for GPI [7]. Nomenclature for the gene loci and iso/allozymes was taken from [8]. Alleles were designated by number with **100* representing the most frequent allele. Subsequent numbers refer to their relative mobility.

- The activities enzymes analyses were performed using revised proceedings by [9]. Was used a GENESYS 2 spectrophotometer at 25°C for activities measures. After each assay the total protein was measured by Bradford method, using a wavelength 595 nm. The graphics were performed in Excel and Origin 6.0 by Windows program, were calculated median and stand deviation and the statistics Student's Test.

4. Results

Conditions of temperature, solved oxygen and pH of the incubation water are in table 1.

Offsprings	Temperature (°C)	Solved oxygen (mg/ l)	pH
1 (couple 1)	25.2	4.24	6.15
2 (couple 2)	25.1	4.27	6.12
3 (couple 3)	25.1	4.43	6.04

Table 1. Temperature, solved oxygen and pH measured of the incubation water in nursery tank.

5. Electrophoresis

Zimograms with phenotypes patterns detected from the couples of *P. argenteus* used for reproduction are in table 2 and figure 1.

	Couple 1		Couple 2		Couple 3	
	Male1	Female1	Male 2	Female 2	Male 3	Female 3
*GPI-A**	**100*	**100*	**100*	**100*	**100*	**100*
*GPI-B**	**-100/50*	**-100*	**-100/50*	**-150/50*	**-150*	**50*
*G6PDH**	**100*	**100*	**100*	**100*	**100*	**100*
*IDHP-A**	**100*	**100*	**100*	**100*	**100*	**100*
*IDHP-B**	**100*	**100*	**100*	**100*	**100*	**100*
*LDH-A**	**100*	**29/100*	**29/100*	**29/100*	**29/100*	**29/100*
*LDH-B**	**100*	**100*	**100*	**100*	**100*	**100*
*MMDH**	**100*	**100*	**100*	**100*	**100*	**100*
*SMDH-A**	**100*	**100*	**100*	**100*	**100*	**100*
*SMDH-B**	**100*	**100*	**100*	**100*	**100*	**100*
*ME-1**	**100*	**100*	**100*	**100*	**100*	**100*
*ME-2**	**100*	**100*	**100*	**100*	**100*	**124*
*6PGDH**	**100*	**88/100*	**100*	**100*	**88*	**100*

Table 2. Phenotypes detected in zimograms from the 3 couples of *P. argenteus* used for reproduction.

