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Historical reconstructions of evolving physiological complexity: O_2 secretion in the eye and swimbladder of fishes

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Summary

The ability of some fishes to inflate their compressible swimbladder with almost pure oxygen to maintain neutral buoyancy, even against the high hydrostatic pressure several thousand metres below the water surface, has fascinated physiologists for more than 200 years. This review shows how evolutionary reconstruction of the components of such a complex physiological system on a phylogenetic tree can generate new and important insights into the origin of complex phenotypes that are difficult to obtain with a purely mechanistic approach alone. Thus, it is shown that oxygen secretion first evolved in the eyes of fishes, presumably for improved oxygen supply to an avascular, metabolically active retina. Evolution of this system was facilitated by prior changes in the pH dependence of oxygen-binding characteristics of haemoglobin (the Root effect) and in the specific buffer value of haemoglobin. These changes predisposed teleost fishes for the later evolution of swimbladder oxygen secretion, which occurred at least four times independently and can be associated with increased auditory sensitivity and invasion of the deep sea in some groups. It is proposed that the increasing availability of molecular phylogenetic trees for evolutionary reconstructions may be as important for understanding physiological diversity in the post-genomic era as the increase of genomic sequence information in single model species.

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Key words: oxygen secretion, Root effect, rete mirabile, choroid, swimbladder, phylogenetic reconstruction.

Introduction

The traditional realm of comparative physiologists is the phenotype. Researchers in this field are generally interested in how organisms work at the organ system, organ, tissue, cellular, organellar and protein levels of organisation. They are fascinated by the wide range of environmental conditions in which organisms exist and how this is matched by the type and capacity of the physiological mechanisms that they employ. In the post-genomic era, the molecular toolbox of several model species is available in the form of fully sequenced genomes and it can be argued that a true integrative view of how organisms function is incomplete without linking genotypes to the encoded phenotypes in the form of proteins, organelles, cells, etc. A major challenge for experimental biologists in the post-genomic era is therefore trying to close the so-called phenotype–genotype gap.

Given the complexity of even a single cell and the emergence of new, unpredictable features at each of the higher levels of biological organisation, it is very difficult, if not impossible, to predict the working of complex physiological systems solely from the underlying genotype. Closure of the phenotype–genotype gap therefore appears a daunting task. Biology, however, unlike physics, can be seen as having already found its single unifying concept, or grand formula: namely evolutionary theory. Thus, looking for the ultimate/evolutionary cause of a physiological mechanism in addition to the proximate/mechanistic cause should help to understand the distribution of particular physiological phenotypes and their associated genotypes in organisms. Thus, the fundamental question for comparative physiologists can be broadened from ‘How does it work?’ (Schmidt-Nielsen, 1997a) to ‘How did it come about to work like it does?’ (Sherwood et al., 2005a).

This review uses the mechanism of oxygen secretion in fishes, which has been intensively studied in the fish swimbladder for over 100 years, to demonstrate the potential insights from such an evolutionary approach. The long generation times of the study species preclude the use of

experimental evolution as a tool, and the analytical approach chosen here is essentially historical, using the comparative method developed in evolutionary biology (Garland et al., 2005). This relies on the availability of phylogenetic trees, which has been greatly improved by the increased capacity and cost effectiveness of DNA sequencing in the post-genomic era.

It is shown how a single, seemingly isolated and exotic, complex physiological mechanism has likely shaped the evolution of the entire respiratory physiology of a group comprising half of all living vertebrates and how it can explain the previously puzzling distribution of certain respiratory phenotypes, ranging from the molecular to whole-organism levels of organisation.

Discovery of O_2 secretion in fishes

The ability of many fishes to concentrate molecular O_2 in their swimbladders, at a level above its concentration in the surrounding water, which is commonly called 'gas secretion', has fascinated physiologists ever since the classic observations 200 years ago by Biot (Biot, 1807). Although this author is usually credited for first finding O_2 concentrations above those in air in the fish swimbladder, Hüfner (Hüfner, 1892) cites work by Brodbelt, who apparently reported the presence of almost pure O_2 in the swimbladder of a swordfish 10 years earlier (in 1797). Nevertheless, Biot appears to have been the first to correlate differences in swimbladder O_2 concentration with the depth at which the fishes were caught (Biot, 1807). Since then, many attempts have been made to unravel the mechanism that, in extreme cases, allows some deep-sea fishes to inflate their compressible swimbladders with 90% O_2 against the hydrostatic pressures several hundred metres below sea level to maintain neutral buoyancy (for reviews of earlier work, see Jones and Marshall, 1953; Alexander, 1966; Fänge, 1966). Eminent late 19th to late 20th century respiratory and comparative physiologists such as Hüfner (Hüfner, 1892), Bohr (Bohr, 1894), Haldane (Haldane, 1922), Krogh (Krogh, 1922), Hall (Hall, 1924), Scholander (Scholander, 1954), Piiper (Krohn and Piiper, 1962; Piiper et al., 1962), Wittenberg and Wittenberg (Wittenberg and Wittenberg, 1974), Schmidt-Nielsen (Lapennas and Schmidt-Nielsen, 1977) and many others have been attracted to the problem. Apart from Krogh (Krogh, 1922), two other Nobel laureates have worked on aspects of the mechanism for swimbladder gas secretion. Thus, von Frisch (von Frisch, 1936) realized the importance of gas secretion in the closed otic (or tympanic) gas bladder of mormyrid fishes for hearing (Stipetic, 1939), and Perutz (Perutz and Brunori, 1982) worked on the molecular mechanism of the exquisitely pH-sensitive O_2-binding characteristics of Root effect haemoglobins (Hbs), which are involved in O_2 secretion (see below).

Mechanism of swimbladder O_2 secretion

Despite the widespread interest in this question and numerous attempts at answers, the basic mechanism behind swimbladder O_2 secretion has only been confirmed during the last four decades. A major breakthrough was achieved after, employing the Krogh principle, a suitable model species had been identified that was particularly amenable for experimentation: the European eel, *Anguilla anguilla* (Steen, 1963b). The mechanism for specifically secreting O_2 relies on three basic components, which are discussed below. The secretion of other gases, whose contribution to swimbladder inflation is usually less than that of O_2, has been described elsewhere (e.g. Pelster and Scheid, 1992) and is not considered here.

Swimbladder metabolism

Steen showed that the glandular swimbladder epithelium of the European eel produces large amounts of lactic acid, which acidifies blood in the swimbladder capillaries and causes a strong decrease in Hb O_2 saturation (Steen, 1963b). His pioneering experiments have subsequently been substantiated and extended on the same species in a series of publications by Kobayashi, Pelster and Scheid (see review by Pelster and Scheid, 1992). It is now clear that anaerobically produced CO_2 from the pentose phosphate pathway significantly contributes to the acidification. In fact, swimbladder metabolism in the European eel appears to be almost completely geared to the production and release of acidic metabolites in the form of lactic acid and CO_2, with a minimum of concomitant O_2 consumption even in the presence of high oxygen partial pressue (P_{O_2}) values (Pelster, 1995). As a result, blood pH in swimbladder capillaries may drop to between pH 7.0 and 6.5 in actively O_2-secreting swimbladders (Steen, 1963b; Kobayashi et al., 1990).

