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Regulation of erythrocyte function: multiple evolutionary solutions for respiratory gas transport and its regulation in fish

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Short title: Gas transport in fish erythrocytes

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Abstract

Gas transport concepts in vertebrates have naturally been formulated based on human blood. However, the first vertebrates were aquatic, and fish and tetrapods diverged hundreds of millions years ago. Water-breathing vertebrates live in an environment with low and variable O_2 levels, making environmental O_2 an important evolutionary selection pressure in fishes, and various features of their gas transport differ from humans. Erythrocyte function in fish is of current interest, because current environmental changes affect gas transport, and because especially zebrafish is used as a model in biomedical studies, making it important to understand the differences in gas transport between fish and mammals to be able to carry out meaningful studies. Of the close to thirty thousand fish species, teleosts are the most species-numerous group. However, two additional radiations are discussed: agnathans and elasmobranchs. The gas transport by elasmobranchs may be closest to the ancestors of tetrapods. The major difference in their haemoglobin (Hb) function to humans is their high urea tolerance. Agnathans differ from other vertebrates by having Hbs, where cooperativity is achieved by monomer-oligomer equilibria. Their erythrocytes also lack the anion exchange pathway with profound effects on CO_2 transport. Teleosts are characterized by highly pH sensitive Hbs, which can fail to become fully O_2-saturated at low pH. An adrenergically stimulated Na^+/H^+ exchanger has evolved in their erythrocyte membrane, and plasma-accessible carbonic anhydrase can be differentially distributed among their tissues. Together, and differing from other vertebrates, these features can maximize O_2 unloading in muscle while ensuring O_2 loading in gills.

key words: Oxygen equilibrium curve (OEC), Bohr effect, Root effect, anion exchange, adrenergically stimulated Na^+/H^+ exchange, carbonic anhydrase, erythrocyte

1. Introduction: All vertebrates originated in an aquatic environment

Vertebrates originated in an aquatic environment. Consequently, they evolved in an environment that is characterized by low O_2 solubility, where O_2 content is only ca. 1/20 to 1/30 of that in an equal volume of surrounding air (depending on temperature) and varies markedly daily and seasonally [1]. The low O_2 availability of water is thought to have been an important selective force in the evolution of respiratory systems in fish. As a result, the O_2 transport system of fish is the most versatile among vertebrates [2-4], with the most extreme adaptation in fishes being the ability to breathe air which occurs in approximately 500 species [5]. Three major alternative solutions in the gas transport by erythrocytes have evolved in fish: that employed by agnathans [6], that used by elasmobranchs [7,8], and that found in teleosts [9]. The pattern employed by elasmobranchs may be the most representative of fish ancestors of the tetrapods and hence mammals [10]. Apart from mammals, all vertebrates have erythrocytes that are nucleated and contain other cell organelles, which may partly explain why many features of the regulation of erythrocyte function, important for the different environmental adaptations in fish, are not utilized in air-breathing mammals with their organelle-free erythrocytes. In mammals these features have either been lost, become vestigial or alternatively, some of these features may have uniquely evolved in specific groups of fishes and thus may never have been present in any mammalian ancestor during the evolution of their O_2 transport system [10-12]. This is an important point to consider when using teleost fishes such as zebrafish (*Danio rerio*) to study O_2-dependent phenomena with a mammalian biomedical application: the zebrafish is a very hypoxia-tolerant tropical cyprinid [13-15]. Although direct link to gas transport cannot be yet made, it is of interest that different from mammals all zebrafish cells exhibit circadian rhythm [16]. In addition to light, oxygen has marked circadian fluctuations [17], and the major oxygen-regulated transcription factor, HIF, shows (circadian) rhythmic behaviour [18-20]. Many of the discoveries on the regulation of erythrocyte function in fish have been made long after the formulation of the general blood gas transport principles in vertebrates, which were mainly centred on mammals (for a thorough old account, see, e.g. Bishop and Surgenor, 1964) [21]. Consequently, more contemporary

findings on erythrocyte function have been interpreted in light of an earlier gas transport framework that may not be appropriate. In this commentary, we consider erythrocyte function in fish in the context of their unique gas transport characteristics.

A couple of examples indicate the marked differences between mammals and fish (and also other vertebrates). First, as a result of retaining mitochondria, fish erythrocytes are capable of aerobic metabolism, and consume significant amounts of O_2 – producing more than 90 % of their ATP aerobically [22-25]. Second, connected with the presence of a nucleus, fish erythrocytes are capable of active gene expression throughout their life span, although gene transcription and translation decrease with age [26,27]. In view of this, after giving a very short background on the basic vertebrate models of gas transport, we consider the different fish solutions in detail with emphasis on their unique features.

2. The basic model of vertebrate oxygen and carbon dioxide transport

Oxygen-carrying pigments are needed to increase the amount of O_2 carried by blood, because without respiratory pigment the amount of O_2 carried would be limited by its low solubility in aqueous media such as plasma. Thus, in the absence of respiratory pigments, the O_2 consumption rate of multicellular animals cannot be high. This is exemplified by icefish, which have secondarily lost Hb entirely from their circulatory system. They are able to live in the absence of Hb largely due to their low metabolic rate in their low-temperature habitat [28,29].

The simplest solution to increase the O_2-carrying capacity of a solution is to have monomeric globins or other monomeric respiratory pigments. However, to be the most effective, O_2 binding at the gas exchanger should occur at as low an O_2 tension as possible and O_2 release at the tissues at an O_2 tension as high as possible. This is difficult to accomplish for monomeric globins with hyperbolic oxygen equilibrium curves (OEC); by having tetrameric globins, where subunit interactions result in a sigmoidally shaped OEC, both the O_2 loading and unloading can be more easily fine-tuned to respiratory requirements.

A standard OEC is presented in Figure 1. As shown over a century ago, increasing the partial pressure of carbon dioxide (PCO_2) decreases Hb-O_2 affinity [30]. When the pH concept was later formalized, the pH-dependent change of O_2 affinity was named the Bohr effect (as an increase in PCO_2 causes a decrease in pH). A single numerical value, P_{50} is often used to describe Hb-O_2 affinity. The value gives the partial pressure of O_2 (PO_2) where Hb is half-saturated, which generally increases as pH decreases, indicating a reduction in Hb-O_2 affinity. A second major discovery in studies of Hb function is now more than 50 years old; in the late 1960's organic phosphates within the erythrocyte were shown to regulate Hb-O_2 affinity [31,32]. Since then, Hb function has been reviewed several times in journals [4,33-35], and books [36-38] to which the reader is referred. The present contribution briefly reviews the basics to provide the background for a discussion on the unique solutions in fish presented here.

In the simplest models, tetrameric Hb molecules exist in two major conformations: the high affinity R(relaxed)-state and the low affinity T(tense)-state. The existence of high- and low-affinity conformations is the prerequisite of cooperative O_2 binding (sigmoidal OEC). Perutz (1990) [6] has discussed cooperativity in detail. Two main factors influence the T-R-state equilibrium. First, the binding of the first O_2 molecules to deoxygenated T-state Hb facilitates the binding of consecutive O_2 molecules (i.e. tends to shift the Hb conformation from the T-state towards the R-state). Second, the binding of protons (H^+s), organic phosphates and other anions (the most important of which appear to be Cl^- [39] and HCO_3^- [40]) stabilize the T-state (these so-called allosteric effectors bind to sites other than the O_2 binding site). The overall OEC of Hb molecules is determined by the probabilities of T- or R-state occurrence; the equilibrium between T- and R-states is virtually instantaneous.