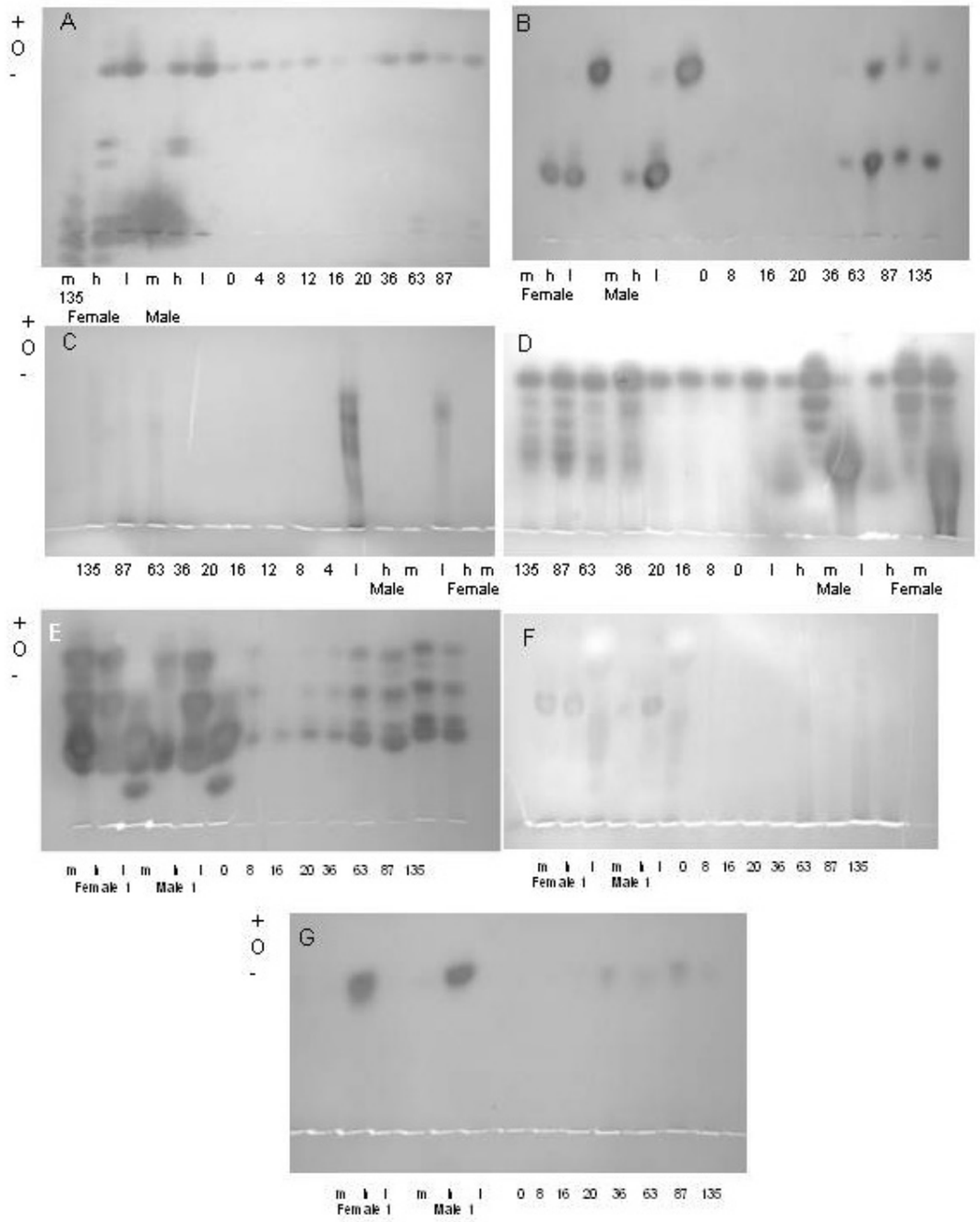


Figure 1. Electrophoretic patterns detected in couple 1 and offspring: A- GPI; B- G6PDH; C- IDHP; D- LDH; E- MDH; F- ME and G- 6PGDH. m - muscle; h - heart; l - liver.

The zimograms showed variant loci: *GPI-B** (**-100*, **200* and, **-300*), 6PGDH* (**88* and **118*), *ME-1** (*124) and, *LDH-A** (**16* and **-16*). Another systems: G6PDH, IDHP and, MDH showed no variation. For the *G6PDH**, *IDHP-A**, *B**, *s-MDH-A**, *B**, *m-MDH** and *ME-1** and *ME-2** loci was not possible to detect the asynchrony or synchrony of gene expression, because the paternal and maternal phenotypes were identical or both had 1 common allele in heterozygotes (table 2).

6. Enzymes activities

The low high ratios L/H are presented in Figure 2. There were detected decreasing of these ratios during the development, what indicated decreasing of B subunits and/or A subunits synthesis. These subunits A synthesis were expected, because the muscle predominant subunit is type A, the product of *LDH-A** was detected late, 36 hours post fertilization, in heteropolymeric form.

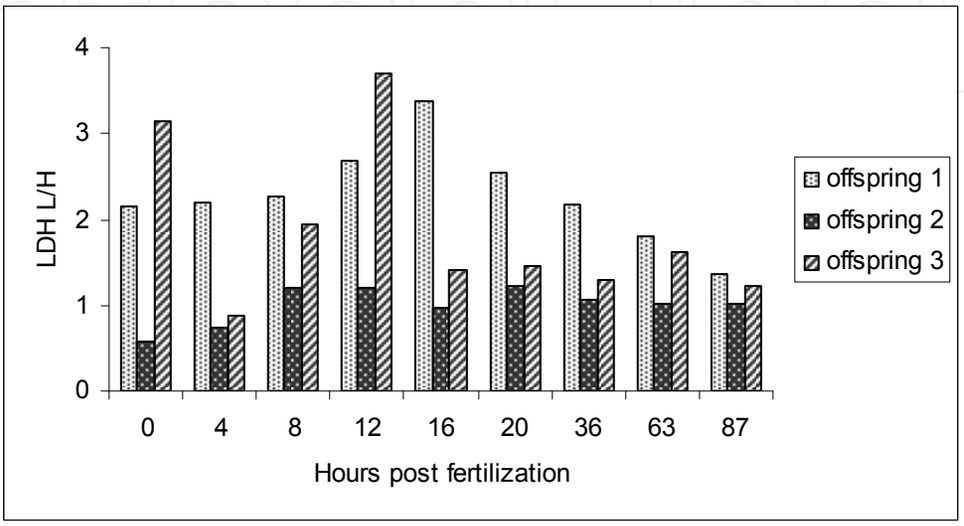


Figure 2. Low/high LDH ratios in different developing phases of offsprings of 3 couples of *P. argenteus*.