Specialised Root effect Hbs

The effect of acidification on Hb O_2 binding is particularly strong in teleost fishes and involves a decrease in the affinity (Bohr effect) as well as cooperativity of Hb O_2 binding (Perutz and Brunori, 1982; Brittain, 1987; Brittain, 2005). The effect is so strong in some fishes such as the Atlantic cod (*Gadus morhua*) that air-equilibrated blood or haemolysates, which are normally close to full Hb O_2 saturation, can release up to 80% of their bound O_2 upon acidification below a certain threshold value of P_{CO_2} or pH (Krogh and Leitch, 1919; Berenbrink et al., 2005). This phenomenon has been called the Root effect after one of the first people to describe it (Root, 1931; Scholander and van Dam, 1954; Pelster and Weber, 1991; Pelster and Randall, 1998). The strength of the Root effect varies between species and can be measured by the degree of acid-induced deoxygenation of functional Hb (i.e. not oxidised or denatured) in blood or in solution at a high reference P_{O_2}, which is conveniently taken as the P_{O_2} of air (e.g. Farmer et al., 1979; Berenbrink et al., 2005) (Fig. 1). In air-equilibrated blood or haemolysates of the European eel, the Root effect causes a maximal reduction of Hb O_2 saturation by ~30–50% upon acidification (Steen, 1963a; Bridges et al., 1983; Berenbrink et al., 2005) and this effect persists at P_{O_2} values higher than those found in air (Steen, 1963a; Bridges et al.,

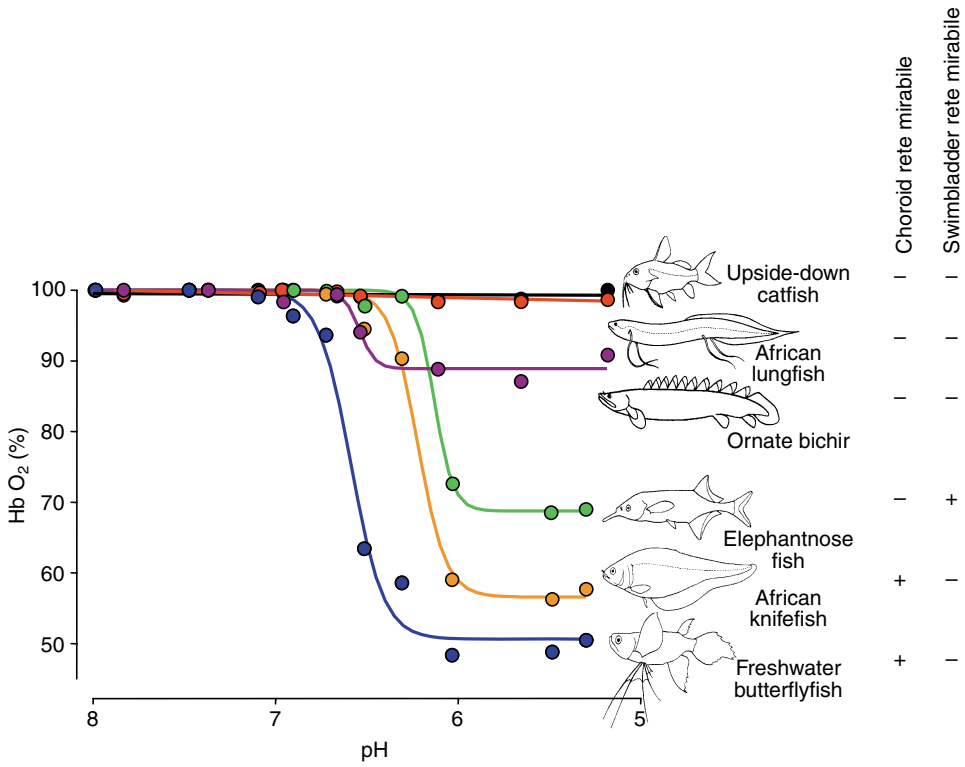

Fig. 1. Variability of the Root effect in haemolysates of selected African fishes. The magnitude of the Root effect is measured as the difference in percentage Hb O_2 saturation between the two plateaus at high and low pH. Presence and absence of choroid and swimbladder retia mirabilia are indicated for each species. Hb O_2 saturation of functional Hb was determined using diluted, air-equilibrated samples (5–15 μmol l^{-1} [Hb_4]) by spectral deconvolution between 500 and 700 nm (Völkel and Berenbrink, 2000). Measurements were performed at 25°C without removal of organic phosphates in 50 mmol l^{-1} Tris HCl buffer (pH 8.0–6.5) and 50 mmol l^{-1} citrate buffer (pH 7.0–5.0) in the presence of 0.1 mol l^{-1} KCl (M. Berenbrink, unpublished). Fish line drawings modified after: Lehrbuch der Speziellen Zoologie. Begruendet von Alfred Kaestner. Band II: Wirbeltiere, 1991©Elsevier GmbH, Spektrum Akademischer Verlag, Heidelberg.

1983; Pelster and Weber, 1990). By contrast, mammalian blood or Hb solutions from dog and rabbit or pig and man, respectively, stay ⩾95% saturated under similar conditions (Bohr et al., 1904; von Ledebur, 1937; Berenbrink et al., 2005).

Vascular counter-current exchange in the rete mirabile

As far as is known today, swimbladder O_2 secretion is invariably linked with the occurrence of an anatomical structure known as the swimbladder rete mirabile. Earlier claims of O_2 secretion in the absence of a rete mirabile (Sundnes et al., 1958) have been refuted by subsequent careful anatomical studies (Fahlen, 1959). The swimbladder rete mirabile comprises a vascular counter-current exchange system that is interspersed in the blood supply of the acid-producing gas gland cells of the swimbladder epithelium. Depending on species, it can comprise just a few or up to several thousand arterial and venous, interdigitated capillaries running in opposite directions, which provides an increased surface area for cross-capillary diffusion exchange of gases and solutes between the arterial supply and venous drainage of the swimbladder epithelium. For the paired swimbladder retia of the European eel, Krogh estimated a total of 116 000 arterial and 88 000 venous capillaries with a total length of 464 and 352 m, respectively, creating a surface area for arteriovenous exchange of 105 cm^2 in a volume as small as a water drop (64 mm^3) (Krogh, 1922).

Haldane first suggested that the rete mirabile might function as a counter-current exchanger for CO_2, increasing the P_{CO_2} of blood in the gas gland, which in turn would cause the release of O_2 from Hb and thereby increase P_{O_2} (Haldane, 1922). However, a simple calculation shows that the O_2 capacity of arterial blood on its own is not high enough to create P_{O_2} values of several hundred atmospheres, even if 100% of the O_2 bound to Hb were released into physical solution upon acidification *via* the Root effect (Jacobs, 1930; Scholander and van Dam, 1954; Brauner and Berenbrink, in press). Jacobs accordingly postulated that the rete mirabile also functions as a counter-current exchanger for O_2 (Jacobs, 1930).