Gas transport in fish is optimized in part through pH effects on Hb function. In this context it is important to emphasize the linked function concept [41]: the effects of H^+s on Hb-O_2 affinity (Bohr effect) and the effects of O_2 on the binding of H^+s (Haldane effect) describe the same phenomenon. Thus, a large numerical value of a Bohr coefficient ($\Delta \log P_{50}/\Delta pH$) is necessarily associated with a

large numerical value of Haldane coefficient, i.e. difference in the acid dissociation constants (pK_a-values) of oxy- and deoxyHb with the overall pK_a of deoxyHb being much higher than that of oxyHb. Often, the effect of pH on Hb-O_2 affinity (usually given as $\Delta\log P_{50}/\Delta$pH) is given in terms of plasma pH values. However, the ratio of $\Delta pH_e/\Delta pH_i$ is less than unity, which means that the Bohr coefficient obtained using plasma pH is smaller than if the value is estimated either based on erythrocyte pH or for Hb solutions i.e. the true value experienced by Hb molecule. The error caused by using plasma pH instead of erythrocyte pH remains constant in mammals regardless of the experimental conditions, since erythrocyte pH cannot be regulated. The same is true for undisturbed fish [42]. However, in many groups of fishes, erythrocyte pH can vary independently from plasma pH (see below), which makes the values for the Bohr coefficient calculated on the basis of plasma pH [43] less meaningful under various stresses and thus such values should be interpreted critically. It is further of note that the effect of pH on the Hb-O_2 affinity may depend on the pH range studied. In the physiologically relevant pH range, a decrease in pH causes a decrease of Hb-O_2 affinity (alkaline Bohr effect). However, in acid conditions (e.g. below pH 6 for human [37]), a decrease in pH may increase Hb-O_2 affinity (acid Bohr effect). Since the alkaline Bohr effect is the physiologically relevant one, we simply refer to that from this point forward as the Bohr effect.

In typical tetrameric Hbs, an increase in temperature decreases the overall O_2 affinity, because Hb-O_2 binding is exothermic. However, the heat of oxygenation of each successive O_2 molecule that binds to the tetramer varies markedly [44]. In addition to the exothermic O_2 binding by haem, the overall temperature effect on Hb-O_2 affinity is affected by the endothermic reactions between Hb and allosteric effectors such as H^+s and organic phosphates [45]. Consequently, the higher the: i) concentration of allosteric effectors, ii) number of allosteric effector binding sites per Hb tetramer and iii) binding affinities of these allosteric effectors, the smaller the temperature-induced decrease in Hb-O_2 affinity. At an extreme, the final result can be a reversed, endothermic overall Hb oxygenation reaction, whereby Hb-O_2 affinity increases with increasing temperature [46,47].

With regard to the transport of CO_2, the end product of aerobic respiration, two properties of most vertebrate erythrocytes are considered to be of primary importance. First, erythrocytes possess high carbonic anhydrase (CA) activity [48,49]. Carbonic anhydrase catalyzes the hydration/dehydration reactions between CO_2 and carbonic acid, which are the rate-limiting steps in establishing the equilibrium between CO_2 and HCO_3^-. Second, the transport of HCO_3^- across the erythrocyte membrane is accelerated by the anion exchange protein, allowing rapid Cl^-/HCO_3^- exchange [50,51]. The rapid Cl^-/HCO_3^- exchange enables the function of the Jacobs-Stewart cycle, utilized in CO_2 transport [52]. In most vertebrates, more than 50% of CO_2 excreted originates from plasma HCO_3^- that must enter the erythrocyte and be converted to CO_2 by the CA-catalysed reaction before it can be excreted across the respiratory epithelium. The usual pattern of CO_2 excretion in vertebrates (Figure 2c) has been described in a number of reviews [9,53-55].

3. The agnathan solution

The jawless (agnathan) vertebrates, comprising modern hagfishes and lampreys as the sole survivors of a once more species-rich group, diverged from the jawed vertebrate lineage some 500 million years ago. Its living descendants differ in important aspects from the general pattern of vertebrate erythrocyte function, including the possession of structurally and functionally different globins and the absence of a rapid anion exchange protein in their erythrocyte membrane.

3.1. Cooperativity of oxygen binding in haemoglobin is generated via monomer-oligomer equilibria

In addition to the mechanism of cooperativity utilized by tetrameric Hbs, co-operative O_2 binding can occur if the aggregation of globins is oxygen-dependent and the O_2 affinities of aggregation states differ. In such a case, aggregated Hbs have a low O_2 affinity and monomeric Hbs have a high O_2 affinity [6]. Aggregation-dependent generation of cooperativity is utilized by the jawless vertebrates, hagfish and lampreys. Early studies on agnathan Hbs failed to see aggregation (and cooperativity) [56],

because dilution favours monomerization [57]. Cooperativity stems from the fact that in the oxygenated state, Hb is monomeric but aggregates upon deoxygenation [57-59]. A schematic depiction of aggregation-dependent O_2 affinity regulation is given in Figure 3. In hagfish it is notable that the different Hb types have markedly different capabilities for aggregation and that the aggregation of some components is influenced by anions like HCO_3^- [60]. Two significant properties of lamprey Hb within erythrocytes are the marked Bohr effect (with a Bohr coefficient of around -1 [56], which contrasts with a much lower mammalian value of around -0.5 [61]), and the reduction in the O_2 carrying capacity of the blood at air saturated O_2 tensions at low pH [56], a phenomenon that is more commonly associated with the blood of teleost fishes [62], where it is referred to as the 'Root effect' (for review see: [10,63]). In contrast, the Bohr coefficient of hagfish Hbs is small – maximally -0.5 [57,60], and depends on the proportions of different subunits with different aggregation properties [60].

On the basis of molecular phylogenetic evidence, it appears that agnathan Hbs with monomer-oligomer equilibria are only distantly related to the tetramer-forming Hbs of other Vertebrata [64], and have actually evolved from cytoglobin-like ancestors [65]. As a consequence, although lampreys have Hb characterized by a pronounced sensitivity to H^+s, resembling Bohr and Root effects of teleost fish, this H^+ sensitivity has probably evolved independently [66]. Another major difference between agnathan and most tetrameric Hbs is that in the former, organic phosphates do not affect Hb-O_2 affinity [67], whereas in the latter, the effect is usually pronounced, and crucial in fine-tuning Hb-O_2 affinity [4,32,68], although some Hbs even in mammals are organic-phosphate-insensitive [69].

3.2. Agnathan erythrocytes do not have rapid anion transport: implications for carbon dioxide transport

In contrast to erythrocytes of other vertebrates, lamprey erythrocytes were shown to regulate intracellular pH actively [70,71]. This was later shown to be associated with the lack of erythrocyte anion exchange activity [72]. A similar absence of rapid erythrocyte anion exchange was reported at the same time for a member of the other agnathan group, the hagfish *Eptatretus stouti* [73]. The lack of an

anion exchanger has a marked influence on CO_2 transport, as plasma HCO_3^- is consequently not available to erythrocyte CA for CO_2 excretion, as is a common characteristic of other vertebrates [55,74-76]. Because of the short transit time of erythrocytes in the gills, and the lack of anion exchange, virtually all excreted CO_2 stems from erythrocyte HCO_3^-. The total amount of CO_2 and HCO_3^- that can be transported within erythrocytes, and thus the efficiency of CO_2 excretion (Figure 2), is very dependent on a high erythrocyte pH, which is much higher in lampreys than hagfishes [76] [about 1 pH unit higher in *Lampetra fluviatilis* than in *Myxine glutinosa* at an extracellular pH of 7.6 [70,77]]. Consequently, lampreys can transport CO_2 much more efficiently than hagfish. This is in keeping with the lifestyles of the groups: whereas hagfish are sluggish and incapable of strenuous activity [78], lampreys undertake long and vigorous spawning migrations [79].

