Differential Expressions of G6PD and Alkaline Phosphatase Isozymes Associated with Ontogeny and Air-breathing Transition in *Channa punctata*

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Abstract

Differential expressions of glucose-6-phosphate dehydrogenase (G6PD) and alkaline phosphatase (ALP) isozymes which are associated with distinct ontogenetic and developmental changes have been monitored using native PAGE. Prehatching organogenesis involving ectoderm and mesoderm coincided with temporal expression of G6PD-6a, G6PD-6b and ALP-2*a* isozymes. Abrupt expression of ALP-3 isozyme and silencing of G6PD-6b occurred as late as 14-15 day post-hatching at the time of the transition to air-breathing. These results demonstrate that repertory of G6PD and ALP isozymes in *C. punctata* is modified to meet the bimodal breathing requirements related to glucose and lipid metabolism despite quite advanced stage of development. Some specific features in the distribution of isozymes of the adult tissue have also been documented.

Introduction

Differential expressions of lactate dehydrogenase (LDH) isozymes in teleosts (Masters and Holmes 1975; Whitt et al. 1975; Whitt 1981; Almeida Val and Luis Val 1993; Klyachko and Ozernyuk 2001) and their profiling during hypoxia (Maxime et al. 2000; Powell and Hahn 2002) have been the subjects of much attention. More recently, stress studies on LDH isozymes have been coupled with free radical-load indicators SOD/catalase isozymes (Luschak et al. 2001, 2005). Rather less information is available on differential expressions of other isozymes that include Glucose-6-phosphate dehydrogenase (G6PD) or alkaline phosphatase (ALP).

In this report we demonstrate that in *Channa punctata*, novel expressions of G6PD and ALP isozymes coincide with the transition to air-breathing. This is unlike LDH isozymes, where the expressions of both A and B subunits were ~12 days ahead of this transition and bimodal respiration was supported by an excess in the level of anaerobic LDH-A (Ahmad and Hasnain 2005). *Channa punctata* is a small hardy fish of economic importance which is assisted by air-breathing organs (ABO) in surviving periods of water scarcity. Its early larvae are, however, exclusively water breather and partitioning into air and water-breathing occur 14-15 day post-hatching when morphological features of the larvae closely resemble adults

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(Banerji 1974). Accessory ABO whose functioning is marked by initiation of air-breathing is a pouch like extension of the gill-chamber known as suprabranchial chamber (Munshi 1962; Hughes and Munshi 1973; Singh et al. 1982).

Materials and Methods

Procurement and breeding of fish

Mature live *Channa punctata* Bloch were brought to the laboratory from a local fish market of Aligarh. They were kept in well-aerated water tanks for acclimation and hypophysectomized employing the standard protocol of Banerji (1974). Twin experiments were carried out at the onset of the monsoon season.

Identification of ontogenetic and developmental stages:

Spawns of *C. punctata* were monitored microscopically at a magnification of 45x. Following identification, various stages were documented on Kodak color film. Selected stages are shown in Fig. 1.

Extract preparation and electrophoresis

Homogenates of a pooled larval stage or adult tissues were prepared in 50 mM Tris-HCl buffer of pH=7.5, centrifuged and analyzed by vertical (10 x 9.4 x 0.01 cm) 7.5 % polyacrylamide gel electrophoresis (PAGE) according to the protocol described earlier (Ahmad and Hasnain 2005).

Histochemical staining of isozyme bands and documentation

This was performed according to the protocol of Shaw and Prasad (1970). Stained gels were fixed in 7 % acetic acid (v/v) and documented by scanning.

Results

Photographs of ontogenetic and developmental stages of *Channa punctata* selected for the present investigations are shown in Fig. 1. Previous record of these events is based on line diagrams (Banerji 1974). Figs. 2 and 3 display the correlation between specific ontogenetic while developmental events and temporal expressions of the investigated isozymes of *C. punctata* are summarized in Tables 1 and 2.

