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Functional Plasticity of Transferrins from Four Air-Breathing Channids (Genus *Channa*: Channidae) and its Relevance to their Survival

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Abstract

Channids are commercially important teleosts which can be cultured in shallow oxygen deficient waters due to accessory air-breathing. The information on metabolic plasticity of channids is inadequate; despite being fundamental to overall survival that determines their commercial output. To find out if transferrin (Tf) of genus *Channa* displays trends in functionality which correlate to their survival in shallow waters with low oxygen, we have compared iron-binding and pH dependent dispensation of bound iron in four channids (*Channa punctatus*, *C. gachua* and; *C. striatus* and *C. marulius*). Transferrin is central to iron metabolism as the main iron transporter and also intimately linked with oxygen-sensing. Our results show that their Tfs retain iron in exceptionally high amounts at acidic pH. Securing free iron at low pH should be imperative if respiratory acidosis occurs, since under low oxygen free iron (as Fe^{3+}) precipitates even at physiological pH. We suggest that retaining bound iron by Tfs at low pH values is a key factor in accomplishing iron homeostasis in channids with impact on fishery output.

Keywords: Genus *Channa*, Paddy-cum-fish culture, obligate air-breathing, Transferrin, pH-dependent iron release.

Introduction

Species of genus *Channa* (Channiformes: Channideae), commonly known as snakeheads or murrels are widely distributed in China, Southeast Asia, Eurasia, Russia and Africa (Wee, 1980; Musikinthorn, 2003). Some of them are commercially important, since they are the component of fish culture in Southeast Asian countries; particularly in shallow oxygen deficient waters and paddy fields (Chakrabarty, 2006; Mehrajuddin *et al*., 2009). These fishes survive aquatic environment of low oxygen availability because the respiratory deficit is compensated by accessory air-breathing (Singh, 1993). The adaptive trait of obligate air breathing by channids has been acquired during the course of long evolutionary descent from Miocene. It was the time when ancestral forms originated in hypoxic waters of marshy extension of Siwalik in Yunan, mainland China (Sahni and Khare, 1977).

Just as in other vertebrates, serum transferrin (Tf) is central to iron recycling in fishes also. Transferrin is bilobal single polypeptide with each lobe having one iron binding domain (Baker *et al*., 2002) and M_r of 70-80 kD (Stratil *et al*., 1983; 1985). Tf is multifunctional protein which contributes to survival of fishes at various stages of life. Iron-binding capacities of Tf phenotypes differently influence sperm motility of carp (Wojtczak *et al*., 2007). It is also reported to protect spermatozoa from mercury and cadmium poisoning (Dietrich *et al*., 2011). Iron binding capacity of Tf polymorphs has been correlated with post-hatching viability of fish larvae, which ultimately influences genetic composition and size of fish population (Hershberger and Pratschner, 1981; Nabi *et al*., 2003). Tfs are part of innate immune response in teleosts (Uribe *et al*., 2011) and restrict microbial growth (Winter *et al*., 1980). In addition, proteolytic fragment of Tf also mediates macrophage induction in teleosts (Stafford and Belosevic, 2003).

This study documents yet another important functional plasticity of highly purified serotransferrin (Tf) from four species of genus *Channa* (Channiformes: Channideae) which is of direct consequence to fishery output, because of implications in the survival. This is the first ever report on better iron retention at low pH values from teleost Tf, which is an adaptive feature essentially required to protect fish from free iron toxicity. The present study on Tf is part of our efforts to correlate proteome with hardy nature of channids. Previously

published reports have demonstrated exceptional heat stability of multiple hemoglobins and parvalbumins (Hasnain and Jabeen, 2001; Ahmad and Hasnain, 2006). LDH isozymes, myofibrillar proteins and polymorphic myosin heavy chain of channids also exhibit functional plasticity (Ahmad and Hasnain, 2005; Arif *et al*., 2007; Ahmad, 2009; Ahmad and Hasnain, 2013). While other fishes may temporarily switch on to expression of heat shock proteins (Kayhan and Duman, 2010), thermostability of proteins appears to be inherent characteristic of air-breathing channids (Hasnain and Jabeen, 2001; Ahmad *et al*., 2007a; Arif *et al*., 2007). The functional modulations and structural stability shown by the above cited channid proteins suggest a wider functional plasticity and proteome stability that encompasses other metabolic processes. Survivorship studies on channids have so far dealt mostly with aspects such as seed production of channids, their biology or diet, metal toxicity and survival of larvae and young stages of *Channa punctatus* or *C. striatus* (Marimuthu and Haniffa, 2006; Haniffa, 2008; Murugan *et al*., 2008; Qin *et al*., 1997; Mehrajuddin *et al*., 2009).