4. The elasmobranchs

A detailed recent review on O_2 and CO_2 transport in elasmobranchs is available [80]. Although elasmobranch Hbs are mostly tetrameric, there is evidence for oxygenation dependent formation of higher polymers and dissociation to dimers [7]. The histidine content of Hb, and thereby the specific hydrogen ion buffer value in the elasmobranchs studied to date is similar to that in mammals [9,80], and the value of their Bohr coefficient is small, usually below -0.5 [80,81]. The organic phosphate binding site appears to resemble that of mammals [82], and their erythrocytes have robust anion exchange [83,84]. Consequently, cartilaginous fishes differ the least among fish from the prototype model of jawed vertebrate gas transport [10-12]. However, there are three specific points which warrant attention with regard to differences in O_2 and CO_2 transport in elasmobranchs as compared to the basic model. First, the plasma osmolality of marine elasmobranchs is about three- to four-fold higher than almost all other vertebrates. While their plasma Na^+ and Cl^- concentrations are much lower than seawater values, their plasma is isosmotic with their seawater environment because of the high plasma urea concentrations, typically up to 0.5 M [85]. Urea at these levels usually denatures proteins, but urea concentrations even up to 5 M appear to have minimal effects on Hb-O_2 transport

function [86]. Although it appears that in most cartilaginous fishes urea has a minimal effect on Hb function [80,86], there are exceptions [87]. Urea tends to antagonize the effect of organic phosphates on Hb function in many but not all cartilaginous fish [80]. Second, some shark species are heterothermic, maintaining the temperature of some tissues, such as the red swimming muscle, up to 10-15^{o}C higher than that of the environment [88,89]. Although many of the associated adaptations are circulatory with a multitude of heat-exchanging *retia mirabilia*, Hb function also appears responsive [80]. In heterothermic fish, the effect of temperature on Hb-O_2 affinity may be reduced or even reversed (as discussed in more detail below [80,81]), and in elasmobranchs specifically the allosteric effector ATP may reduce or reverse the temperature-effect [90]. Third, there appear to be unique features in the CO_2 transport of elasmobranchs. Both erythrocytic and plasma-accessible CA in gills appear to play a role in CO_2 excretion [80,91], whereby some plasma HCO_3^- can be dehydrated to CO_2 directly from the plasma compartment, without entering the erythrocyte, probably by membrane-bound extracellular gill cell CA [92]. The model of CO_2 excretion in elasmobranchs is schematically represented as Figure 2c.

5. The teleosts

5.1. The Root effect: extreme pH sensitivity of the oxygen equilibrium curve.

Although the effect of pH on Hb-O_2 affinity is a property of virtually all Hbs, many teleost Hbs are characterized by extreme pH dependence so that Hbs fail to become fully O_2-saturated at low pH values regardless of the O_2 tension [12,62,63,93-96]; Fig. 4), a phenomenon known as the Root effect. When the pH becomes so low that the Root effect is induced, the cooperativity of O_2 binding eventually disappears, and Hill's n can even become less than 1 [97], which is suggestive of large subunit heterogeneity in the O_2 affinity of the T-state (α and β) globins [63].

The physiological role of the Root effect in teleost Hbs can be considered as an acid-triggered mechanism by which oxygenated Hb can release substantial amounts of its bound O_2 even in the presence of a high PO_2 [96]. According to the classical viewpoint, the effect is utilised to generate

supra-atmospheric PO_2 during one or both of the following processes: (1) the secretion of O_2 into the teleost swim bladder for buoyancy regulation and/or (2) the improved O_2 supply to the often avascular and relatively thick teleost retina. In both locations, the Root effect is thought to be elicited by local tissue acidification and co-occurs with vascular counter-current gas exchangers, the swim bladder *rete mirabile* and the choroid *rete mirabile*, respectively. These *retia mirabilia* allow for any O_2 released via the Root effect to diffuse back from the venous to the arterial side of the *rete mirabile* and thereby multiply the initial increase in its partial pressure. Similarly, the increase in CO_2 that is associated with the initial blood acidification, can be kept localised by its back-diffusion from the venous to the arterial side of the *rete mirabile*. While the roles of the Root effect in O_2 delivery to the swim bladder and eye are generally well accepted in the literature, a large scale evolutionary reconstruction of the origins of the Root effect and the swim bladder and choroid *retia mirabilia* has suggested that the Root effect evolved before the first occurrences of either the retinal or the swim bladder O_2 secretion mechanism [12], raising the question about the initial roles of the Root effect that may confer selective advantages.

The high pH dependency of teleost fish Hbs is also reflected in the magnitude of the Bohr coefficient, which often exceeds -1, even when calculated relative to plasma pH [97-99]. The structural basis of the marked pH dependency of teleost Hbs has been reviewed earlier [96,97], and here we focus on how the erythrocyte properties of teleost fish, in light of the highly pH sensitive Hbs, affect gas transport.

The initial evolutionarily adaptive role of the Root effect may have been to facilitate O_2 unloading generally in working muscle of teleost fish [100,101], making it later possible to utilize the property to give greatly enhanced O_2 secretion to the eye and swim bladder. Oxygen uptake and delivery is maximized by a high Hb-O_2 affinity at the gills and a low Hb-O_2 affinity in the tissues. In simple terms this means that when pH decreases in the muscle to induce the Root effect, the PO_2 in unloading is markedly increased. Since the rate of diffusion depends directly on the partial pressure gradient, this speeds up the diffusion of O_2 to working muscle markedly.

To set the stage, one needs first to consider how the large Haldane effect of teleost fish Hb, i.e. the markedly higher pK_a of deoxy- than oxyHb, can theoretically influence arterial and venous erythrocyte pH in the absence of acid loads. Since the CO_2/HCO_3^- buffer system plays a reduced role in water breathers and Hb is the major non-bicarbonate buffer in their blood, erythrocyte pH in arterial blood would be close to the overall pK_a value of oxyHb and that in venous blood close to the higher overall pK_a value of deoxyHb in the absence of any external acid loads from the tissues, i.e. at constant CO_2 tension. The marked increase of erythrocyte pH appears to stop at 50% Hb-O_2 saturation [102]. In resting conditions the deoxygenation-induced increase in erythrocyte pH is also transmitted to plasma pH. This means that theoretically venous blood can have a pH value that is higher than that of arterial blood even in the presence of an overall acid load. The gradient between erythrocyte and plasma pH in selected conditions is illustrated in Figure 5.

Induction of the Root effect requires a significant, species-dependent, decrease of erythrocyte pH. Assuming that metabolism causes a tissue acid load, which is adequate to cause a 30 % decrease of the maximal O_2 saturation, that the Hb concentration is 100 g/L and that arterial blood is virtually fully O_2-saturated, then the release of O_2 to the capillary network can be adequate to increase the O_2 tension to 500 mmHg – to a value more than ten times greater than what is found in the absence of significant acid load in rainbow trout (*Oncorhynchus mykiss*) [103]. Consequently, the diffusion gradient for O_2 transfer could be increased dramatically in fish with Root effect Hbs. Such an increase will drastically increase the O_2 available to swimming muscle, whereby the Root effect would markedly favour O_2 delivery to the muscle of strenuously exercising teleost fish. Notably, the above is only a theoretical calculation and assumes that blood is in a closed system. While that is true only for blood in the arterial and venous system, the capillary circulation is at best a semi-open system, since the volume (of fish) which is in contact with tissue capillaries is limited. The only truly open part of the circulation is the gill circulation, where blood is in close contact with the near infinite volume of water in the ambient environment.