The unique vascular anatomy of the bipolar swimbladder rete mirabile in the European eel has enabled measurements of gas and solute concentrations in blood before and after passing the arterial and venous capillaries (Steen, 1963b; Kobayashi et al., 1990). These classic studies have confirmed that the rete mirabile acts as a counter-current exchanger for CO_2 and lactate or lactic acid, and that part of the Root effect can thereby already be elicited in arterial rete capillaries (Pelster and Scheid, 1992). Back-diffusion of O_2 from venous to arterial capillaries of the rete has not been demonstrated in these experiments, presumably because the rate of O_2 diffusion into the swimbladder under the particular conditions was so large that the P_{O_2} of the blood entering the venous part of the rete mirabile did not provide a high enough diffusion gradient for counter-current O_2 exchange (Pelster and Scheid, 1992). Nevertheless, O_2 back-diffusion in the rete mirabile has still to be postulated for the many cases where the P_{O_2} of swimbladder gases exceeds the P_{O_2} that can be theoretically generated by the Root effect after acidifying arterial blood.

O_2 secretion in the fish eye

So intimate appears the correlation between O_2 secretion and the presence of gas-exchanging retia mirabilia in fishes that another, previously unknown, site of O_2 secretion was predicted and confirmed in the fish eye by Wittenberg and Wittenberg

(Wittenberg and Wittenberg, 1962; Wittenberg and Wittenberg, 1974). This site was solely predicted from the presence of a vascular counter-current system, known as the choroid rete mirabile, behind the retina of several fish species (Fig. 2A–D). These authors measured P_{O_2} values in excess of air saturation, in some cases even above 100 kPa, in the vitreous humour close to the retina of several fish species that possessed a choroid rete mirabile. By contrast, measurements on fishes and tetrapods without this structure only yielded values close to mixed venous blood (Wittenberg and Wittenberg, 1962; Wittenberg and Wittenberg, 1974). The ocular system is poorly investigated and it is clear that important functional differences must exist compared with the swimbladder (Wittenberg and Haedrich, 1974; Berenbrink, 1995; Bridges et al., 1998). But it is commonly assumed that lactic acid release by the retinal pigment cell layer acidifies blood in the choriocapillaries and causes an increase in P_{O_2} *via* the Root effect, which is then multiplied by counter-current exchange in the choroid rete mirabile, analogous to the swimbladder mechanism (Wittenberg and Wittenberg, 1962; Wittenberg and Wittenberg, 1974; Pelster and Weber, 1991; Pelster and Randall, 1998). In a detailed study, Waser and Heisler have recently demonstrated that the Root effect is an essential component of the O_2 secretion mechanism in the eye of the rainbow trout, *Oncorhynchus mykiss* (Waser and Heisler, 2005). In contrast to the swimbladder, where O_2 is secreted for buoyancy regulation, ocular O_2 secretion is thought to be advantageous for supplying the vigorous O_2 demand of the often avascular retina of the fish eye (Wittenberg and Wittenberg, 1962; Wittenberg and Wittenberg, 1974) and therefore ultimately for vision (Berenbrink et al., 2005).

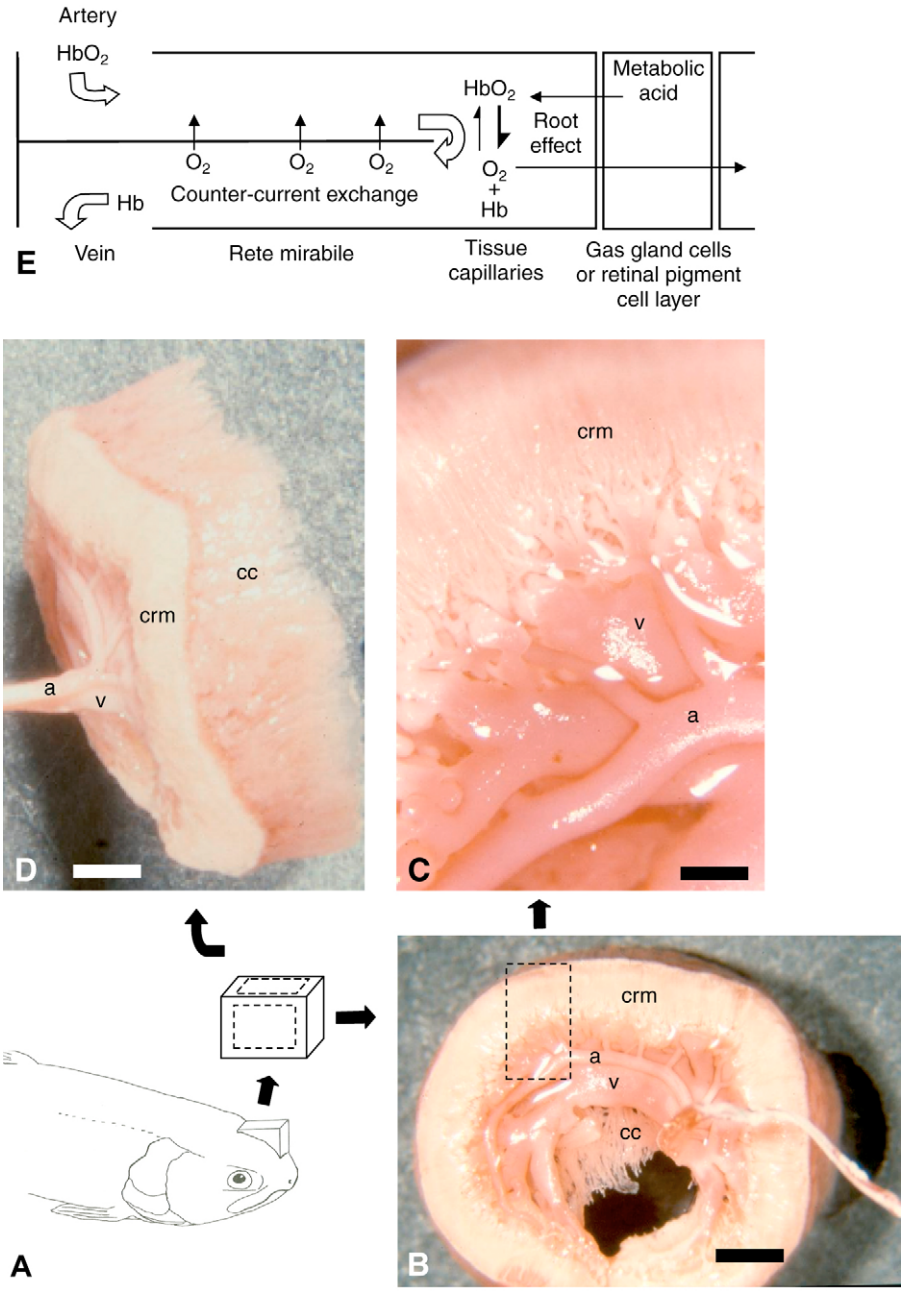

Fig. 2. The choroid rete mirabile in the eye of the rainbow trout. (A) Schematic drawing to illustrate the orientation of the vascular corrosion cast from which photomicrographs were taken. (B) View of vascular corrosion cast of left eye from inside the orbit. The opening in the lower centre presents the entry point of the optic nerve. (C) Area indicated by the broken rectangle in B at higher magnification, showing parallel array of intermingled arteries and veins in the choroid rete mirabile. (D) Dorsal view (anterior side is at the bottom) showing the connection between choroid rete mirabile and the dense network of choriocapillaries, which underlies the retina. Abbreviations: a, v, branches of the ophthalmic artery and vein, respectively; crm, choroid rete mirabile; cc, choriocapillaries. Scale bars, 2 mm in B and D, and 500 μm in C. (E) Simplified diagram of the mechanism for specific O_2 secretion in the eye and swimbladder of fishes. The three essential components are indicated. See text for further explanation. A–D modified from Berenbrink (Berenbrink, 1995).