Materials and Methods

Source of Samples

Live fish were procured from local suppliers of Aligarh and nearby delivery centers (latitude 27°30′ N; longitude 79°40′E) of the state of Uttar Pradesh (India). *Channa punctatus* (Bloch), *Channa gachua* (Hamilton) known as 'spotted and dwarf snakehead', respectively are small fishes. Specimens used in this study were in the range of 12-18 cm in length and ~80-100 gm in weight. In this study 20-38 cm long young specimens of *Channa striatus* (Hamilton) and *C. marulius* (Bloch) weighing ~150-800 gm were used. Within the specified size range specimens of all four channids were immature. Sex discrimination is not possible at these stages. Commercially available human transferrin (Fluka biochemica, Switzerland) has been included as the reference.

Following laboratory acclimatization of live catches for 16 h at 12-15°C, blood was collected from anaesthetized fish. Tricane methosulfate (MS222) in a concentration of 20mg/L anaesthetized fish within ~3 min.

Isolation and Storage of Sera

Blood was taken from live fish specimen by cardiac puncture using sterilized plastic syringe equipped with #23 needle. Serum was pipetted out from the clotted blood and centrifuged at 3,000 rpm for 10 min to sediment contaminating blood corpuscles. Clear serum was collected discarding sedimented blood corpuscles. Sera were analyzed immediately or stored at -20°C until further analysis.

Native Polyacrylamide Gel Electrophoresis (PAGE)

For routine screening of serum samples, 7.5% non-denaturing PAGE was carried out in SDS-free discontinuous buffer system as reported previously (Nabi *et al*., 2003). Acrylamide-linker ratio was 30:0.8. The upper gels were 3% in acrylamide-bis and 0.125 M Tris-HCl (pH 6.8) and lower 7.5% gels were in 0.375 M (pH.8.6). Tris-glycine (25 mM-0.25M) of pH 8.3 was the running buffer. A pre-run in 1x upper gel buffer facilitates sharp entry into gel. The native gels were stained with Coomassie Brilliant Blue (CBB R-250) at room temperature. Following electrophoresis, gels were stained in CBB-R-250 (250mg) destained with 10% acetic acid containing 5 ml methanol. Commercially obtained human Tf was used as the reference transferrin.

Identification Tf Bands (Electromorphs) in Native Gels

Just before loading, 4µl serum sample was mixed with 1 µl of 0.1M ferrous ammonium sulfate solution. Following electrophoresis, gels were incubated for 30 min in hydroxylamine hydrochloride solution (100 mg/50 ml in 7% acetic acid). Subsequent addition of specific stain solution (25mg Nitroso-R/ml of distilled water) developed Tf as deep green bands (Møller and Naevdal, 1966).

Purification of Individual Tf Band (Electromorph) to Homogeneity

Serum samples with identical phenotypes were pooled to purify Tf by a two-step protocol. Firstly, partial purification was achieved by precipitating Tf in the range of 55%-95% saturation of ammonium sulfate (Ahmad *et al*., 2007b). The precipitate was exhaustively dialyzed against four changes of distilled water. For preparative native PAGE, partially purified and dialyzed Tf samples were loaded on to a wide single slot preparative gel and run according to native PAGE protocol essentially as described above. Band identified as Tf were subsequently cut out and protein was electroeluted as per standard protocol (Nabi *et al*., 2007; Ahmad and Hasnain, 2013). Electroeluted samples of each Tf electromorph or isoform was crosschecked for homogeneity by native PAGE. Identical Tf isoforms were pooled and lyophilized for subsequent experiments.