The obtained ratios between the glycolytic enzyme LDH and MDH from different phases of offspring developing from each pair, as well the medians calculated by the 3 couples together offspring showed the greater oxidative metabolism/malate aspartate shuttle until 87 hours post fertilization are in Figure 3.

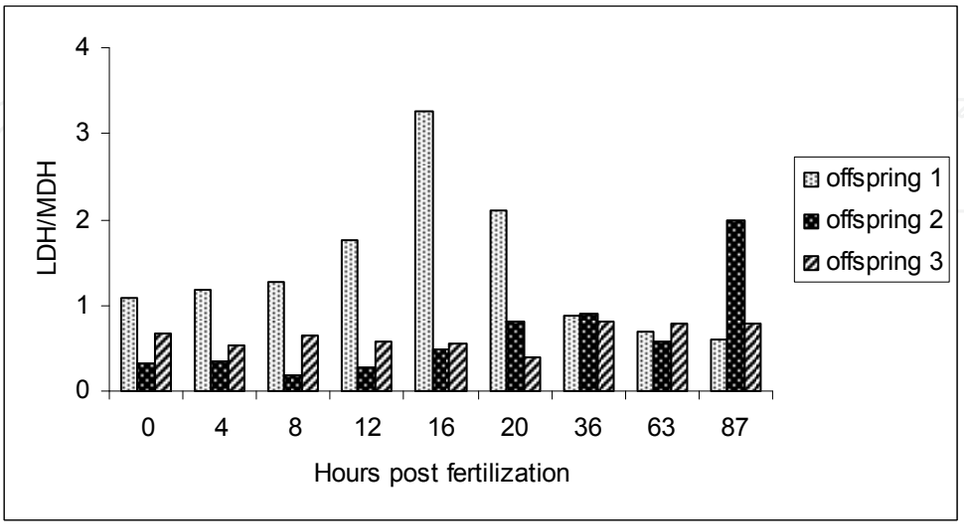


Figure 3. LDH/MDH ratios in different developing phases of offsprings of 3 couples of *P. argenteus*.

Differently of the observed for the LDH/MDH ratios, the LDH/CS observed ratios, for the 3 progenies, as well as the ratios obtained by the median of each enzyme for the 3 issues from the 3 couples presented all the values greater than one, and the greatest values in the stages between 12 and 16 hours post fertilization, decreasing after that (Figure 4). The PK/CS ratios and its medians observed were not great when compared with LDH/CS ratios, showing the maximum values at 16 hours post fertilization (Figure 5). The median value obtained for the 4 enzymes activities, showed (Figure 6).