Open questions in the study of O_2 secretion

The preceding section identified the three essential components of the mechanism for O_2 secretion in the swimbladder (and, by analogy, in the fish eye; Fig. 2E): (1) the metabolic capacity of swimbladder gas gland cells to strongly acidify blood in the swimbladder capillaries, (2) the presence of Root effect Hbs that can offload O_2 upon acidification, even in the presence of high P_{O_2} values, and (3) a rete mirabile, which allows CO_2 and

O_2 back-diffusion into the arterial supply of the gas gland cells and which thereby localises and multiplies the initial increase in the partial pressures of these gases.

However, although the formidable physiological capacity for O_2 secretion in the swimbladder has been known now for 200 years, many details of the process are still unclear. For example, while several transport mechanisms for acid–base equivalents across swimbladder gas gland cells have been identified in the past decade, their interaction is only beginning to be understood (Pelster, 2004). Similarly, while the importance of the vagus nerve for stimulating swimbladder O_2 secretion was demonstrated more than 100 years ago (e.g. Bohr, 1894), the regulatory mechanisms controlling the acid production rate of gas gland cells are largely unknown (Pelster, 2004).

Likewise, 25 years after the explanation of the Root effect as an exaggerated Bohr effect of human HbA (Perutz and Brunori, 1982), this interpretation is now increasingly challenged, Thus, the search for the molecular mechanism(s) of the Root effect still continues, despite the sequencing, crystallographic characterisation and modelling of an increasing number of fish Hbs with and without a Root effect. Earlier work tried to explain the molecular mechanism of the Root effect by changes in one or very few key amino acid residues in fish Hbs, which at low pH would greatly stabilize the low O_2 affinity T(ense)-state conformation of haemoglobin over the high affinity R(elaxed)-state conformation (Perutz and Brunori, 1982). This classical model assumed that the O_2 affinities within the T- and R-states were fixed. Subsequent studies of deep sea fish Hbs, which can release O_2 even at very high P_{O_2} for swimbladder filling, indicated the presence of two roughly equal fractions of O_2 or CO-binding sites with markedly different ligand binding affinities (Noble et al., 1986). This was interpreted as subunit heterogeneity elicited by low pH and explained the decrease in the cooperativity of Hb O_2 binding found in many Root effect Hbs at low pH. In other words, after the first half of subunits has been occupied, low pH appears to essentially block further O_2 binding to the other half of the subunits. At least in tuna (*Thunnus thynnus*) Hb, the latter are probably the β-chains (Yokoyama et al., 2004).

Many fishes further express several Hb isoforms in their red blood cells, some of which may show a strong Root effect whereas others show none at all (e.g. Pelster and Weber, 1990). Thus, the overall maximal Root effect in red blood cells can further be modulated by varying the fraction of these functionally different Hbs.

With more and more structural and functional data now available, it is increasingly clear that interspecific differences in the mechanism(s) of the Root effect may exist. Thus, as in the Bohr effect of human Hb, the C-terminal histidine of the β-chains may be responsible for about 50% of the total Root effect in Hb of common carp (*Cyprinus carpio*) (Parkhurst et al., 1983). On the other hand, structural studies on Hbs of the Antarctic fishes *Trematomus* (formerly *Pagothenia*) *bernaccchii* and *T. newnesi* and on tuna Hb indicate the absence of a dominant role for this residue in the Root effect (Ito et al., 1995; Yokoyama et al., 2004; Mazzarella et al., 2006a; Mazzarella et al., 2006b).

Similarly, the Root effect is generally seen as caused by a stabilisation of the low O_2 affinity T-state conformation of Hb by protons. Yet in Hb of the spot (*Leiostomus xanthurus*), proton binding to the R-state has been suggested to create positive-charge clusters, which destabilise the high-affinity R-state and cause the switch to the T-state (Mylvaganam et al., 1996). The major Hb of *T. newnesi* has a very similar R-state structure to spot Hb but shows no Root effect, questioning the generality of this explanation (Mazzarella et al., 1999).

At least in *T. bernacchii*, tuna and HbC of *T. newnesi*, a large part of the Root effect can be explained by the sharing of a proton and formation of a strong hydrogen bond between two pairs of aspartyl residues of the α and β chains at low pH in the T-state but not R-state conformation of the protein (Ito et al., 1995; Yokoyama et al., 2004; Mazzarella et al., 2006a). Recently, it has been suggested that this interaction of aspartyl residues is the minimal permissive requirement for the Root effect in fish Hbs and that several histidinyl residues, which may differ between species, act as modulators of the Root effect afforded by the interaction of the aspartyl residues (Mazzarella et al., 2006a; Mazzarella et al., 2006b).

The view is emerging that the mechanism of the Root effect is different from that of the Bohr effect of human Hb and may be the result of several, additive mechanisms, whose contribution can differ between species (Yokoyama et al., 2004; Bonaventura et al., 2004; Brittain, 2005; Berenbrink et al., 2005; Berenbrink, 2006; Mazzarella et al., 2006a/b??).

Notwithstanding gaps in our knowledge about all the intricacies of the system, the mechanism of swimbladder O_2 secretion has become a standard example of a complex and integrated physiological system in textbooks of animal physiology (Hill et al., 2004; Randall et al., 2002; Schmidt-Nielsen, 1997b; Sherwood et al., 2005b; Willmer et al., 2005; Withers, 1992).

Steps in the evolution of O_2 secretion

This section addresses the ultimate or evolutionary cause behind swimbladder O_2 secretion. In other words, how did it evolve to work in the way it does? How could such a complex system evolve and how can the increasing availability of molecular sequence information in the post-genomic era help to answer this question?

It appears highly unlikely that even the three basic components of the system originated simultaneously. If, however, the components evolved stepwise, what were the selective advantages for these steps, given that only the three components together allow O_2 secretion? Is there a particular sequence in which the components had to be acquired, and which came first, the swimbladder or the ocular system?