Neuraminidase Digestion

Purified Tf isoform was digested with neuraminidase according to the protocol of Stratil *et al*., (1983). The ratio of Tf to neuraminidase was 2:1 and the digests following 24 h of incubation were analyzed by SDS-PAGE outlined below.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Molecular weights (M_r) of pure Tf preparations were estimated following SDS-PAGE in the system of Doucet and Trifaro (1988). In this system, upper stacking gel was 4% and contained 70 mM Tris-HCl (pH 6.7), 5% glycerol and 4 mM EDTA. Separating gel was 10% in 0.2 M Tris-0.1 M glycine. All buffers including running buffer contained 0.04% SDS. Top reservoir buffer was 0.04 M tris-0.06M glycine (pH 8.5) while the bottom reservoir buffer was one time diluted top reservoir buffer. SDS-PAGE gels were silver-stained. Native as well as SDS gels were scanned using Gel-Pro analyzer (Media Cybernetics, USA; Version 3.0).

Isoelectric Focusing (IEF)

The *p*I values of different Tfs were determined against the marker proteins (Pharmacia Biotech, Sweden) following focusing on 1 mm thick polyacrylamide gel containing 5%T, 3%C in Pharmalyte of pH range 3-10 containing 10% glycerol. The focusing was performed at 1500 V, 50 mA for 2 hrs. Other details of the protocol were as per the guidelines in Supplier's manual.

Iron Binding Analysis

Diferric Tf isoform of *C. punctatus*, *C. gachua*, *C. striatus*, *C. marulius* and human were stripped of iron according to Palmour and Sutton (1971) by exhaustive dialysis against 1.0 M citric acid. Dialysis against distilled water with 4 changes within 24 hrs followed. The apotransferrin of each species was dissolved at a known concentration (1.0µg/ml) in 25 mM Tris-HCl (pH 7.5) that also contained 30mM $NaHCO_3$. Iron binding of Tf was monitored by successive addition of FeNTA (0.1 µg / µl) at 470 nm on Genesys-10 UV-Visible Scanning Spectrophotometer (Thermospectronic, USA). FeNTA has the composition : 0.19 g nitrilotriacetic acid per 3 ml of distilled water; 1.0 M NaOH (2 ml); 0.5 M ferric chloride (2 ml); all in 1 L final volume of distilled water. Atoms of iron (Fe) bound per molecule of Tf were calculated as reported by Welch (1990).

Release of Iron from Diferric Transferrins at Different pH Values

Buffers of pH values from 6.5 to 2.0 were used to monitor release of bound iron as the function of pH. Aqueous solution of diferric Tfs from each of the four *Channa* species and human Tf (100 µl at 50 µg/100 µl) were added to 300 µl buffer of desired pH (Welch, 1990). Buffers were 0.2 M sodium acetate in the range of pH 5.5 to 3.7, and 0.2 M glycine-HCl was used for pH 2.5. Tris-maleate buffers of pH 7.0-6.5 were used for pH above 5.5. For each Tf, the decrease in absorbance at 470 nm was noted and plotted as percentage against different pH values.

Results

Identification of Tf Variants in Channids by Native PAGE

During routine screening of sera for Tf variants, gels were developed with CBBR-250 staining. However, as described under Materials and Methods, the selected replica native gels were also developed with specific staining to identify Tf bands in CBB-stained gels. The criterion for channids has been validated by partial biochemical characterization of Tf variants in *Channa punctatus* (Nabi *et al*., 2003; 2007). The sera of individual species were pooled for purification. The purified Tf bands from each *Channa* species were homogeneous when examined with native PAGE analysis, as shown in Figure1A and in IEF profiles (Figure 2). Native PAGE profiles of total serum (ts) with purified Tf band (pTF) of different species are compared in Figure 1A. Stacking of purified pTf coincides with pre-identified location of a Tf band in PAGE profiles of phenotypes from pooled ts (Figure 1A). The individual purified Tf from each of the four channids was subjected to further biochemical characterization, including iron-binding and pH dependent-release of bound-iron.

