Oxygen delivery to the fish eye: Root effect as crucial factor for elevated retinal $P_{O_2}$

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Accepted 6 September 2005

Summary

Although the retina has one of the highest metabolic rates among tissues, certain teleost fishes lack any vascular supply to this organ which, in combination with the overall thickness of the organ, results in extremely long diffusion distances. As the only way to compensate for these obstacles, oxygen partial pressure ($P_{O_2}$) in the eyes of such fish is elevated far above atmospheric values. Although not supported by any direct evidence, the enhancement of $P_{O_2}$ is considered to be related to the Root effect, the release upon acidification of Hb-bound $O_2$ into physical dissolution, possibly supported by countercurrent multiplication similar to the loop of Henle.

The present study evaluates the magnitude of intraocular $P_{O_2}$ enhancement under tightly controlled physiological conditions, to directly confirm the involvement of the Root effect on intraocular $P_{O_2}$ in the retina of rainbow trout Oncorhynchus mykiss. Intraocular $P_{O_2}$ was determined with special polarographic microelectrodes inserted into the eye. $P_{O_2}$ profiles established in vivo by driving electrodes through the entire retina yielded average $P_{O_2}$ values between 10 mmHg (1.3 kPa) at the inner retinal surface and 382 mmHg (50.9 kPa) close to the outer retinal limit (Bruch’s membrane). According to estimates on the basis of the diffusion distances determined from sections of the retina (~436 μm at the site of $P_{O_2}$ measurement) and literature data on specific oxygen consumption, the in vivo determined values would be sufficient to cover the oxygen demand of the retina with some safety margin.

For a clear and direct in-tissue-test as to the involvement of the Root effect, an isolated in vitro eye preparation was established in order to avoid the problem of indirect blood supply to the eye from the dorsal aorta only via the pseudobranch, a hemibranch thought to modulate blood composition before entry of the eye. Any humoral effects (e.g., catecholamines) were eliminated by perfusing isolated eyes successively with standardized red blood cell (RBC) suspensions in Ringer, using trout (with Root) and human (lacking any Root effect) RBC suspension. To optimize perfusate conditions for maximal Root effect, the Root effect of trout RBCs was determined in vitro via graded acidification of individual samples equilibrated with standardized gas mixtures. During perfusion with trout RBC, $P_{O_2}$ at the outer retinal limit was 99 mmHg (13.2 kPa), but fell by a factor of 3.3 upon perfusion with human RBC in spite of higher total oxygen content ($T_{O_2}$ 2.8 for trout vs 3.9 mmol l$^{-1}$ for human RBC). Upon reperfusion with trout RBC, $P_{O_2}$ was restored immediately to the original value. This regularly observed pattern indicated a highly significant difference ($P=0.003$) between perfusion with trout (with Root effect; high retinal $P_{O_2}$) and perfusion with human (no Root effect; low retinal $P_{O_2}$) RBC suspension, thus clearly demonstrating that the Root effect is directly involved and a crucial prerequisite for the enhancement of $P_{O_2}$ in the retina of the teleost eye.

Key words: rainbow trout, Oncorhynchus mykiss, ocular oxygen partial pressure, avascular retina.

Introduction

Oxygen ($O_2$) transfer to the site of consumption within tissues takes place in animals generally by simple diffusion along a diffusion gradient. Thus, oxygen partial pressure ($P_{O_2}$) in the tissues is expected to be lower than environmental $P_{O_2}$. This general rule, however, does not apply to a few known instances in teleost fish species. $P_{O_2}$ is extremely high in the swimbladder of deep swimming fish (Nielsen and Munk, 1964), and $P_{O_2}$ values in the eyeballs of several teleost species have also been reported to exceed atmospheric $P_{O_2}$ values (Wittenberg and Wittenberg, 1961, 1962; Fairbanks et al., 1969). While secretion of $O_2$ into the swimbladder serves the adjustment of buoyancy but is metabolically irrelevant, the extraordinary feature of superatmospheric $P_{O_2}$ values in the eye is thought to compensate for the special conditions of $O_2$ delivery to retinal tissues. Passive diffusion of $O_2$ to meet the demands of thick and completely avascular retinae in some teleosts is possible only by establishing a high $P_{O_2}$ diffusion gradient (Wittenberg and Wittenberg, 1974). This task is aggravated by the retina possessing one of the highest metabolic activities among tissues (Anderson, 1968; Yu and Cringle, 2001).
Oxygen content and supply in teleost eyes