In line with the above prediction, we have observed that the O_2 tension of blood in the caudal vessel of striped bass (*Morone saxatilis*) was significantly increased (from 118.6 to 173.3 mmHg) and supra-atmospheric after 5-min chasing [104]. The pH of sampled blood after exercise was markedly reduced (by 0.3 pH units) during exercise, and may be sufficiently low to induce the Root effect in this species.

In conclusion, the drastically increased O_2 unloading potential due to the extreme pH dependence of Root effect Hbs in teleost fish may represent the basis for a highly efficient O_2 transport system relative to that found in air-breathers, which is also coupled with a more efficient respiratory gas exchange system (counter current exchange [2]). However, the use of Root effect Hbs for acid-triggered, sustained and drastic increases in muscle PO_2 values is of benefit only, if the pH effects in the erythrocyte can be reversed in the time it takes such tissue capillary blood to reach the O_2 uptake site in the gills. Recent work on salmonids in particular, referred to in the following section, suggests that this is made possible by the β-adrenergically stimulated sodium/proton (Na^+/H^+) exchange in their erythrocytes [105] .

5. 2. The adrenergically stimulated, oxygen-dependent sodium-proton exchanger of erythrocytes

Erythrocyte pH can be rapidly increased in teleost fish through an adrenergically stimulated Na^+/H^+ exchange [12,42,106]. Adrenergically stimulated Na^+/H^+ exchange has also been found in erythrocytes of amphibians [107-109]; however, activation in these cells is not rapid [109] precluding a role in the short term regulation of O_2 transport. The adrenergic Na^+/H^+ exchange activity of teleost erythrocytes is strongly oxygenation dependent [110-112], and results in rapid net H^+ extrusion following stimulation [113,114]. The increase in erythrocyte pH_i is dependent upon the absence of CA in the plasma which would otherwise short-circuit this response by rapidly catalysing the conversion of plasma HCO_3^- and extruded H^+s to CO_2 [113,114]. Also, a marked effect of the Na^+/H^+ exchange on erythrocyte pH requires that the buffering capacity of Hb is low. Indeed, the buffering capacity of teleost Hbs is much lower than that of other vertebrate groups [115]. The extremely rapid H^+ extrusion and its O_2 dependency

appear unique for teleost erythrocytes, although O_2 dependency in the adrenergically stimulated Na^+/H^+ exchange of the amphibian *Bufo marinus* [109] and in other ion transport pathways of vertebrate erythrocytes [116-119] has been described. Common to those ion transport pathways is that they seem to be involved in volume regulation, which does not appear to require as rapid a response as that in the O_2 transport cascade. The O_2 dependency of Na^+/H^+ exchange appears to be generated not by molecular oxygen but by hydroxyl radicals [120], suggesting that oxyradicals are not only toxicants but also important signalling molecules in animals [121].

Characterization of the β-adrenergic receptors of teleost erythrocytes yielded a surprise: the receptors were clearly of the β_3-subtype [122]. Since this receptor subtype, characterized both genetically and pharmacologically, is associated with thermoregulation and fat metabolism in homeothermic vertebrates [123] but in teleost fish with regulation of O_2 transport in stress, it represents an intriguing example of how the same receptor type can evolve to regulate completely different functions [124].

5.3. The simultaneous presence of Root effect and adrenergic Na^+/H^+ exchange maximizes oxygen unloading from erythrocytes but enables effective oxygen uptake in gills

Although the Root effect and marked pH dependency of Hb-O_2 affinity may increase O_2 unloading during a generalized blood acidosis (such as occurs in hypoxia or exhaustive exercise), O_2 uptake at the gills would decrease if erythrocytic pH_i did not recover prior to gill entry. However, recent studies on rainbow trout have shown that the erythrocytic pH recovery occurs well within the venous transit time of the erythrocyte from the tissues back to the gills [105] as a result of the adrenergic stimulation of erythrocyte Na^+/H^+ exchange, whereby pH_i is largely independent of pH_e and consequently O_2 loading in gills can be maintained. While the activation of erythrocyte Na^+/H^+ exchange ensures O_2 loading in the gills, it could prevent any pH decrease upon further acid load in tissues due to tight regulation of pH_i. However, the effect of Na^+/H^+ exchange on erythrocytic pH_i can be reduced by speeding up the extracellular hydration/dehydration reactions between HCO_3^- and

CO_2, as their rate in comparison to the rate of H^+ extrusion determines the extent of the change in pH_i [114,125]. Thus, a decrease in erythrocyte pH_i at the tissues depends mainly on the activity of extracellular (i.e. plasma-accessible) CA: the greater the activity, the greater the reduction in pH_i and consequently the decrease in Hb-O_2 affinity and increase in O_2 unloading. In keeping with conditions that could maximize O_2 delivery in the presence of erythrocyte Na^+/H^+ exchange activation, plasma accessible CA is absent in the gills of most teleosts that have been studied [126,127], but present in some tissues such as the muscle and the heart [101,128,129] of some species. The differential distribution of plasma accessible CA (absence in the gills and presence in the tissues) creates conditions for greatly enhanced O_2 unloading that could more than double O_2 unloading with no change in tissue perfusion [100]. The potential of this system has been demonstrated in rainbow trout blood both *in vitro* [130] and *in vivo* [101], as well as in other salmonids [131]. Most recently the potential has been demonstrated in a more derived teleost, cobia (*Rachycentron canadum*; Shu and Brauner, unpublished). Functional evidence for the role of this system in enhancing O_2 unloading has been demonstrated in Atlantic salmon (*Salmo salar*), where injection of a plasma accessible CA inhibitor (C18) in fish swimming at a moderate speed induced a 30% increase in cardiac output to compensate, and at higher swimming speeds fish collapsed (Harter, T.S., Zanuzzo, F.S., Supuran, C.T., Gamperl, A.K. and Brauner, C.J., unpublished). Clearly more studies are required to determine just how widespread this system is among teleosts and its functional importance given that there are more than 25000 teleost fish species with marked differences in the presence and activity of the erythrocyte Na^+/H^+ exchange of those species where it has been investigated [12,112,132]. Furthermore, the differential tissue distribution of CA has mainly been studied in salmonids.

In conclusion, maximal efficiency of O_2 transport in teleosts requires that the high pH dependency of Hb-O_2 affinity, adrenergic activation of erythrocytic Na^+/H^+ exchange and differential distribution of plasma-accessible CA function in concert (Fig. 6). Alone, each of the phenomena could cause problems for gas transport, but together, they form a highly efficient system that differs markedly

from other vertebrates. How the different components have evolved has been discussed by Randall et al., 2014 [127] and Harter and Brauner, 2017 [133], but remains a fruitful area for further investigation.