Evolution of increased metabolic acid production

Differences in the capacity for lactic acid production are frequently observed in different tissues of vertebrates, and *a*

priori there appears to be no reason why the retinal pigment cell epithelium or the swimbladder epithelium should not be able to evolve a higher capacity for lactic acid production, either to augment aerobic ATP production in the metabolically active retina or to avoid diminishing gaseous O_2 concentration and decreasing buoyancy in a swimbladder filled by air-swallowing at the surface. Hence, the metabolic changes in the retina or swimbladder epithelium may initially only have been of a quantitative nature, not involving a fundamental change in metabolic pathways. Interestingly, uncoupling between glycolysis and a lack of tissue oxygen is seen in several other quite diverse vertebrate tissues such as the mammalian retina (Winkler, 1981) and the rattlesnake tailshaker muscle (Kemper et al., 2001). This indicates that high lactic acid production rates in the presence of ample oxygen may evolve more easily than one would predict based on the much higher ATP yield per mole of glucose when pyruvate is channelled into the citric acid cycle and oxidative phosphorylation rather than transformed to lactic acid. Because studies comparing the metabolism of the retina or swimbladder of species with and without O_2 secretion are lacking, it is assumed in the following that the production and release of acidic metabolites into the blood supply of the swimbladder or retina was not a limiting step in the evolution of O_2 secretion.

Evolution of a rete mirabile

Retia mirabilia occur in different anatomical locations in many vertebrate groups, suggesting that this deviation from the normal vascular pattern arises frequently, such that it may be genetically fixed by natural selection as soon as it provides an animal with an advantage that ultimately increases its fitness (Carey, 1973; Block et al., 1993). Examples include the heat-exchanging retia mirabilia in the head of some endothermic teleosts and sharks or some birds and mammals that are employed in selective brain heating or cooling, respectively (Linthicum and Carey, 1972; Block and Carey, 1985; Jessen, 2001). Other examples include the vasa recta of the mammalian kidney, the vascular arrangement in the placenta of some mammals, the heat-conserving retia mirabilia in the extremities of several birds and mammals (Scholander, 1958) and the rete mirabile in the spermatic cord of many mammals, which protects the testes from overheating (Harrison and Weiner, 1949).

The retial arteries of heat exchangers frequently have rather large diameters, sometimes up to several hundred micrometres (Block and Carey, 1985). They therefore appear poorly equipped for gas exchange with neighbouring veins (Carey, 1973). By contrast, choroid and swimbladder retia mirabilia consist of smaller vessels of capillary dimensions (Krogh, 1922; Scholander, 1954; Wittenberg and Wittenberg, 1974). Theory predicts that without a mechanism to elevate tissue P_{O_2}, such gas-permeable retia mirabilia risk shunting O_2 away from the tissues, as blood from the respiratory organ entering the arterial part of the rete has a higher P_{O_2} than blood leaving the tissues in the venous part of the rete (Kobayashi et al., 1989). Under these circumstances, physically dissolved O_2 can diffuse down the arteriovenous concentration gradient across the increased surface area of the rete mirabile, short-circuiting tissue O_2 supply. Thus, evolution of a gas-permeable rete mirabile before the evolution of a Root effect appears disadvantageous.

Evolution of the Root effect

A Root effect without a swimbladder or choroid rete appears equally disadvantageous. Like other water-breathing animals, teleost fishes generally have a low capacity blood CO_2/bicarbonate buffer system (Heisler, 1986). In addition, their Hb, which usually constitutes the major non-bicarbonate buffer component in the blood, has a reduced number of surface histidine residues compared with that of most other vertebrates, which results in lower specific buffer values of teleost Hbs (Jensen, 1989; Berenbrink et al., 2005). Taken together, this means that teleost blood is easily acidified under conditions such as exercise-induced lactic acid production. In the presence of a Root effect, there is the risk that the resulting drop in pH causes incomplete Hb O_2 saturation in the gills and impairs tissue O_2 supply. Quite unlike other vertebrates, some teleosts with a Root effect Hb are able to protect their red blood cell pH and thereby Hb O_2 saturation under these conditions by β-adrenergic activation of a Na^+/H^+-exchanger (βNHE) in their red blood cells (Nikinmaa, 1992; Berenbrink and Bridges, 1994). However, others, like the European eel, are not (Romero et al., 1996). Thus, without a rete mirabile in the swimbladder or eye, and the associated benefits of ocular or swimbladder O_2 secretion, the possession of a Root effect appears not only superfluous but even dangerous.

Hence, the problem is that a rete mirabile and Root effect appear only beneficial when they occur together and that each alone seems disadvantageous. Accepting their simultaneous evolution as unlikely, which came first and what was the selective advantage? In this context it would obviously help to know when, relative to the Root effect, low Hb buffer values and the βNHE evolved, because these two factors are likely to influence how seriously a Root effect might impair adequate Hb O_2 loading in the gills under general acidosis.

Phylogenetic trees and evolutionary reconstruction in comparative physiology

Retia mirabilia, Root effect, βNHE and Hb buffer value obviously do not leave their mark in the fossil record. The only way to study their evolution, therefore, is to take the relevant information from living species and reconstruct the evolution of each feature back to the ancestors on a phylogenetic tree, using parsimony or maximum likelihood methods (Maddison and Maddison, 2000; Garland et al., 2005). This allows identification of when and how often a feature evolved and whether its absence in a group is due to the trait never having evolved or due to a secondary loss. Moreover, comparing the evolution of two or more features can identify instances of correlated evolution. Crucially, by comparing the relative sequence in which its component parts arose, the steps leading to the evolution of a complex