According to IUPAC-IUB nomenclature (1971), G6PD isozymes are numbered from anode to cathode and closely stacking bands have been recognized as subforms of a particular G6PD isozyme (Champion and Whitt 1976; Chingjiang and Schröder 1984; Whitt 1981). Based on this, *C. punctata* three classes G6PD-5, -6 and -7 were recognized in the relative order of decreasing electrophoretic mobilities from anode to cathode (Fig. 2). Among them, G6PD-6b is the major embryonic activity from 13 h post-fertilization to 24 h post-hatched larvae (Fig. 2a, Table 1). The G6PD-6a and G6PD-6b appear as distinct activities 13 h post-fertilization, which gradually intensify up to 24 h post-hatching. At a low intensity, they persist in extracts of larvae up to 11 days. The slowest migrating single G6PD-7 isozyme

occurs from fertilized egg onwards (Fig. 2a). This isozyme co-stacks with the single band of hexokinase (results not included here) in larval stages and tissues of adult *C. punctata.*

Two banded G6PD-5 isozyme, exists as a strong activity in the brain, eye and liver (Fig. 2b). As the bands of low intensity, it is present in other tissues of *C. punctata*, as well. Gut and muscle lack single banded G6PD-6 and G6PD-7 and/or hexokinase of the identical mobility, which are rather strong to moderate activities in other tissues (Fig. 2b).

The PAGE patterns of alkaline phosphatase isozymes are shown in Fig. 3. Following the scheme of Whitmore and Goldberg (1972), activities are numerically marked as ALP-1 to ALP-3 from cathode to anode. Ontogenetic patterns reveal the existence of two activities of alkaline phosphatases, ALP-1 and ALP-2 (Fig. 3a). The ALP-1 is a single band of approximately equal intensity in ova and all the embryonic stages persisted up to 24 h post hatching (Fig. 3a). The ALP-2*a* showed temporal expressions which gradually intensified to become a predominant activity in 3-day old larval extract (Fig. 3a). The ALP-2*b* is present from the egg onwards and follows a trend similar to ALP-2*a*. Low intensity ALP-3 is also detected in 15 day old larval homogenates (Fig. 3b, Table 1).

The ALP-3 is detected as a slurred band in almost every tissue showing a moderate expression. Maximum activity of ALP-2*a* is noted in the brain, eye and heart while that of ALP-2*b* is in the kidney and ovary (Fig. 3b). Trace activity of ALP-2*b* is also observed in the liver and muscle. Least anodal ALP-1 is detected in tissues other than the brain and shows maximum activity in liver and muscle but differs in electrophoretic mobility (Fig. 3b).

Table 1. Relative level of activities of G6PD and ALP isozymes estimated by densitometric analysis of individual lanes by Scion Imaging. The notions are : high activity (+++); intermediate activity (++); low activity (+); present in traces (±) and total absence (-).

	Fertilized egg	7 h	13 h	19 h	22 h	**Hatchling**	12 h	24 h	3 days	11 days	15 days	30 days
Glucose-6-phosphate dehydrogenases												
G6PD-7	+	+	+	+	+	+	+	+	+	+	±	±
G6PD-6b	-	-	+	+	+	+	+	+	±	±	-	-
G6PD-6a	-	-	+	+	+	+	+	+	±	±	±	±
G6PD-5b	+	+	++	++	++	++	++	++	±	±	±	±
G6PD-5a	+	+	++	++	++	++	++	++	±	±	±	±
Alkaline phosphatases												
ALP-1	+++	+++	+++	+++	+++	+++	+++	+++	+	+	±	±
ALP-2*a*	-	-	+	+	+	+	+	+	++	++	-	-
ALP-2*b*	+	+	+	++	++	++	++	++	-	-	±	±
ALP-3	-	-	-	-	-	-	-	-	-	-	±	±

Table 2. Temporal expression of a specific isozyme and its correspondence with the distinct change (s) in the morphological features.