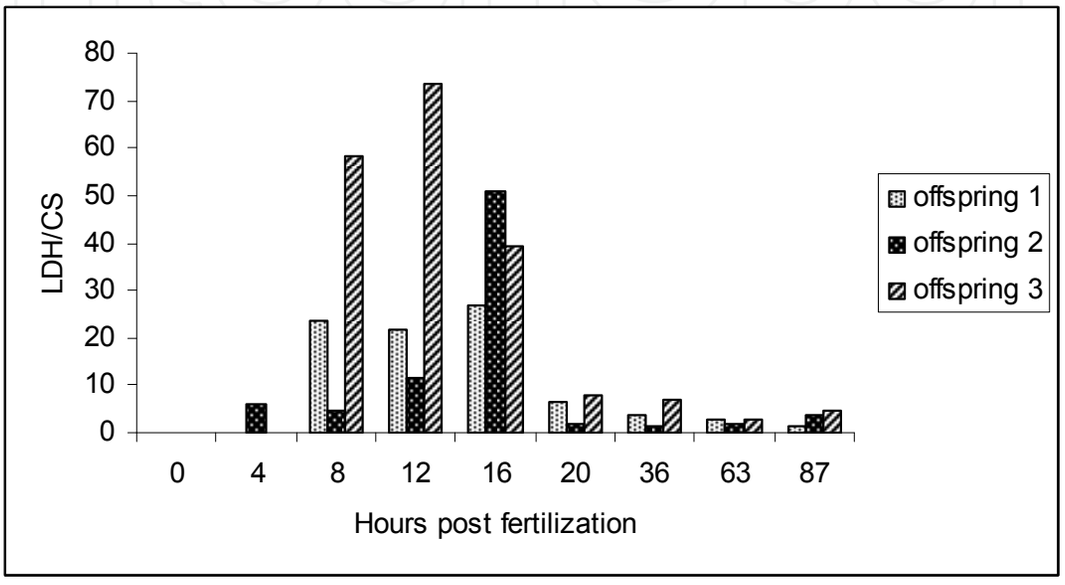


Figure 4. LDH/CS ratios in different developing phases of offsprings of 3 couples of *P. argenteus*.

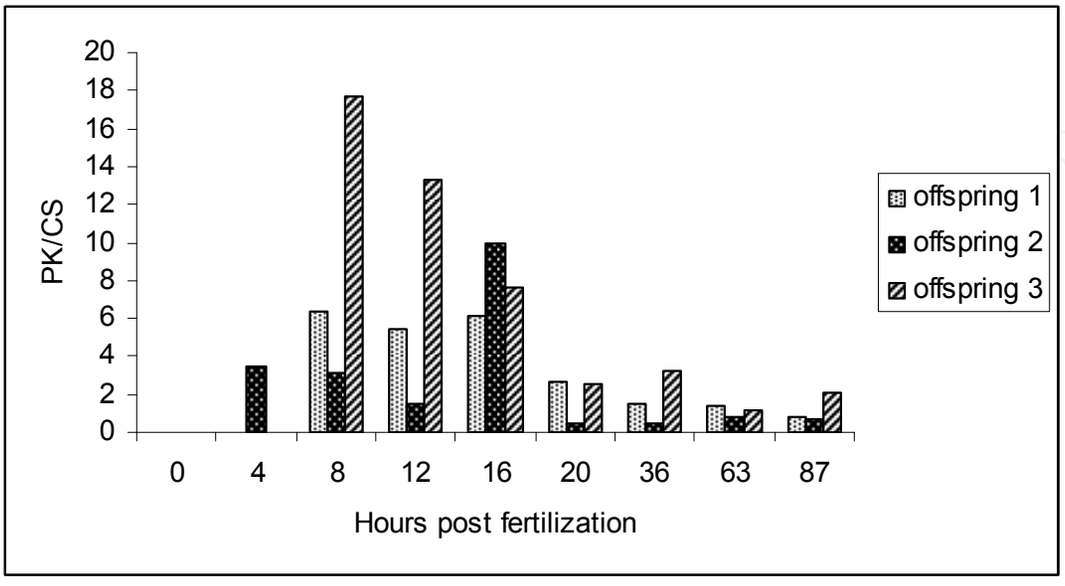


Figure 5. PK/CS ratios in different developing phases of offsprings of 3 couples of *P. argenteus*.