5.4. Temperature effects on teleost haemoglobins: ectothermy and heterothermy

It has been known for over a century that some species of tuna can maintain parts of their body at a temperature much higher than that of the ambient environment [134-136], a feature shared by some sharks [88], as discussed above and known as regional heterothermy. The swimming muscle in the body core has a high temperature, which permits improved muscle performance, but the temperature at the body surface and the gills is close to ambient. The marked difference in temperature between the body surface and the core can be maintained by the organization of blood vessels in *retia mirabilia* in which arteries with warm blood from the core flow counter-current, and in close proximity to the veins with cold blood from the body surface [137]. This system allows for effective heat conservation in those tissues. If the effect of temperature on Hb-O_2 affinity were similar to that of other vertebrates, this could lead to a pronounced loss of O_2 from arteries to veins within the *retia mirabilia* so that the associated peripheral tissues could suffer from O_2 lack. As early as 1960 it was observed that at 50% saturation, the O_2 affinity of tuna Hb was not affected by temperature [138]. It was later observed that in bluefin tuna blood, a reverse temperature effect exists, where Hb-O_2 affinity actually increased with increasing temperature at saturations above 50% and decreased at low saturations [139,140]. While the reversed temperature effect appears counterproductive for O_2 release and O_2 consumption in core muscle, this trait is thought to minimize the loss of O_2 from arteries to veins in *retia mirabilia.* Interestingly, the blood of some ectothermic marine fishes lack the effect of temperature on Hb- O_2 affinity [47,99], suggesting that a pre-existing trait was utilized in the evolution of Hb function of regionally heterothermic fish.

Regional heterothermy in fishes may involve the whole core of the fish being regulated above ambient temperature, opah (*Lampris guttatus*) as the most extreme case [141] or only a specific tissue such as the eye/brain area as in billfish [46,142]. It is usually thought that in regional heterothermy the

effects of temperature on Hb-O_2 affinity are reduced or reversed [45,139]. At least two factors influence the overall heat of oxygenation: exothermic O_2 binding to the haem groups of Hb and the endothermic dissociation of allosteric effectors (mainly H^+s and organic phosphates) from the Hb [45,46]. Because both enthalpies differ depending on the oxygenation state of Hb, the apparent heat of oxygenation also depends on Hb-O_2 saturation [140].

Apart from the evolution of Hb function in response to temperature in regional heterothermy, the ambient temperature to which fish are adapted appears to have had little influence on functional characteristics of Hb. This is important to note, when considering the possibilities of fish to adapt to increased temperatures, brought about by climate change. It was initially thought that fish from extremely cold, Arctic and Antarctic, habitats, where the temperature is stable and can be close to -2°C, would have reduced temperature dependency of Hb-O_2 affinity [143,144]. However, more recently it has been shown that temperature sensitivity of Hb-O_2 binding in Antarctic fish appears similar to temperate fish [145]. A complicating factor in evaluating the effect of temperature on Hb-O_2 binding is that, e.g., the enthalpy of the binding of ATP to Hb is influenced by pH [146], and pH is affected acutely by a change in temperature and following temperature acclimation, both of which can influence the apparent relationship between temperature and Hb-O_2 affinity.

6. Evolutionary convergence of responses to achieve similar organismic traits

Similar to adrenergically-stimulated erythrocytes in teleost fishes, erythrocyte pH is also largely independent from plasma pH in the agnathan lampreys. In the latter this feature is mainly due to the presence of constitutively active O_2-dependent Na^+/H^+ exchange [147] in the absence of rapid Cl^-/HCO_3^- exchange [72] which results in a high erythrocyte pH [70,71,148]. This is in contrast with the other agnathan group, hagfish, which also lack the anion exchanger [73], but have low erythrocyte pH because of minimal Na^+/H^+ exchange or other H^+ extrusion mechanisms [149]. Thus, in lampreys and teleosts, although the mechanism through which erythrocyte pH is to some degree independent of plasma pH is different, the final outcome is similar (convergent evolution). The evolutionary reasons for this

convergent evolution are necessarily speculative. However, at least two possibilities are plausible. First, in both groups of water-breathers, intensive short-term bouts of exercise occur. Since the erythrocyte pH_i (and thereby Hb-O_2 affinity) can be maintained high in gills, the exercise-induced metabolic acidosis does not appreciably reduce O_2 binding in gills. Second, both teleost fish and lampreys are characterized by highly pH-sensitive Hbs with low buffer values [9]. It is plausible that because of this they cannot afford to lose the ability to regulate erythrocytic pH_i via Na^+/H^+ exchange. Regardless of the reason, although the lamprey and teleost fish lineages diverged about 500 million years ago [150], O_2 transport by erythrocytes has converged to enable similar organismal function.

7. Erythrocytes – not only for gas transport: roles in buffering and cell signalling

The above has concentrated completely on respiratory gas transport. However, while this is undoubtedly the primary function of erythrocytes, they possess many other important properties. First, as Hb is a very abundant protein with many histidine residues that can donate or accept H^+s, Hb is a very important extracellular buffer as long as the erythrocytic Hb is accessible to extracellular acid loads. The buffering capacity of Hb is proportional to the histidine content, which is markedly reduced in teleost fish, but high in lungfish, elasmobranchs and tetrapods [11,12,115]. Among the agnathans, the histidine content is distinctly higher in hagfish than in lampreys [9,57], which is also reflected in their respective H^+ buffering capacities [151]. It is probable that the reduction of Hb histidine content and thus H^+ buffering capacity in both lampreys and teleost fish may be to benefit O_2 transport characteristics (see above). Haemoglobin has a pronounced role in rapid extracellular buffering as long as erythrocytes have rapid anion exchange [76], as is the case for all vertebrates except agnathans [72,73]. Because HCO_3^- transport across the agnathan erythrocyte membrane is exceedingly slow [74,75], erythrocytic Hb cannot rapidly buffer extracellular acid loads. Although a number of explanations are possible, perhaps this limited ability to buffer an extracellular acid load is associated with a more temperate distribution where metabolic rate and thus metabolic acid loads

are reduced. This antitropical distribution is clearly seen for lampreys, which tend to have freshwater ammocoetes (usually river-resident lamprey larvae) with a low thermal tolerance [152].

Another role for Hb is its influence on redox balance and nitrite-nitrate-nitric oxide equilibrium. This may have an influence on the regulation of erythrocyte adrenergic Na^+/H^+ exchangers and other oxygenation-dependent ion transporters. For example, potassium transport in crucian carp erythrocytes appears to have two different oxygenation-dependent sensors [119]. One appears to be a hydroxyl radical sensor [121,153], where Hb may be the primary regulator of hydroxyl radical level. Based on mammalian studies, Hb appears to be a biological Fenton reagent with deoxyhaemoglobin as the form responsible for hydroxyl radical generation [154]. So far, the sensing mechanism of the other O_2 sensor described has not been clarified.

Mammalian studies also suggest that Hb is an oxido-reductase, largely depending on its level of oxygenation [155]. It plays an important role in regulating vasodilatation and vasoconstriction by influencing nitric oxide (and nitrite) level. When Hb is oxygenated, it scavenges NO and the vessels are constricted. When Hb is deoxygenated, it reduces nitrite to form NO, whereby vessels dependent on NO signalling are dilated [155]. Marked formation of NO from nitrite occurs in the erythrocytes of a teleost fish, carp [156]. Further, the nitrite reductase activity correlates with the Hb-O_2 affinity, and changes when the erythrocyte NTP level changes [157], the latter being modulated in response to hypoxia in fish [68]. In view of these findings Hb appears to play a part in NO-dependent signalling. This is appropriate, as NO-signalling plays a role in vascular tone, i.e. regulating O_2 transport to capillaries. Another signalling molecule, hydrogen sulphide, has also been demonstrated to be especially important in regulating the vasculature in response to hypoxia [158,159]. Not surprisingly, Hb regulates the sulphide turnover both in fish and humans [160,161]. There are significant species-dependent differences both in the formation of sulphaemoglobin [162] and in the effects of sulphide on membrane ion transport [163] in the erythrocytes of teleost fish, but the physiological significance of these differences has not been evaluated. The involvement of Hb in sulphide

signalling may be a reason for the continuous presence of ferric methaemoglobin, which does not carry O_2, but enables reversible sulphide reactions [160,161]. Also another gaseous signalling molecule, CO, interacts and is regulated by Hb at least in mammals [164]. The overall picture that emerges is that gaseous signalling plays an important role in erythrocyte function and that Hb is important in its regulation. However, so far this remains a little studied area, which would clearly be worthy of further investigation.