| Stage #	Characteristic feature	Expression of isozyme gene
13-h post-fertilization	Clear optic cups and formation of notochord	Appearance of G6PD-6a, -6b, ALP-2*a*
24-h post-hatching	Pectoral fin bud and buccal invagination prominent	Isozymes of G6PD and ALP except ALP-3 has maximum intensity
3-day post-hatching	Yolk completely absorbed, heart shaped abdomen	Intensity of G6PD-5, -6 declined; ALP-2*b* disappears
15-days post-hatching	Initial sign of respiration	G6PD-6b disappears; ALP-3 appears
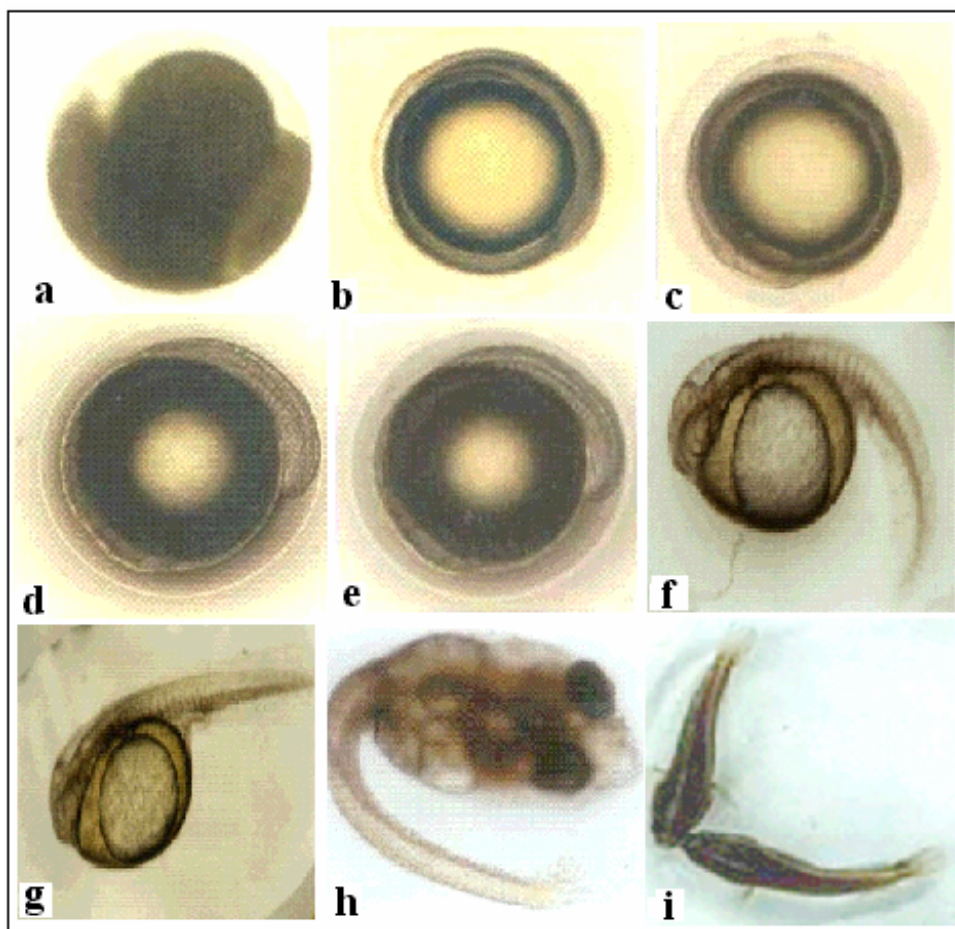
Fig. 1. Post-fertilization morphological and developmental changes in ovum and larvae of *Channa punctata* Bl. at 45x. The figures are: fertilized egg (a) and subsequent changes at 7 h (b); 13 h (c); 19 h (d); 22 h (e) and hatchling (f). Post-hatching developmental stages showing 12 h (g), 11 days (h) and 15 days old larvae (i).
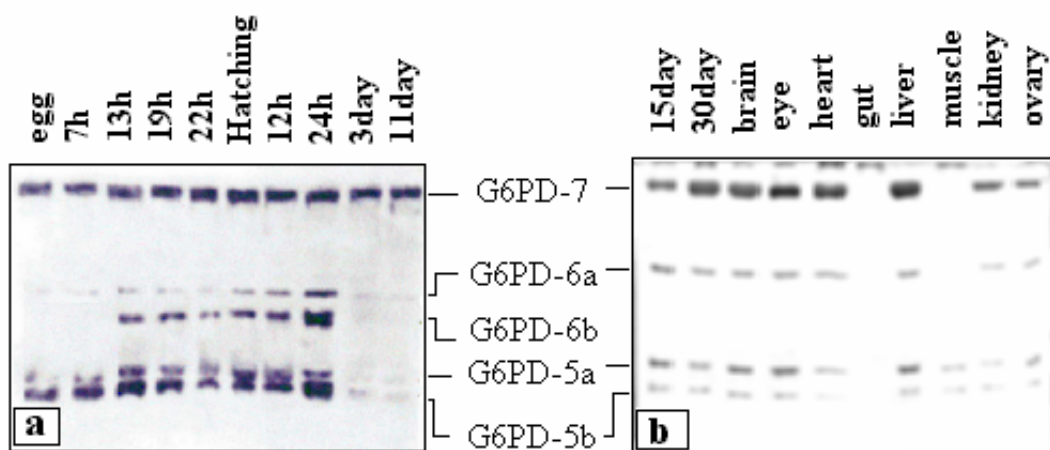