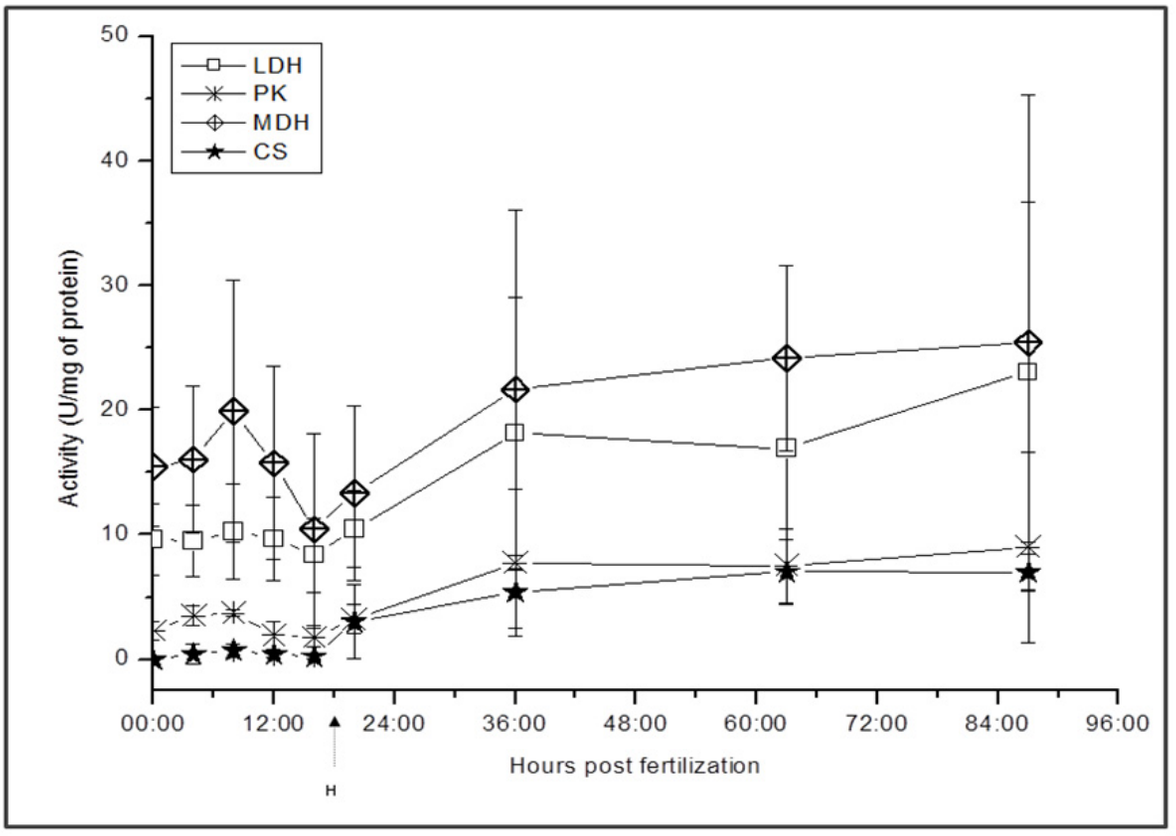


Figure 6. Quantitative glycolytics and e oxidatives average enzymes alterations in the early ontogeny of the offsprings of the 3 couples of *P.argenteus*, and H= hatching; 36 hours: starting *LDH-A**.

7. Discussion

Temporal iso/allozymes expression during early ontogeny of *P. argenteus.*

The investigation of gene expression in vertebrate's embryos indicated that developing program is determined in part by the transcripts of matter genes during the egg maturation and the other part by the expression of the embryos' genes after the fertilization [10].

The later *GPI** heteropolimeric detection at 16 hours post fertilization, indicates the both A and B subunits were simultaneously synthesized in many cells of embryos. These B homodimers were firstly detected, late at 36 hours post fertilization, like [11] also detected late expression for this locus in *L. cyanellus,* between 25 and 38 h.p.f.. According these authors, during the development of the *L. cyanellus,* the A homodimer levels were constant before the B subunits levels have been increased. According to [12] *P. scrofa*, detected the B homodimer activity after 12.5 hours after fertilization. According [13], A/B ratios change due the miotonic differentiation. This hypothesis can be accepted since the first skeletal muscle contractions were visible in *L. cyanellus*, *P. scrofa* and *P. argenteus* simultaneously at early subunits B expression.

The G6PDH from adults and their offspring presented one monomorphic locus liver restricted. In ontogenetic studies the single band was detected 36 hours post fertilization, revealing the late expression, like was observed by [11] in *L. cyanellus* at between 11 and 14 h.p.f.

The IDHP (IDH $NADP^+$- linked) catalyzing the carboxylation of 2-ketoglutarate, is not a strictly a Krebs cycle enzyme. Nevertheless, it is sometimes utilized by organisms to generate NADPH for fat biosynthesis. Under this conditions the carbon source for the reaction is usually glutamate, which is transaminated 2-ketoglutarate, the immediate substrate for the cell simultaneously generates isocitrate and thus helps to augment the pool of Krebs cycle intermediates (anaplerotic reactions) [14]. On the adult animals studied the expression of the IDHP loci was bidirectional divergent due the *IDHP-A** is skeletal and heart muscle predominant and the *IDHP-B** is liver restricted. The *IDHP-A** locus had an early expression while the *IDHP-B** had a late expression at 36 h.p.f.