8. The functions of "new" globins

Tetrameric Hb is at very high concentration within erythrocytes with most fish expressing multiple isoforms [165]. However, in addition, fish erythrocytes transcribe at least cytoglobin, globin x and neuroglobin [166]. In particular, a high mRNA level of the gene encoding neuroglobin was found. In three-spined stickleback, neuroglobin transcription in erythrocytes was the most active of any tissue, and even more active than that of Hb [166]. The function of neuroglobin is far from clear, but it has been suggested to play a role in redox or NO regulation [167] and in regulating free sulphide levels [168]. As discussed above, all of these signalling systems may be active in erythrocytes and can involve Hb. Thus, neuroglobin formation in teleost erythrocytes may be related to redox, NO or sulphide regulation. Globin x is a membrane-bound globin [169], which can also take part in either redox [170] or NO [171] regulation. Another plausible function for globin x would be in the regulation of adrenergic Na^+/H^+ exchanger. It was proposed that Hb may be a regulator of the erythrocyte adrenergic Na^+/H^+ exchanger [172]. However, properties of bulk Hb did not fit the requirements for the O_2 sensor [118]. A minor, membrane-bound globin with O_2 affinity different from bulk Hb, such as globin x, on the other hand, could be involved.

9. Conclusions

From the preceding sections it is clear that the erythrocyte functions of fishes are underpinned by various unique mechanisms, which are vastly different in the different fish groups. These erythrocyte functions often deviate from the prototype textbook dogma. Thus when using fish in environmental

research, their phylogenetic relationships need to be taken into account. When fish are used with biomedical questions in mind, it must be remembered that their O_2 transport system can be far more efficient than that in mammals because of the need for efficient O_2 extraction from their low-O_2 environment. It is not well understood how the O_2-dependent phenomena beyond gas transport are different in zebrafish and humans. Perhaps the major difference between human and fish erythrocytes is that the former do not possess nuclei and other cellular organelles. Consequently, while mammalian erythrocytes are devoid of gene expression and produce energy anaerobically, fish erythrocytes are aerobic, and many of their adaptations can involve active protein production. Notably, however, the effectiveness of gene expression decreases with the age of the erythrocyte [27], adding age-dependent selective removal of erythrocytes to possible regulatory mechanisms behind gas transport. Such selective removal of erythrocytes would affect, e.g., the adrenergic responsiveness of the erythrocytes [173], and affect the seasonality of the responses of erythrocytes [174].

Conflict of interest

There is no conflict of interest.

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Figure captions

Figure 1 a. O_2 equilibrium curves with O_2 saturation (%) of Hb on *y*-axis and the partial pressure of O_2 (kPa) of the Hb solution on *x*-axis. Often, the O_2 affinity of Hb is given as the P_{50} value, which is the partial pressure of O_2 at which Hb is 50% O_2-saturated. The effect of decreasing pH is shown by a right shift of the O_2 equilibrium curve from A to B. The numerical value for the pH effect (Bohr coefficient) is usually given as Δ log P_{50} value/ΔpH. **b.** The interaction between O_2-binding Hb subunits (*n*; sigmoidality) is given by the Hill plot, where the *y*-axis is the logarithm of the ratio of the oxygenated Hb fraction (*S*) over the deoxygenated Hb fraction (1-*S*) [log (S/(1-S))] and the *x*-axis is the logarithm of the partial pressure of O_2). 1. gives the log P_{50} value for Hb in the *R*-state , 2. the slope of the line is *n* (interaction between O_2-binding Hb subunits), 3. gives the log P_{50} value for *T*-state Hb, and 4. the overall log P_{50} value (=the same as the log P_{50} value derived from OEC).

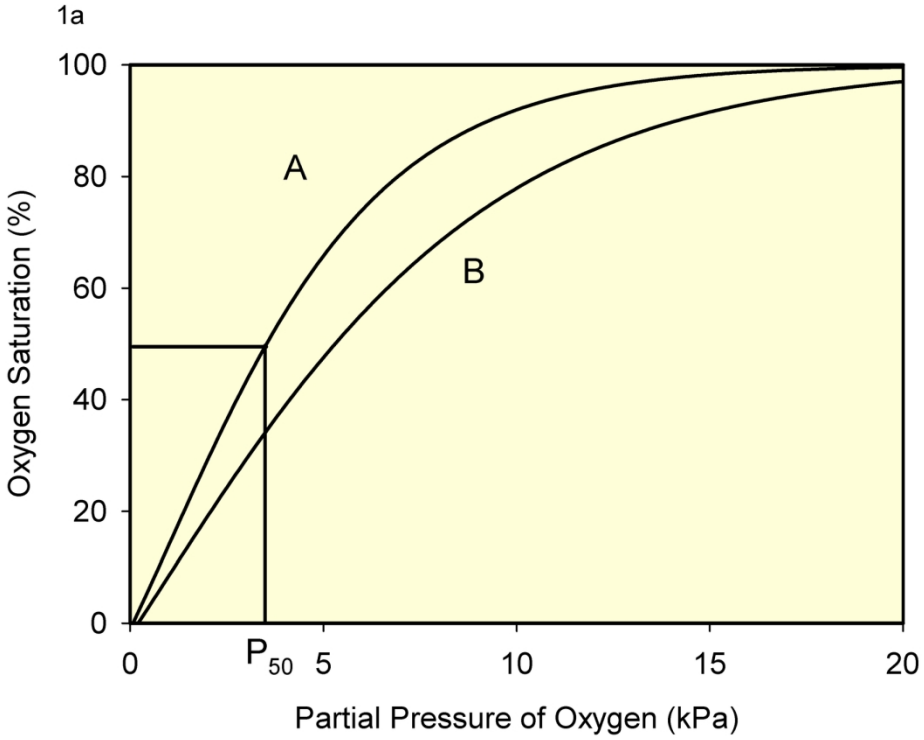
Figure 2. Schematic representations of basic patterns of CO_2 excretion in vertebrates. In the agnathans, hagfish (a) and lamprey (b), sharks (c) and teleosts (d). In the agnathans, the red blood cells lack Cl^-/HCO_3^- exchange. In the hagfish, carbonic anhydrase (CA) catalyzes the conversion of HCO_3^- to CO_2 from both the plasma compartment (via plasma accessible CA; CA-IV) and the erythrocyte. In lamprey, CA-IV is absent and all HCO_3^- converted to CO_2 is from the erythrocyte and oxygenation of Hb provides H^+ for this reaction through a large Haldane effect. In sharks, the presence of CA-IV permits CO_2 directly from the plasma compartment and erythrocyte Cl^-/HCO_3^- exchange permits plasma HCO_3^- to enter the erythrocyte for conversion to CO_2. This pattern represents the general vertebrate pattern of CO_2 excretion in vertebrates and is similar in air-breathers. In teleosts, CA-IV is absent and all CO_2 excreted is through the erythrocyte where a tight coupling of O_2 uptake and CO_2 excretion exists due to the presence of a large Haldane effect.