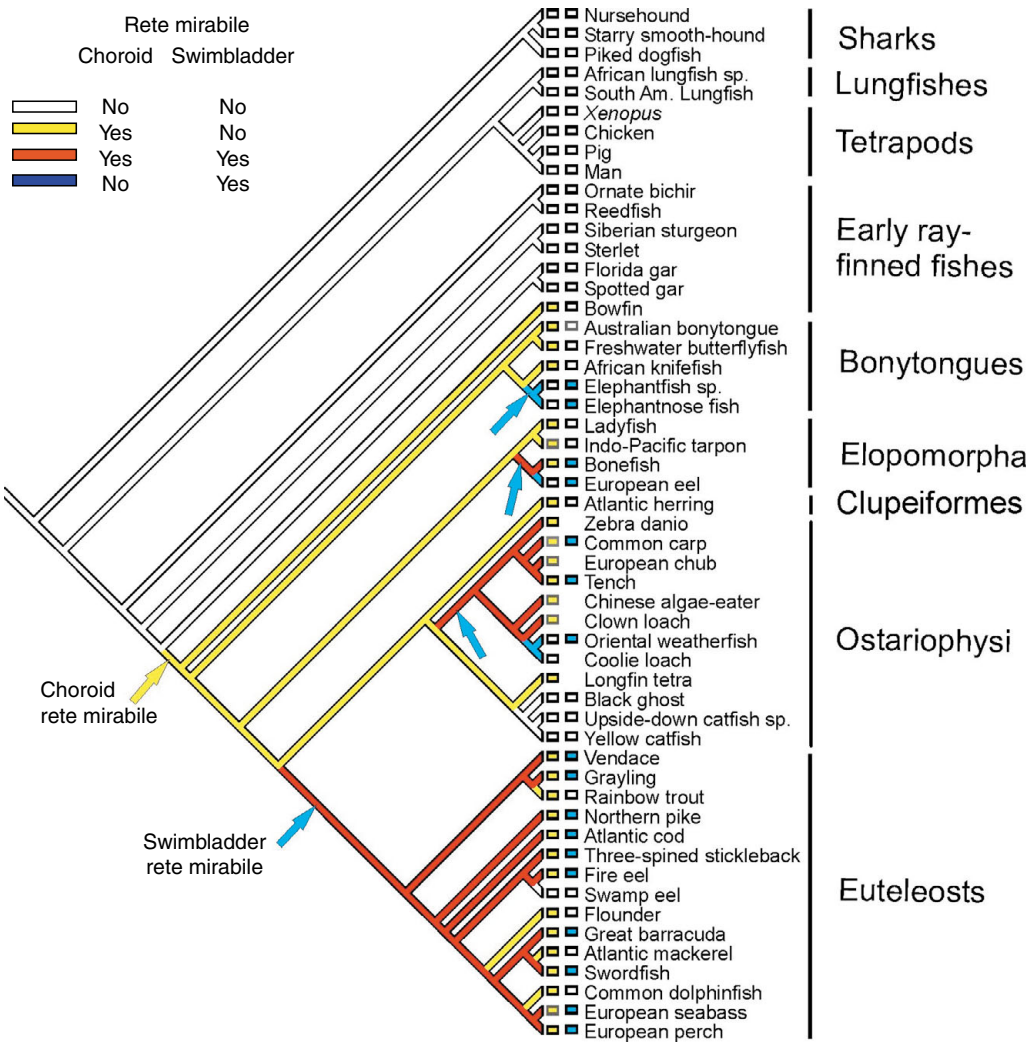

Fig. 3. Evolution of swimbladder and choroid retia mirabilia in jawed vertebrates. Blue and yellow boxes mark the presence of swimbladder and choroid retia mirabilia in living species, respectively. Open boxes indicate the absence of the respective structure, whereas no box indicates missing information. The status of ancestral species has been reconstructed on a composite branching diagram by maximum parsimony. Yellow and blue branches indicate the presence of the choroid and swimbladder rete mirabile, respectively. White and red branches indicate the absence and presence, respectively, of both structures in an ancestral species. The yellow arrow indicates the branch segment along which the choroid rete mirabile first evolved. The blue arrows indicate the subsequent evolution of a swimbladder rete mirabile in four separate groups. Modified after Berenbrink et al. (Berenbrink et al., 2005).

physiological system can be identified, and pre-dispositions and constraints can be inferred. An elegant example of this approach is the study of the evolution of endothermy in the suborder of scombroid fishes (mackerels, tunas and billfishes) (Block et al., 1993).

Until quite recently, extending such analyses to larger groups, such as a whole class or even several classes of vertebrates, would have been seriously hampered by the lack of a clear understanding of higher-level phylogenetic relationships. With ever-increasing computing power and high-throughput, low-cost sequencing techniques the situation has greatly improved in the post-genomic era. Rather than single genes or parts thereof, multiple concatenated genes and whole mitochondrial genomes are now commonly used to unravel phylogenetic relationships, and dealing with large data sets from as many as 100 species no longer causes serious computational problems (Murphy et al., 2001; Miya et al., 2003). In addition, the use of derived shared molecular markers such as the presence or absence of insertions and deletions in coding sequences, nuclear introns and alternatively spliced transcripts (Venkatesh et al., 2001) or the analysis of retroposon insertion patterns (Nishihara et al., 2006) is now possible on a large scale, promising tools for solid phylogenies for any group of interest to comparative physiologists.

Reconstructing the evolution of O_2 secretion

Recently, the evolution of swimbladder and choroid retia mirabilia, the Root effect, red blood cell βNHE and Hb buffer values has been reconstructed on a composite phylogeny of jawed vertebrates (Berenbrink et al., 2005). The study suggests that, of the two structures, the choroid rete mirabile evolved first, about 250 million years ago, and that it evolved only once, namely within the ray-finned fishes in a common ancestor of the bowfin *Amia calva* and teleosts, after it had diverged from the gar lineage (Lepisosteidae) (Fig. 3). Although most living teleosts possess a choroid rete mirabile, the structure has been secondarily lost in several unrelated groups. These include elephantfishes (Mormyridae), eels (Anguilliformes), catfishes (Siluriformes), some loaches (Cobitoidea) and swamp eels (Synbranchoidei) (Berenbrink et al., 2005) (Fig. 3). Interestingly, some members of these groups are noted for their nocturnal habits, small eyes, benthic lifestyle, murky aquatic environment or finding their food predominantly using chemical rather than