On this study the LDH was product from two loci: the polymorphic *LDH-A** in skeletal muscle with two variant alleles **-16* and **29* and, the monomorphic *LDH-B** predominant in liver and heart. In the electrophoretic studies realized by the 3 offspring, the B subunit was the only one band observed in all stages of developing in the same level. This early expression of the B subunit was described for other teleosts as well as for other vertebrates [15; 16; 17]. The *LDH-A** loci product, the A subunit had been detected 36 h.p.f. in heteropolimeric form. Author [15] detected this locus activity at 34 h.p.f. it can be related to firstly muscle larvae contractions and has an important biochemical role, at anaerobic energetic modulation, that could be one requisite to muscle cells differentiation.

The sMDH-A* liver predominant, and muscle predominant sMDH-B* were non monomorphics and did not presented the typical temporal divergence, probably reflecting the heart loci expression, like detected by author [18] in P. scrofa. In L. cyanellus, the homodimer sMDH-A* and heterodimer sMDH-B were detected at the same time, early of development. B homodimer was visible at 28-31 h.p.f. [11].

There are two forms from vertebrate's malic enzyme (ME), the mitochondrial (less anodic mME) and citosolic (more anodic sME) [19]. In this work we called the ME-2 for mME and ME-1for sME . The ME-2 detected in muscle and heart appeared at 36 h.p.f. and the ME-1 liver-restricted was detected at 63 h.p.f. These enzymes provide the Krebs cycle with metabolic intermediates and play an important role in muscular gluconeogenesis *in situ* [20]. In the adult electrophoretic analysis the *ME-1** isoform product was detected only in liver, and the *ME-2** in heart and skeletal muscle, bidirectional divergent expression pattern. During the ontogeny both loci showed later expression: the *ME-2** at 36 hours post fertilization and the *ME-1** at 63 hours post fertilization.

The 6PGDH liver-restricted enzyme, showed a new variant allele, the **88*. And only one band for this locus was detected at 16 hours post fertilization. Thus there are two loci

detected at 16 hours post fertilization: the *GPI-B** muscle predominant and *6PGDH** liver restricted, from glycolytic and 6-phosphogluconate pathways respectively. On this moment the larvae movements were stronger inside of the egg, two hours before the hatching. Were detected, at the early stage of development, enzymes from glycolysis represented by GPI and LDH, the Krebs cycle by IDHP, mMDH, sMDH, gluconeogenesis, and lipogenesis shuttle malate-aspartate in the mitochondria by sMDH and ME activities. The Krebs cycle, it is important to mention, not only provides the catabolism of energy compounds, but also serves in the formation of equivalent reducing required to other metabolic pathways in the α-ketoglutarate generation of anabolic ways precursors like as oxaloacetate, (precursors in the amino acids formation) and citrate (which can be diverted to the fatty acids synthesis). Only ME, whose principal function is the provision of equivalent to reducing the fatty acids synthesis did not take any of his two loci expressed since the beginning of development.

The ADH and SOD, enzymes related to detoxification, studied in adults, showed no embryonic activity.

With 16 (6PGDH) and 36 (G6PDH) h.p.f. has been detected by the activity of the 6-fosfogluconate, important NADPH generator which is used in the synthesis of fatty acids, and pentose for the synthesis of nucleic acids. Author [21] analyzing the initial ontogeny of the *Erymizon sucetta*, there ova and in the early stages of their development, high levels of G6PDH and 6-PGDH suggesting that much of the glucose available free at that time, be directed towards the pathway of 6-fosfogluconate.

In *P. argenteus*, the asynchrony from paternal alleles were detected within 2 loci only at crossings where the parents had not alleles present in mothers, *GPI-B** and *6PGDH** and maternal alleles in the *GPI-B** during *P. scrofa* ontogeny asynchrony during activation of paternal alleles for *GPI-B** was also detected [12]. Beyond this locus, [1, 15, 18] show asynchrony paternal in: *LDH-A**, *sMDH*-A* and *B** and *GPI- A** in *P. scrofa*. Author [22] found, in studies of early ontogenetic development of zebra fish, *Danio rerio*, that the expression of the mRNA of the way ADH3 of alcohol dehydrogenase is of maternal origin, there asynchrony of expression between maternal and paternal genes.

This some late expression loci could be a result of its regulation on molecular and cellular levels as proposed by [10] and [23]. The molecular events that lead to patterns of ontogenetic early enzyme and isozyme exp**res**sion are under genetic control, direct or indirect, and represent the gene regulation in its broadest sense [24, 25]. Although this gene regulation occurs in the embryo, many genes appear to be cast from the first moments of the early ontogeny, ensuring that the basic enzymatic machinery is in operation in the most critical phases of the development process, in which the body just gets energy from the egg reserve and formed all the structures needed for their survival in the subsequent phases of life.