Fig. 2. Zymograms showing temporal schedule of expression of Glucose-6-phosphate dehydrogenase (G6PD) isozymes in *Channa punctata*

(a) During ontogeny : Lanes 1-5 = fertilized egg, 7 h, 13 h, 19 h and 22 h embryo), in hatchling (lane-6) and post-hatching advanced larval stages (lanes: 7-10 = 12 h, 24 h, 3 days and 11 days old) of *C. punctata.*

(b) During development (lanes: 1 & 2=15 and 30 day old developed larvae) along with the zymograms of the adult tissues (lanes: 3-10 are brain, eye, heart, gut, liver, muscle, kidney and ovary, respectively).
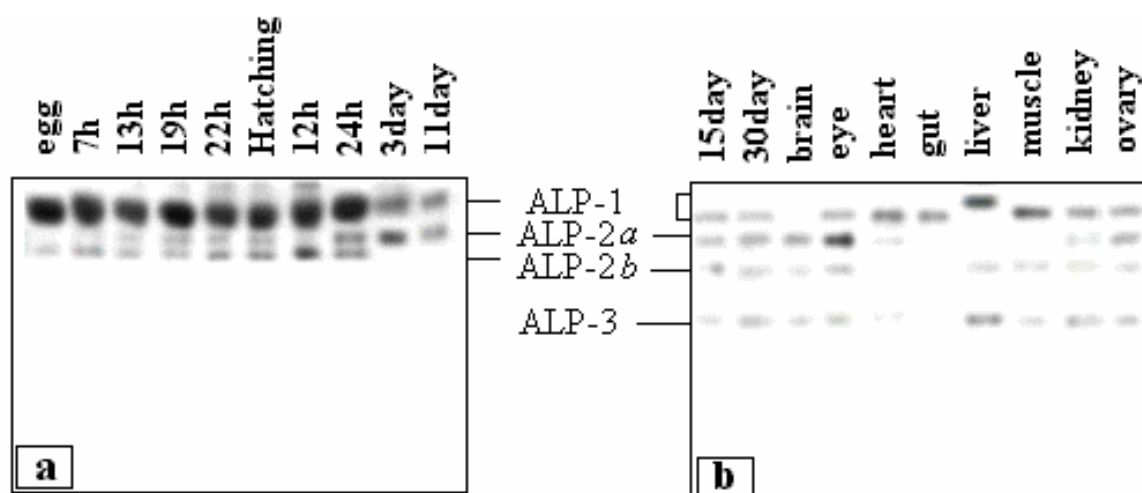
Fig. 3. Zymograms showing temporal schedule of expression of alkaline phosphatases (ALP) activity in *Channa punctata*.
(a) Ontogeny and various developed stages (sequence of lanes is same as in Fig. 2a).
(b) Differential expression of ALP isozymes during development (lanes: 1 & 2) and the zymograms of tissues of adult *C. punctata* (lanes: 3-10). Sequence is same as given in Fig. 2b.