Figure 3. The generation of cooperativity by aggregation/dissociation reactions in lamprey Hbs. Monomers have the highest O_2 affinity, and oxygenation favours dissociation of oligomers to monomers. Dissociation of oligomers to monomers is also favoured by an increase in pH and dilution.

Figure 4. O_2 equilibrium curves of rainbow trout **A.** at resting plasma pH (7.65) and **B.** at pH 7.40, where the Root effect has been induced (i.e. O_2 saturation approaches asymptotically a value below full saturation, in this case maximal O_2 saturation is 69%, i.e. Root effect 31%; data from [98]).

Figure 5. Arterio-venous pH difference (dpH) in rainbow trout **A.** theoretically, if the only thing affecting blood pH were the difference in pK_a value between oxy- and deoxyHb (data based on [175]), **B.** in normoxic conditions, and **C.** in moderate hypoxia (40% air saturation) (data for b and c from [103]). A decrease in the negative value of dpH (as compared to A) or an increase in its positive value indicates acid input to blood.

Figure 6. Schematic representation on how the presence of Root effect Hb, adrenergic Na^+/H^+ exchange of erythrocyte membrane, and the absence of carbonic anhydrase in the gills and its presence in the muscle allow to secure O_2 loading at gills, but increase O_2 unloading in muscle of strenuously exercised rainbow trout. In gills, there is no plasma-accessible carbonic anhydrase. Thus, the adrenergically activated Na^+/H^+ exchange increases erythrocyte pH after the blood has left the muscle. Consequently, the Hb-O_2 affinity increases and O_2 loading in gills remains effective (i.e. Hb reaches close to full saturation) despite plasma acidification. From gills, blood flows to working muscle, which has plasma-accessible carbonic anhydrase. Consequently, erythrocyte pH decreases, even though the adrenergic Na^+/H^+ exchange of membrane is active. The pH drop can be adequate to induce the Root effect, whereby O_2 is released from Hb and increases the partial pressure of O_2 and speeds up diffusion to O_2-requiring structures. The changes occur at physiologically relevant time scales.



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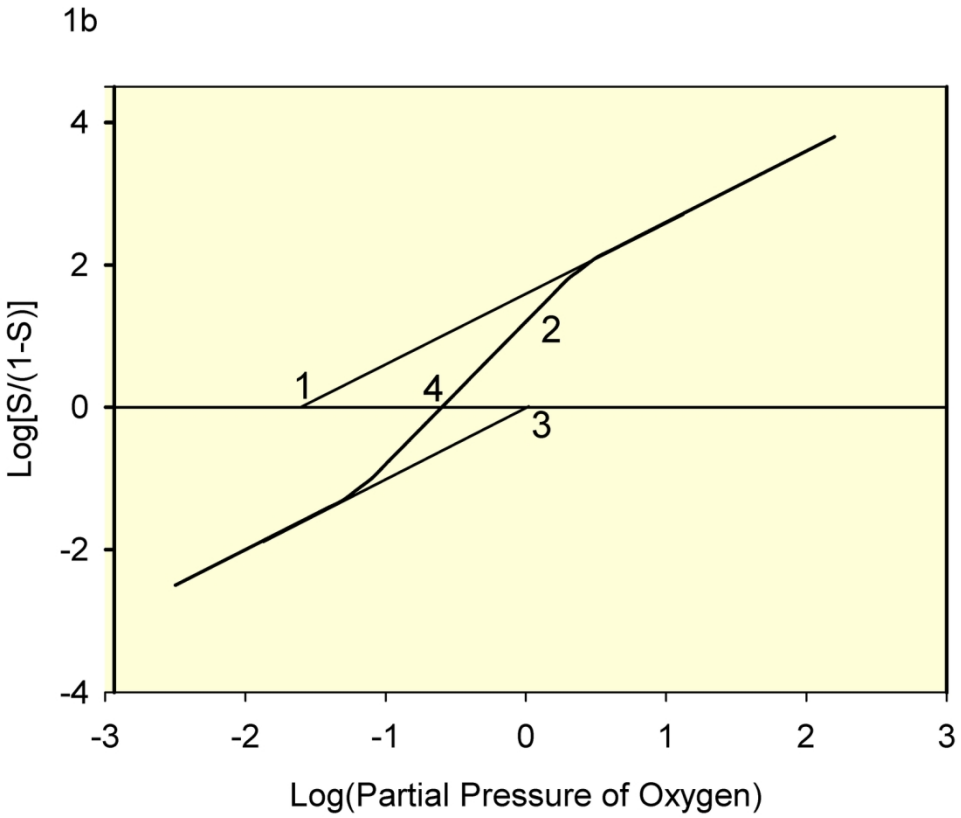

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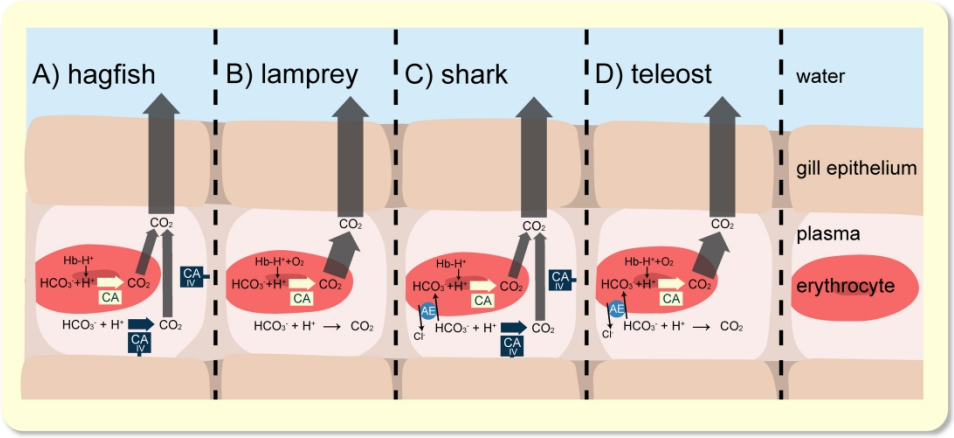