Effect of Neuraminidase Digestion on Molecular Weight (M_r) of Tf Isoforms

SDS-PAGE patterns of purified Tf isoforms of all four channid species are shown in lanes 3, 5, 7 and 9 of Figure 2B, which demonstrates that M_r of purified Tfs do not differ substantially, since they stack as a band of 71-72 kD. Following removal of carbohydrate moiety by neuraminidase digestion, a reduction of similar magnitude is observed in M_r values of each channid transferrins (lanes 4, 6, 8 and 10). As compared to M_r of 80 kD calculated for human Tf (lane 1), the M_r range of channid Tf is typically lower. This is also evident from digested human Tf Figure. 2 (lane 1d), which stacks as a band of higher M_r as compared to glycoprotein-free states of channid Tfs (lane-2) stacking as bands of ~66-67 kD. The results confirm that channid Tfs consists of sialic acid as an essential constituent of carbohydrate moiety.

Isoelectric focusing (IEF) Profiles of Purified Tf Preparations

The IEF profiles of purified Tf band of all four channid species are shown in Figure 2. Each preparation focused as the single band with pI value of 4.5 for *C. striatus* and *C. marulius* and 4.7 for *C. punctatus* and *C. gachua*. The selected pTf bands of

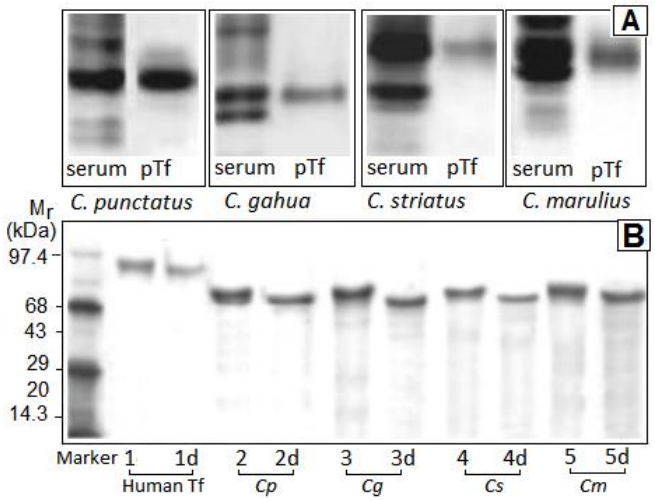

Figure 1. A: Native PAGE profiles showing homogeneity of purified Tf (pTf) of *Channa punctatus*, *Channa gachua*, *Channa striatus* and *Channa marulius.* For each *Channa* species, lane 1 shows relevant portion of PAGE profiles of total sera (ts) and lane 2 purified Tf (pTf) of corresponding species. pTf was used for biochemical characterization.
B: Comparison of SDS-PAGE profiles of purified control pTf with neuraminidase digested pTf. Lane 1, 3, 5, 7 and 9 are undigested controls of human pTf and of *C. punctatus*; *C. gachua*; *C. striatus* and *C. marulius*. Digested pTf lanes 2, 4, 6, 8 and 10 of each species show the distinct reduction in M_r values as compared with corresponding undigested controls. M is the molecular weight marker. All the lanes were developed with silver stain.

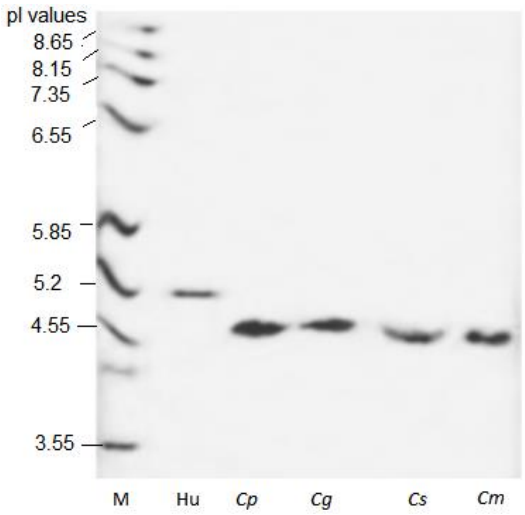

Figure 2. Isoelectric focusing patterns of pTf of *Channa* species and human pTf. Lanes from left to right are : 1, IEF markers (M); 2, Hu (purified human Tf); 3, *C. punctatus*; 4, *C. gachua*; 5, *C. striatus*; and 6, *C. marulius*. Gels were 5% in polyacrylamide, 1 mm thick and contained 10 % glycerol. Pharmalyte of pH range 3-10 was used. The focusing was performed at 1500 V (50 mA) for a period of 2 hours.

each species are either homozygous or devoid of discernible *p*I differences under conditions applied in this study.