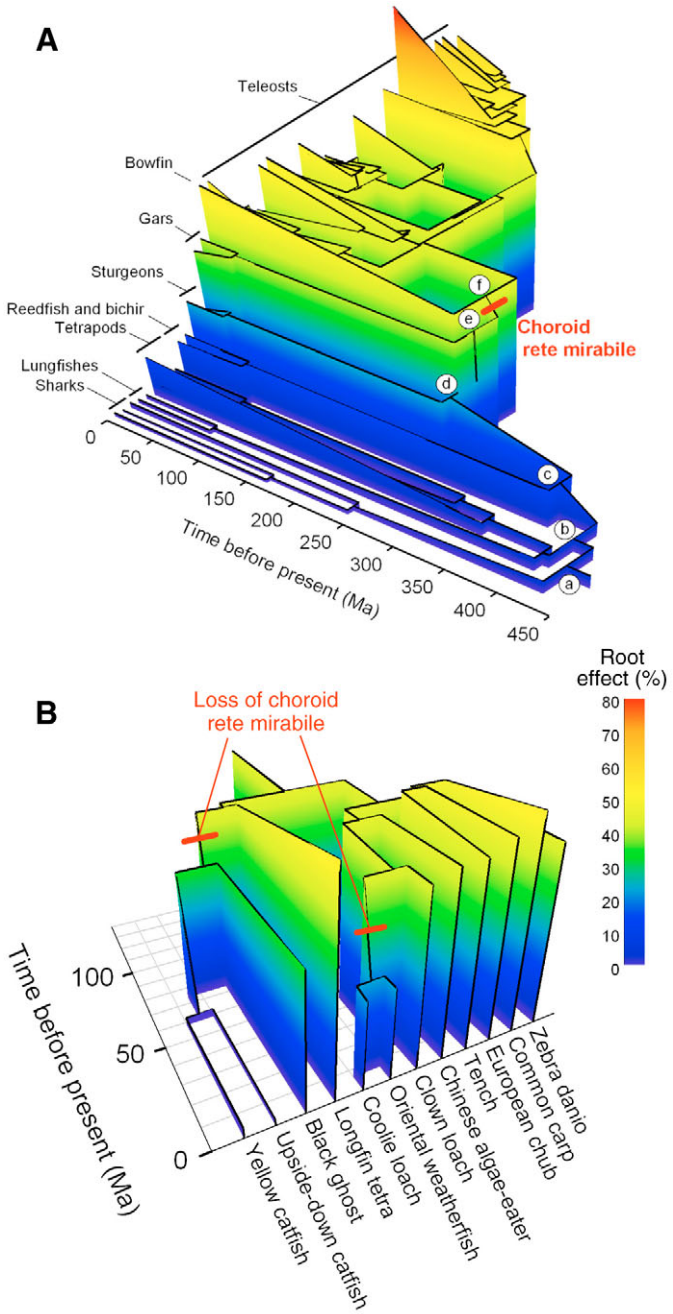

Fig. 4. Evolutionary reconstruction of the Root effect in jawed vertebrates. The underlying phylogenetic tree is based on the species and branching pattern shown in Fig. 3. The Root effect has been colour coded and its magnitude in ancestral species has been reconstructed on the *z*-plane of the structure by linear parsimony from values measured in living species as shown in Fig. 1. (A) The three-dimensional structure has been rotated to visualise the gradual increase of the Root effect in early ray-finned fishes (nodes c–f) after their ancestors diverged from the lineages of sharks (a) and lobe-finned fishes (including tetrapods, b). The red bar indicates the origin of the choroid rete mirabile in the branch leading to the bowfin and teleosts only after the Root effect had increased. (B) Enlarged part of the structure in A after rotation, showing two examples of secondary reductions of the Root effect in Ostariophysi. The Root effect is only ever reduced when the choroid rete mirabile has been lost. The latter is indicated by red bars. The oriental weather loach still has a swimbladder rete mirabile, whereas the two catfishes lack both types of rete (Fig. 3), consistent with a complete loss of the Root effect in the latter group. Ma, million years. Modified from Berenbrink et al. (Berenbrink et al., 2005).

visual senses (e.g. Nelson, 1994). Indeed, reconstruction of Root effect evolution suggests that all living or ancestral species with a choroid rete mirabile also have a Root effect of about 40% or more (Berenbrink et al., 2005) (Fig. 4). Given the close functional association of the two components in living species, this suggests that those ancestors also had the ability to secrete O_2 in their eyes and that loss of the choroid rete mirabile in several groups was each time associated with a change in the O_2 supply characteristics to their eyes.

The above study also showed that any of the several secondary reductions of the Root effect only occurred when the choroid rete mirabile had been lost (Fig. 4B). This result was statistically highly significant and suggests that natural selection maintains the Root effect in species with a choroid rete mirabile. As discussed above, a rete mirabile without a Root effect is not only insufficient for O_2 secretion but also carries the danger of short-circuiting normal tissue O_2 supply by O_2 back-diffusion. Thus, loss of the Root effect in species still possessing a choroid rete mirabile appears evolutionarily constrained not only because of the benefits of the Root effect in terms of O_2 secretion but also because it ameliorates the inherent danger of possessing a gas-permeable rete mirabile.

The same considerations also lead to the prediction that the choroid (and swimbladder) rete mirabile should only have evolved in species where the Root effect was already present. In fact, this is exactly what has been found (Berenbrink et al., 2005). Reconstruction of Root effect evolution in the teleost lineage indicates its gradual increase from about 5% in their last common ancestors with cartilaginous fishes and lobe-finned fishes (e.g. sharks and lungfishes, respectively; nodes a and b in Fig. 4A) to 15% in their last common ancestor with the most basal ray-finned fish lineages, the Polypteriformes (reedfish and bichirs; c in Fig. 4A). This is followed by Root effects of ~25% and then 40% in the last common ancestors of teleosts with sturgeons and gars, respectively (d and e in Fig. 4A). Importantly, in none of these ancestors is a choroid or swimbladder rete mirabile reconstructed.

If the Root effect did not originally evolve because of its function in ocular or swimbladder O_2 secretion, what was its original selective advantage and how was its negative side effect, the danger of impaired Hb O_2 saturation in the gills under general blood acidosis, compensated for?

It is possible that the blood gas transport characteristics in early ray-finned fishes were not quite as vulnerable during acidosis as they are in teleosts. This was not due to possession of a red blood cell βNHE because this mechanism only evolved in advanced teleost groups, after they had diverged from the more basal teleost group of bonytongues (Osteoglossomorpha) (Berenbrink et al., 2005). Decreased

vulnerability against acidosis may rather have been due to higher specific Hb buffer values in early ray-finned fishes. Thus, living members of ancient ray-finned fish lineages, such as reedfish and sterlet, which already show a small Root effect, still have elevated Hb buffer values compared with living teleosts. The evolutionary reconstruction indeed confirms that the Root effect originally evolved in the presence of high Hb buffer values. Thus, the low blood pH values necessary to elicit the Root effect may rarely have been achieved in early ray-finned fishes, because their buffer properties were more similar to those of present-day sharks and lungfishes than to teleosts (Berenbrink et al., 2005; Berenbrink, 2006; Brauner and Berenbrink, in press).

Because buffer properties of proteins are largely dependent on the number of accessible surface histidine amino acid residues, their buffer properties can be estimated from primary amino acid sequences and structural information. For Hb, this approach is facilitated by (1) the conserved three-dimensional structure of the globin fold and the contact sites between the globin monomers and (2) the rich source of vertebrate Hb sequences in the protein and nucleotide databases, such that a strong correlation between predicted and measured Hb buffer values has been established (Berenbrink et al., 2005; Berenbrink, 2006).

Thus, an important and, until recently, largely unrecognised phenotype, which bears on the basic characteristics of the blood gas transport system in vertebrates, can be directly predicted from the genotype. This approach has been used to estimate Hb buffer values in ancestral vertebrates as well as in the elusive living coelacanth *Latimeria chalumnae* (Berenbrink, 2006). Surprisingly, expanding the evolutionary reconstruction by using a larger dataset of over 70 vertebrates indicates that, apart from ray-finned fishes, other vertebrate groups such as passeriform birds have also undergone a significant evolutionary decrease in Hb buffer values for as yet poorly understood reasons (Berenbrink, 2006).

It appears then that the negative aspect of the Root effect in ray-finned fishes was less severe when it first originated than it was after Hb buffer values had declined. But what was the selective advantage of the Root effect and also of the decrease in Hb buffer values? As mentioned above, Root effect Hbs are characterized by a strong decrease in Hb O_2 binding affinity and cooperativity with decreasing pH. It is the latter property that sets the Root effect apart from the Bohr effect found in other vertebrate Hbs (Brittain, 1987) and that may be primarily responsible for incomplete Hb saturation at low pH in air-saturated blood or Hb solutions. However, the decrease in cooperativity is most severe at low pH values (Brittain, 1987), which may not be reached *in vivo* in the presence of high Hb buffer values and without acid back-diffusion across a rete mirabile. It is conceivable, therefore, that it was the decrease in Hb O_2 affinity with pH alone that was selected for in early ray-finned fishes. Under this scenario, the associated decrease in O_2-binding cooperativity at lower pH was just a by-product without any physiological consequences, and the well-known advantages of a strong Bohr effect for efficient blood O_2 transport were behind the evolution of Hb properties in early ray-finned fishes. Indeed, the evolution of the Bohr effect and the Root effect are strongly correlated in ray-finned fishes, and evolutionary reconstruction suggests that the Bohr effect increased independently in ray-finned fishes and lobe-finned fishes (including tetrapods) from the rather low Bohr effect of their last common ancestor with elasmobranchs (Berenbrink et al., 2005). This is consistent with the emerging view that the Root effect is not just an exaggerated Bohr effect of human HbA (Perutz and Brunori, 1982) but is largely based on an entirely different molecular mechanism (Ito et al., 1995; Mylvaganam et al., 1996; Yokoyama et al., 2004; Bonaventura et al., 2004; Berenbrink et al., 2005; Berenbrink, 2006; Mazzarelli et al., 2006a; Mazzarelli et al., 2006b).