Discussion

Ova and all developmental stages of *C. punctata* float near the water surface, while those of air-breathing catfish, *Heteropneustes fossilis* sink to the bottom (Sherwani et al. 2001). This may be attributed to a broader perivitelline layer which absorbs more water to impart buoyancy to *C. punctata* ova.

Slurred resolutions of G6PD and ALP isozymes have been attributed to glycosylation of these isozymes (Whitmore and Goldberg 1972; Champion and Whitt 1976; Basaglia 1989). It is obvious that just as in starch gels (Chippari-Gomes et al. 2003), glycosylation interferes with crisp resolution during electrophoresis in polyacrylamide gels as well (Figs. 2 and 3).

Both of the investigated isozymes are metabolically important. While G6PD catalyzes first reaction in pentose-phosphate pathway which provides adequate levels of NADPH for reductive synthesis reactions, ALP is intimately related to lipid metabolism. Our results demonstrate that during differentiation in *C. punctata*, metabolic demands concerning these isozymes are met in three ways: (i) by novel temporal expressions typically represented by G6PD-6a, -6b and ALP-2*a* isozymes; (ii) by an increase in already existing isozyme G6PD-5a and G6PD-6a activities, and (iii) by silencing G6PD-6b isozyme expression. Out of them, G6PD-6a, -6b and ALP-2*a* display a correspondence with the differentiation and organogenesis involving ectoderm and mesoderm (Table 2).

The results also show that the most striking post-hatching adaptive change, that is, the transition to bimodal respiration requires the expression of a new alkaline phosphatase activity, the ALP-3 isozyme. This is in contrast with LDH, where 48 hr post-fertilization expression of anaerobic A_4 isozyme is ~12 days ahead of the air-breathing transition (Ahmad and Hasnain 2005). Moreover, due to disappearance of G6PD-6b in 15 day old transitional larvae, zymograms become adult tissue-like (Fig. 2b). In this respect, our results are similar to a

previous report on platyfish, where new G6PD isozymes characterized adults and lacked isozymes typical of the young stages (Scholl 1973). Parallel with the expressions of above isozymes, relative intensities of the two banded 'house keeping' G6PD-5a also displayed a decrease. Collectively, the differential profiles of isozymes clearly indicate a modification in regulation of metabolism correlated with bimodal breathing following transition to air-breathing as late as ~12 day post-hatching. At this advanced stage of development, the external morphology of larvae shows close resemblance with that of the adults (Fig.1i).

Lipid metabolism until 3rd day post-hatching was intimately associated with G6PD-5a, -5b and G6PD-6a, -6b, ALP-1 and ALP-2*b* isozymes. Since these isozymes declined with complete absorption of yolk, they obviously have a role in yolk absorption, while ALP-2*b* finally disappeared. The G6PD-6a, due to persistence in pre-airbreathing larvae, must be an essential requirement in pentose-phosphate pathway up to air-breathing. In adult fish also, G6PD isozymes display tissue specificities. For instance, G6PD-5a and G6PD-5b isozymes are absent in gut and thus they are not essential activities of this shunt in it. Similarly, gut along with skeletal muscle lacked G6PD-6a and G6PD-7 indicating independence from essentiality of these isozymes (Fig. 2b).

Appearance of ALP-2*a* at 13 h post-fertilization (similar to ALP-2*b* discussed above) and its gradual intensification suggests a correlation with the hydrolysis of lipids (Table 1). Low levels of ALP-3 were detectible either in transitory larvae (15-day old) or more developed stages which followed this transition (>30 days old). It occurs exclusively in all the adult tissues except gut, specifically in lipid-rich brain, ovary, eye, heart and kidney of *C. punctata*. In this respect, it differs from the reported cases of water-breather trout and guppy, where lipid hydrolyzing phosphomonoesterases are restricted to kidney and intestine only (Whitmore and Goldberg 1972; Cvancara and Huang 1978).

Conclusion

In conclusion, the schedule and regulation of G6PD and ALP gene expression in *C. punctata* exhibited substantial species specificity with respect to specific ontogenetic stages and tissue distribution. More significantly, our results for the first time demonstrate that despite the advanced state of development and growth, a modification in regulatory patterns of these isozymes accompanies the switchover to bimodal respiration.

Acknowledgements

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