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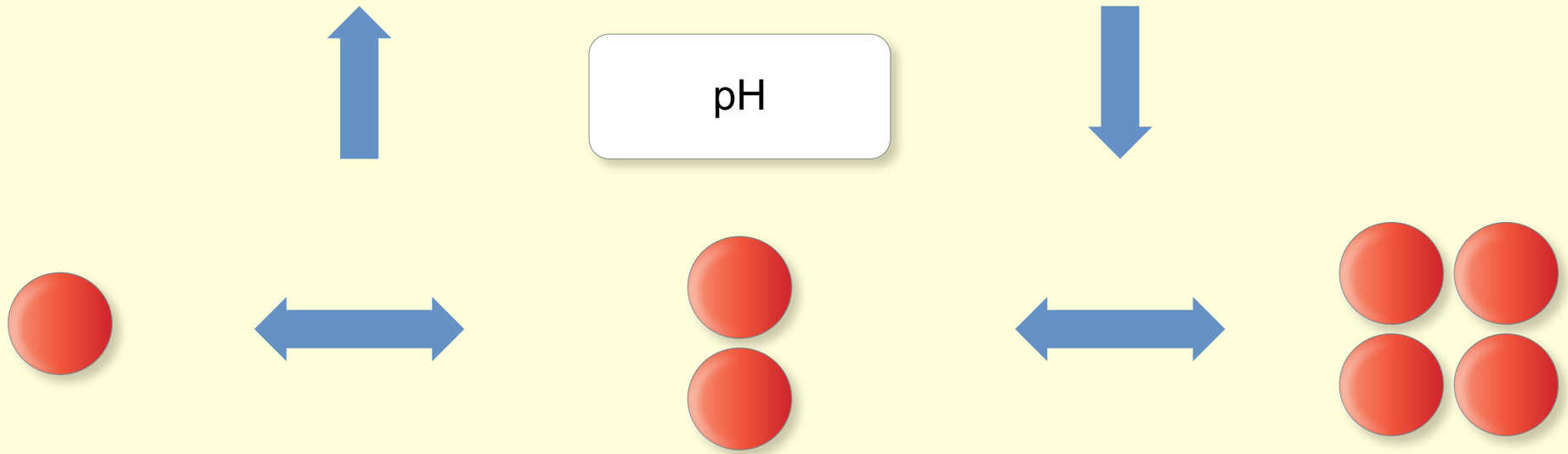
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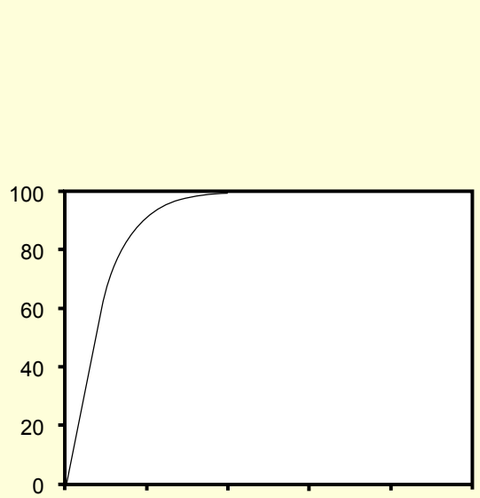
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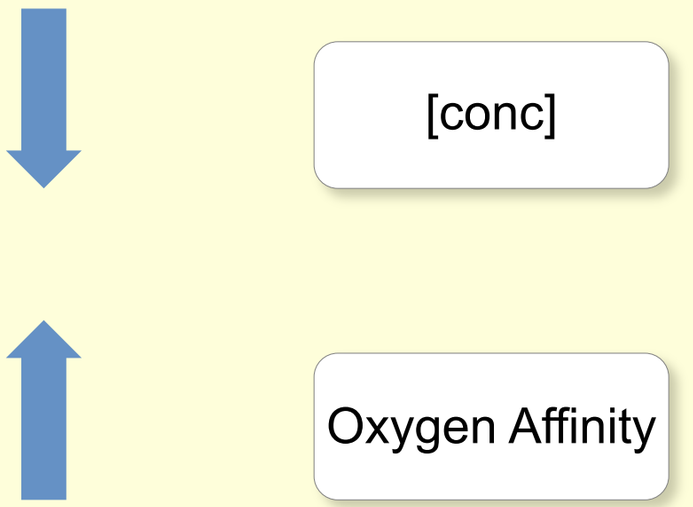
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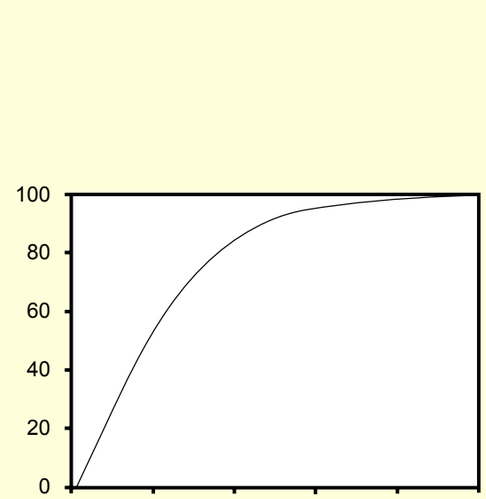
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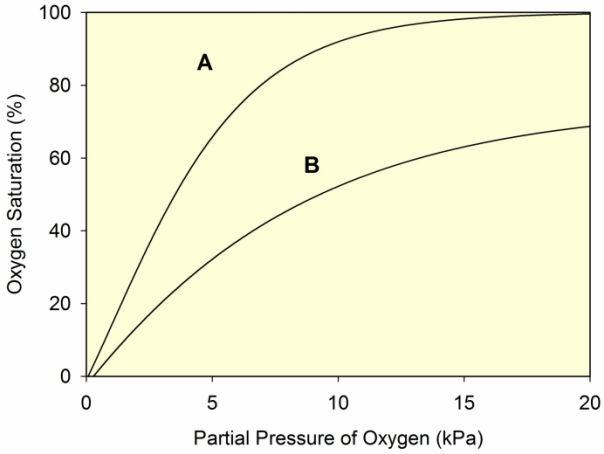

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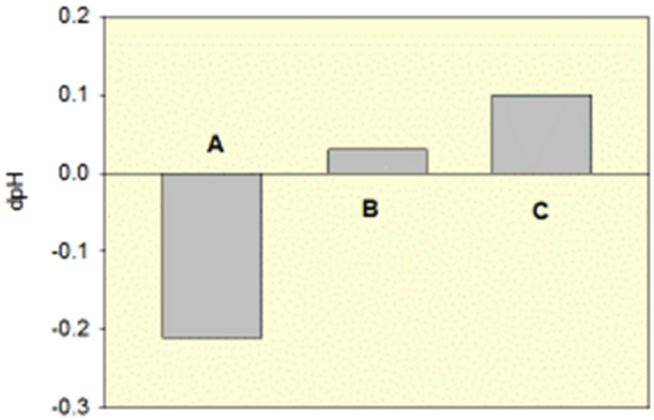
209x296mm (300 x 300 DPI)



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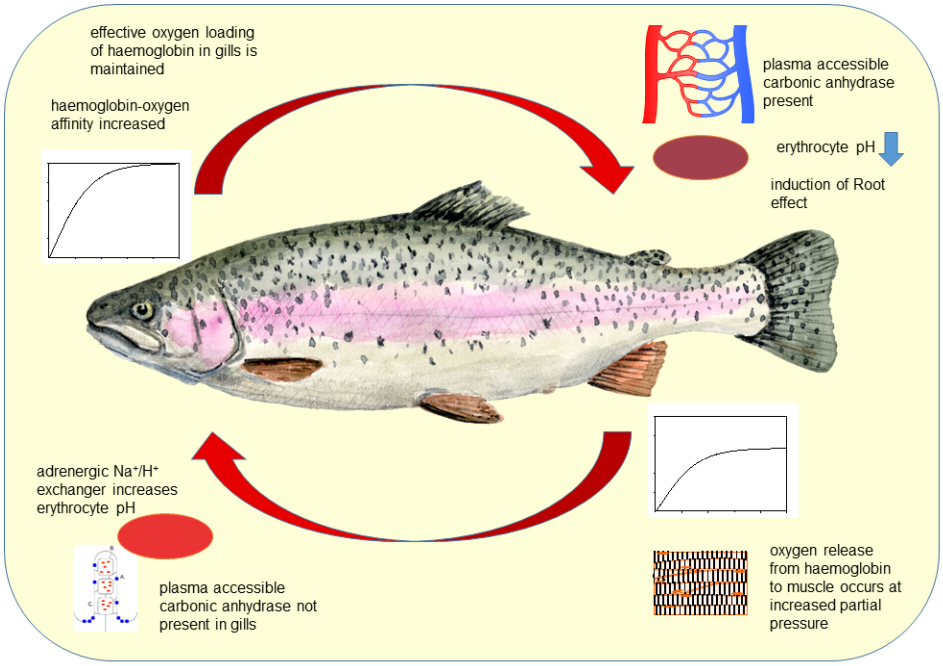

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275x190mm (96 x 96 DPI)