Iron Binding

When purified apo-Tfs were titrated with FeNTA in the presence of bicarbonate ion, absorption at 470 nm followed a sigmoid path of increase with an end-point corresponding to ~2 atoms of iron/ mol (Figure 3). Each iron-binding curve was species-specific in terms of profile and iron saturation capacity. The requirement of iron saturation in the decreasing order was : *C. punctatus* >*C. gachua* >*C. marulius* >*C. striatus* (Figure 4).

Release of Bound Iron from Diferric Tfs

The most remarkable characteristic of diferric fish Tf is that it retains >20% bound iron even at pH 3.0 (Figure 4A-B). At this pH, human Tf is stripped off the bound iron. Among channids, Tf of *C. gachua* retains highest amount (%) of bound iron at acidic pH. The shapes of iron release curves of *C. gachua* and *C. punctatus* transferrins are similar. Likewise, shape of iron dissociation curve of *C. marulius* Tf

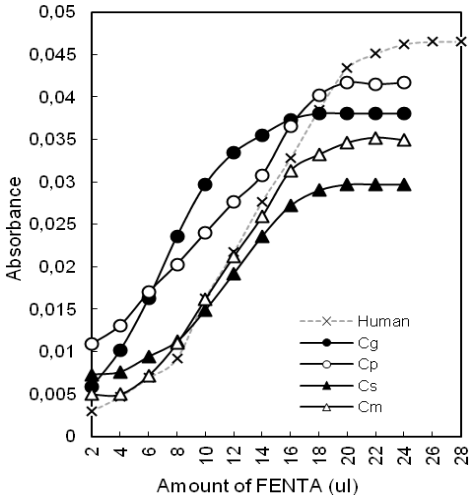

Figure 3. Iron-binding curves of purified apoTfs of four channids and human. Apotransferrin pTf (0.5 mg/ml) of *C. punctatus*, *C. gachua*, *C. striatus*, *C. marulius* and human was dissolved in 25 mM Tris-HCl (pH 7.5) that also contained $NaHC0_3$. Iron binding was monitored at 470 nm on Spectronic-1001 following successive addition of FENTA (0.1 µg/µl) until Tf was totally saturated. Atoms of iron (Fe) bound per molecule of Tf were calculated according to Welch (1990). FENTA has the composition: 0.19 g per 3 ml of distilled water; 1.0 M NaOH (2 ml); 0.5 M ferric chloride (2 ml); all in 1 L of H20.