Apparently the asynchrony for father genes expression is more common that the mother because the egg or ovum brings a wealth of content cytoplasmic, and organelles. According

[26] cases of asynchrony are rare in offspring from couples of the same species, and can be caused by genetic changes in regulatory loci.

8. Enzymatic activities

According to [27], fat acids, glycogen and adenilic nucleotides (ATP, ADP e AMP) are the more important energetic substrates from mature egg.

The LDH low/high ratios calculated in the initial phases of the early ontogeny of *P. argenteus* (of 0 the 135 hours after the fertilization), average L/H values of each phase of the initial ontogeny of the 3 couples sample that in the interval of 0 to the 12 hours after fertilization occurred the biggest inhibition of the LDH and, therefore, the biggest amount of B subunits. After this period, occurs the reduction of this inhibition and reasons L/H, produced, probably, for the synthesis of subunits, whose detection occurs in electrophoresis 36 hours after the fertilization.

The analyses of the reasons between indicating enzymes of anaerobic metabolism and the MDH must be seen with exceptions when we try to measure the level of aerobic metabolism, a time that this enzyme is involved also in the malate-aspartate shuttle and, at least part of its activity, it must be related with this function. When we used laccolitic enzyme in the reason is the LDH, average values shows predominance of the oxidative metabolism / malate aspartate shuttle up to 87 hours after the fertilization, with its higher value with 8 hours after the fertilization when it is differentiated head extremity and the tail extremity. After this phase, considering the average reasons, an increase of the glycolytic metabolism occurred, and in the morphogenetic development, the movements if they intensify inside of the egg. When the glycolytic enzyme of the reason was the PK, in all phases and in the averages of these ratios we observed predominance of the oxidative metabolism and/ or malate-aspartate shuttle. The biggest reason was detected 36-87 hours after the fertilization when was detected an increase of the glycolytic metabolism.

The observed values in the offspring of the 3 couples of *P. argenteus*, for LDH/CS and average of these (even so not homogeneous, what it can have been consequence of the crude extract use), had disclosed predominance of the anaerobic metabolism in all the analyzed phases, being this, bigger between 12 and 16 hours after fertilization decaying after that. The values of PK/CS and average of these not so high how much of LDH/CS, they after show to its maximum (anaerobic metabolism) 16 hours post fertilization.

The water of the nursery used for the *P. argenteus* development, contained on average 4.3 mg oxygen/liter, more than what the water where the adult fish had been collected, therefore the water that comes of the barrage passes for aeration process before arriving at the nurseries. Thus, the higher enzymatic activity, in all the phases of the initial ontogeny of the 3 couples of *P. argenteus*, kept in these conditions, was of the LDH, characterizing the predominance of the anaerobic metabolism. The second more active enzyme was the

MDH what it would characterize high activity of the function malate-aspartate shuttle. The aerobic metabolism, characterized for the activity of the CS, lowest of the 4 analyzed, it was remained low. Soon after the hatching, the activities of 4 enzymes had suffered increase what it would be in accordance with the active movement of the larvae soon after hatching according to [28] with increase of the carbohydrates metabolism described for [29].

Studies carried through with the teleost *Misgurnus fossils* [30] show the dependence, during its initial development, of the use of the stored glycogen, as energy source. If it will be possible to surpass these results for other species of teleosts, this would be the only substratum of glycolysis in the embryos. According to [25], glycolysis and the phosphogluconate way are the more important energy sources for the biosynthetic activities and maintenance of the embryo morphology. The glucose is, doubtlessly, important in the period between the fertilization and after-hatching, as indicated for the high levels of B subunits from LDH and A subunits from GPI, as well as the high activity of the LDH, during this period and for the appearance of the delayed subunits, in *P.argenteus, P.scrofa* [1, 12, 15, 18] and in *E. sucetta* [21].

The subunits B predominance since the first phases of the initial ontogeny of *P.argenteus*, kinetically adjusted to the aerobic metabolism, in the lactate oxidase function, would supply, it would supply to private the shuttle function malate-aspartate of the MDH, enzyme with the highest activity detected here.

The adults of this species are migratory in time of piracema (November to February) and the São Francisco basin river rough relief and the fish have to use of essentially anaerobic metabolism (it has pulled out) to cross these obstacles.

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