The mechanism for the Bohr–Root effect in teleosts involves fewer histidine residues than that in human HbA (Yokoyama et al., 2004; Lukin and Ho, 2004) and this may have allowed an evolutionary decrease in Hb histidine content and thus Hb buffer value in early ray-finned fishes. Such a reduction conceivably increases the efficiency of a given acid load to elicit the Bohr effect, such that already small increases in lactic acid or CO_2 production rates in a tissue can cause a relatively strong pH decrease and enhanced O_2 release *via* the Bohr effect (Berenbrink et al., 2005; Berenbrink, 2006).

To conclude the above section, the following steps that led to the origin of the complex system of O_2 secretion can be conceived. It started with the evolution of a kind of Bohr effect in early ray-finned fishes by a mechanism that was different from the mechanism in tetrapods and involved a strong decrease in Hb O_2 binding cooperativity at low pH values. This was the Root effect, whose properties at low pH values were initially probably not relevant, because the Hb of early ray-finned fishes had a high Hb buffer capacity, and pH values low enough to cause significant Hb deoxygenation, even at high P_{O_2}, may rarely have been encountered. Subsequently, Hb buffer values gradually decreased, presumably because this increased the efficiency by which an acid load changed Hb O_2 affinity in the tissues and allowed O_2 off-loading. Under these conditions, the apparently relatively facile mutation of a rete mirabile in the blood supply of the retina was genetically fixed, because it allowed back-diffusion of acid that was produced in the metabolically very active retina and thereby made it possible to achieve low enough pH values to depress not only Hb O_2 affinity but also cooperativity. This allowed a higher fraction of O_2 to be released into physical solution and improved retinal O_2 supply. However, in parallel with the advantages of ocular O_2 secretion, the system had become more vulnerable against general acidosis. This was likely the driving force for the evolution of a red blood cell βNHE in advanced, highly visual teleosts that protected Hb O_2 loading in the gills against the Root effect under general acidosis (Berenbrink et al., 2005).

So, the widespread and extraordinary physiological capacity for O_2 secretion, which has been studied in relation to the swimbladder and buoyancy regulation for 200 years, originally

evolved in the eye of fishes and for an entirely different purpose. Evolutionary reconstructions further show that the mechanism for O_2 secretion is closely linked to the evolution of the Bohr effect and reduced Hb buffer values in teleost fishes and to the occurrence of the unique βNHE in their red blood cells. The distribution of these features had previously been largely unexplained and, since teleosts comprise about 24 000 described species (Nelson, 1994), it is true to say that ocular O_2 secretion has shaped the evolution of blood respiratory gas transport characteristics of half of all living vertebrates (Berenbrink et al., 2005).

Repeated evolution of swimbladder O_2 secretion

In contrast to the choroid rete mirabile and ocular O_2 secretion, the subsequent evolution of a rete mirabile and associated O_2 secretion in the swimbladder occurred several times independently (Fig. 3). It is as if the possession of Root effect Hbs and low Hb buffer values pre-disposed species for the evolution of this mechanism. Examples of groups who independently evolved a swimbladder rete mirabile are the elephantfishes (Mormyridae) and the group comprising bonefishes, halosaurs, spiny eels and true eels (Albuliformes + Anguilliformes). Both groups show a conspicuous increase in species number compared with their closest relatives without a rete mirabile (25- to 100-fold higher, respectively), which is consistent with adaptive radiation after the acquisition of the rete mirabile (Berenbrink et al., 2005). In elephantfishes a swimbladder rete mirabile is found in each of two paired vesicles, which develop as buds from the anterior part of the larval swimbladder, become separated, completely closed and situated close to the inner ear during development. These otic bladders are important for increased auditory sensitivity (Stipetic, 1939; Yan and Curtsinger, 2000; Fletcher and Crawford, 2001). Keeping these bladders inflated against diffusional loss may be the selective advantage of gas secretion in this group in addition to buoyancy control. However, until now, the actual O_2 content in the otic or tympanic bladder of elephantfishes has not been determined, and O_2 secretion is only inferred by the possession of a rete mirabile and a Root effect and the general difficulty to keep closed, perfused gas pockets in tissues inflated without a mechanism for gas secretion (Berenbrink et al., 2005; Piiper et al., 1962).

In contrast to their close relatives, which do not have a swimbladder rete mirabile (Fig. 3), members of Albuliformes + Anguilliformes have extensively radiated in the deep sea (Nelson, 1994), suggesting that the ability to control buoyancy by swimbladder O_2 secretion has obviated the need to travel to the surface and replenish swimbladder volume by air intake through the oesophagus and has thereby allowed expansion into this new habitat.

Swimbladder O_2 secretion evolved in at least two more groups, some Ostariophysi (e.g. carp relatives and loaches) and the euteleosts (e.g. salmonids, cod, swordfish, perches; Fig. 3). Compared with their enormous species diversity, relatively few species of the two groups have been investigated and phylogenetic relationships within these groups are currently not well resolved (Miya et al., 2003; Saitoh et al., 2003). In addition, swimbladder retia mirabilia or the entire swimbladder appear to have been frequently lost (e.g. rainbow trout and swamp eel, respectively; Fig. 3). It is therefore difficult to identify exactly when and how often O_2 secretion evolved in Ostariophysi and Euteleosts and what the major selective advantages may have been. For example, in some Ostariophysi, the volume of the swimbladder is greatly reduced and it no longer appears involved in buoyancy control. However, it is never completely lost, presumably because all Ostariophysi have a mechanical connection between their swimbladder and the inner ear, which aids in hearing (Alexander, 1966). Whatever the selective advantage of a swimbladder may be for a given species, it can generally be assumed that the predation risk from aquatic, aerial or wading predators is reduced when air intake at the water surface can be avoided by the ability to keep the swimbladder inflated through secretion of O_2.

In 200 years of research on the mechanism of O_2 secretion, using the Krogh principle and finding a suitable experimental model species has perhaps been the single most significant step in advancing our knowledge of the system. The availability of improved phylogenies in the post-genomic era and the use of modern techniques for evolutionary reconstructions have now led to the realisation that swimbladder O_2 secretion evolved at least four times independently and was influenced by changes in blood gas transport characteristics that were connected to the earlier evolution of ocular O_2 secretion. Together with the frequent secondary loss of the ocular and swimbladder mechanism, this allows the identification of teleost species with every possible combination of presence and absence of the ocular and swimbladder O_2 secretion mechanism (see colour code in Fig. 3). This raises the exciting opportunity to compare related species pairs, which differ in the presence of one of the mechanisms, to assess the relative importance of the different components of the system for ocular and swimbladder O_2 secretion. As an example of such an analysis, Berenbrink et al. have shown that ocular O_2 secretion is more significantly associated with higher Root effects than swimbladder O_2 secretion (Berenbrink et al., 2005).

This review indicates that the increasing availability of molecular phylogenetic trees for evolutionary reconstructions may be as important for understanding physiological diversity in the post-genomic era as the increase of genomic sequence information in model species.

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