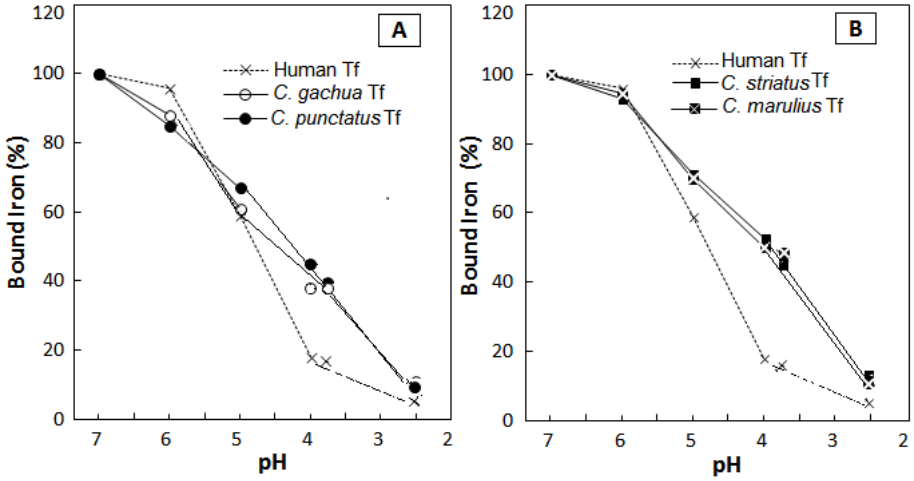

Figure 4. The pH dependence profiles of iron release from the diferric transferrins of four *Channa* species. Human Tf run as comparative control shown as dotted line. **A.** Typical iron release plots of pTf of *C. gachua* and *C. punctatus.s.* **B.** Typical iron release plots of pTf of *C. striatus* and *C. marulius.* Aqueous solution of diferric Tfs from each of the four fish species and human Tf (100 µl of 0.5mg/ml Tf) were added to 300 µl of 0.2 M buffers of various pH values. Buffers were : Tris-maleate (pH 7-6.5), sodium acetate (pH 5.5 to 3.7) and Glycine-HCl (pH 2.5). For each Tf, the decrease in absorbance at 470 nm was noted and plotted as percentage against pH values. All determinations are average of duplicate assays of 2 different preparations.

resemble closely with that of *C. striatus* Tf. A tendency towards semi-biphasic release of iron is observed for the channid Tfs; however, there is no published report on iron release of a fish Tf to compare our results. The most important point that validates iron-release profiles of channid Tfs, is that profile of human Tf obtained in this study is in agreement with already published profile of human Tf (Welch, 1990).

Discussion

Tf polymorphs from highly polymorphic *Channa punctatus* have been purified and further characterized biochemically (Nabi *et al*., 2007). No such data are available on three other channids selected here. Although, Tfs of all *Channa* species investigated here were polymorphic, the objective of the present study was biochemical comparison of one Tf electromorph each from pooled sera of *Channa* species. Properties of Tf which were purified to homogeneity (Figure 1A, pTf lanes) will be described and discussed here.

Partial biochemical characterization confirms that purified Tfs of *Channa* species are similar to other vertebrate transferrins (Figure 1B). The

approximate M_r values of channid Tfs (Figure 1B) are in agreement with the general range of 70-81 kD, documented for vertebrate Tfs (Stratil *et al*., 1983; Welch, 1990). Most of vertebrate serotransferrins are glycoproteins with the exceptions of some cyprinids among teleosts (Stratil *et al*., 1983). Purified Tf of each channid resembled teleost homologues as a monomeric glycoprotein (Stratil *et al*., 1985) and displayed no apparent interspecies differences of molecular weights (71-72 kD). However, as compared to M_r of 80 kD calculated for human Tf (Figure 1B, lane 1), the M_r of channid Tf is typically low, which also applies to its glycoprotein-free state (Figure 1B: lanes with suffix-d run next to control Tfs). The same Tf preparation of each *Channa* species that is run as the control (Figure 3, lanes 2, 3, 4 and 5) was subjected to digestion with neuraminidase. The average M_r of digested channid Tf was calculated as ~66-67kD. The reduction in molecular mass of neuraminidase digested Tf (due to removal of sialic acid residues) confirmed that Tf of channid serotransferrin are glycoprotein with carbohydrate moiety consisting of sialic acid. Following digestion, a reduction of almost similar magnitude occurred in M_r of human Tf also.

Purified band of Tf from each species focused as a single band in IEF profiles (Figure 2). Identical *p*I values of 4.5 were obtained for *C. punctatus* and *C. gachua* Tfs, and 4.7 for *C. striatus* and *C. marulius* Tfs. IEF value sharing by Tfs indicates similarity between their primary structures. However, the *p*I range of 4.5-4.7 indicates that all four channid Tfs have relatively high acidic amino acids contents, as compared to carp transferrins or human reference, which have higher *p*I values of 5.0 and 5.13, respectively (Valenta *et al.*, 1976; Welch, 1990).

An absorption maximum of iron-Tf complex at 470 nm indicated typical vertebrate stoichiometry of iron binding and sigmoid saturation course suggests cooperativity. Therefore, biochemical characteristics and iron binding profiles of channid Tfs, including synergistic bicarbonate requirement, are typical of a vertebrate bilobal Tf molecule, wherein one site per lobe binds one atom of iron (Schlabach and Bates, 1975; Aisen *et al*., 2001; Byrne *et al*., 2010). Upon saturation, similar to human Tf (used as a reference Tf), each channid Tf also binds ~2 atoms *per se* (Figure 3). However, Tf molecules of channid are relatively more efficient, as evident from species differences in iron saturation levels. Tfs of *C. gachua* and *C. punctatus* are saturated at higher iron concentrations than *C. striatus* and *C. marulius* (Figure 3). In other words, Tfs of the former two species can bind more iron as compared to later two species. The iron-binding capacity of Tfs has potential implications in fish life from larval stage survivorship to general health by way of immune responses and resistance to bacterial infections.

What makes channid transferrins strikingly different from other known Tfs is the pH dependent release of bound-iron (Figure 4). This functional specificity highlights that it is an alternative mechanism of protecting cell from iron toxicity when oxygen availability is low. Diferric channid Tfs retained ~20% iron at as low pH as 3.0, where human as well as other mammalian Tfs retain negligible amount of iron (Welch, 1990). Retention of bound iron at such low pH values has not yet been reported for any other vertebrate Tf. However, the shape of iron release curves is semi-biphasic that represents two different gradients which merge at certain pH values, as reported for some other vertebrate Tfs (Welch, 1990). Interestingly, trends displayed by iron-release gradients of Tfs of *C. striatus*-*C. marulius* and of *C. gachua*-*C. punctatus* are at par with the structural affinity displayed by *p*I values of each pair (Fig. 4A). As per the differences in pH stabilities, N-terminal iron binding site releases iron more readily at higher pH (5.5-6.0), while iron release from C-site occurs at pH 4.5 to 5.0 (Hirose *et al*., 2000; Aisen *et al*., 2001; Lambert *et al*., 2005). A biphasic iron release profile indicates differences in iron retention capabilities of N and C lobes of transferrins at a specific pH. Therefore, substructural environment of Tf domains involved in iron release in *C. punctatus* and *C. gachua* is more similar as compared to that of *C. striatus* and *C. marulius*. This is in agreement with the observations on other biochemical characteristics of Tfs of these species being reported in this study.

The tolerance of hypercapnic-acidemia in *Channa argus* (Ishimatsu and Itazawa, 1983) and down-regulation of Tf in killifish under low oxygen availability (Gracey *et al*., 2001) strongly suggest probable existence of other mechanisms which counter hypoxic stress. A switchover to anaerobic metabolism in *C. punctatus* under asphyxia is an already documented example of existence of alternate mechanisms of sustaining low oxygen availability (Ahmad and Hasnain, 2005).

As suggested by the data here, retention of bound iron by Tf at low pH values is one of such mechanisms which would prevent iron toxicity during acidosis under low oxygen availability. Even if acidosis occurs due to hypoxia-induced metabolic stress and/or Tf expression down-regulated as its consequence (Gracey *et al*., 2001), iron toxicity at acidic pH values can be circumvented by an efficient iron-retention capability of Tf available in the system (Figure 4). Functional plasticity of channid Tfs has thus been a crucial factor during adaptive evolution of channids as air-breathers which conferred upon them sustenance to oxygen-deficient ambience.

The O_2-dependent regulation of expression of Tf, which is also linked to haemoglobin gene expression and several iron homoeostasis genes, is essentially part of the multistep transcriptional control mechanism. It involves regulatory hypoxia response elements (HRE), factors (e.g. hypoxia inducible factor or HIF), hydroxylases and proteases and acid/base homoeostasis adenylyl cyclases (Gracey *et al.*, 2001;

Wenger, 2002; Law *et al*., 2006; Rytkönen *et al*., 2007; Tresguerres *et al*., 2010; Chepelev and Gilmore, 2011).

Roles so far assigned to Tf of various teleosts are crucial to their survival at several stages of life which ultimately affects their population size. As mentioned under Introduction, determining sperm motility (Wojtczak *et al*., 2007), protecting sperm from metal toxicity (Dietrich *et al*., 2011) and assisting survival (Hershberger and Pratschner, 1981) have been correlated to differential functionality of Tf polymorphs. We suggest that in case of channids also Tf protects fish life from iron toxicity and from iron-mediated free radical damage as in other vertebrates and fish species (Baker *et al*., 2002, Carriquiriborde *et al*., 2004). The observed functional plasticity has potential implications for fishery output of natural populations as well as of cultivated *Channa* species.

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