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P02 can be elevated by acidification of blood possessing a Root effect (Root, 1931; Root and Irving, 1943; Brittain, 1987; Pelster and Randall, 1998). With lowering of pH, the amount of haemoglobin-associated O2 in the blood of some teleost fish is reduced and O2 transferred into physical dissolution, which may result in considerable elevation of P02 on the basis of the low aqueous O2 solubility. This primary effect of P02 elevation may suffice to produce the elevated P02 values observed in teleost eyes, but is too small to adequately pressurize the swimbladder in deeper waters. Even if the whole amount of O2 bound to haemoglobin (Hb) in the arterial blood of a teleost fish (about 5 mmol l−1) could be released upon acidification, P02 and thus hydrostatic pressure would rise by only about 2800 mmHg (373 kPa).

Any elevation of P02 in excess of that produced by the single-pass Root effect is thought to be brought about in the swimbladder by a counter-current blood vessel arrangement, the rette mirabile. The primary effect of P02 enhancement through the Root effect and acidification by the gas gland at the tip of the vessel system can be boosted by back-diffusion of blood gases, metabolites and possibly also HCO3− from the venous capillaries into the arterial vessels of the rette mirabile or vice versa (Kobayashi et al., 1989, 1990), greatly enhancing the initial effect by counter-current multiplication (Kuhn et al., 1963), but also minimizing gas and metabolite loss from the location, similar to the conditions in Henle’s loop of the kidney. Purportedly, the choroid rette mirabile underlying teleost retinæ has a similar function in elevating ocular P02.

Lack of alternative mechanisms makes the Root effect the most likely candidate to be responsible for initial elevation of P02. This notion is supported by the in vitro demonstration of reduced O2-carrying capacity of Hb at low pH and high P02 (Root, 1931; Root and Irving, 1943; Scholander and van Dam, 1954; Hamann, 1990; Pelster and Weber, 1990), the pattern of distribution of the Root effect almost exclusively to teleost fish with swimbladder gas secretion and high P02 values in the eye (Farmer et al., 1979; Pelster and Weber, 1991; Pelster and Randall, 1998), the evolutionary co-development of certain morphological and physiological traits deemed essential for gas secretion (Berenbrink et al., 2005), and by the observation of higher venous than arterial P02 values in the eel gas gland (Steen, 1963; Kobayashi et al., 1990). All these data, however, represent indirect evidence; direct confirmation of the postulated chain of mechanisms is still lacking.

The basis for an evaluation of the mechanistic process in the eye of teleosts is even scarcer than for the swimbladder. Apart from a few studies in trout on the dependence of high ocular P02 on carbonic anhydrase activity (Fairbanks et al., 1969, 1974; Hoffert and Fromm, 1973) and the relevance of high P02 for vision (Fonner et al., 1973; Hoffert and Ubels, 1979) little evidence is available. Moreover, reported control values (arterial pH and P02) much below physiological ranges raise questions as to the conditions of experimental animals or employed methods (Fairbanks et al., 1969, 1974).

The lack of relevant direct evidence as to the involved mechanisms may reflect difficulties in accessing the supply vessel of the teleost eye (Waser and Heisler, 2004), but may also be related to the complicating factor of indirect blood supply of the teleost eye from the dorsal aorta (DA) via pseudobranchial artery, pseudobranch and ophthalmic artery (Müller, 1839). The function of the interconnected gill-like pseudobranch is still unknown (Bridges et al., 1998; Kern et al., 2002; for a review, see Laurent and Dunel-Erb, 1984), although ‘a role for vision’ (Müller, 1839) and ‘a role in altering blood chemistry to support oxygen secretion in the eye’ have been postulated (Bridges et al., 1998). Following these suggestions, key parameters in the ophthalmic artery blood carrying the ocular supply may deviate significantly from the DA site.

This study is aimed at analysing the intraretinal conditions of O2 supply, in particular at evaluating intraretinal P02 of rainbow trout under controlled physiological conditions, as well as at a direct test of the contribution of the Root effect for ocular P02 enhancement. For this purpose a number of experimental series were conducted: (1) measurement of retinal diffusion pathways, (2) in vivo determination of intraretinal P02 with particular emphasis on blood gas and acid–base homeostasis, and on eventual effects of intraocular hydrostatic pressure changes, (3) determination of the Root effect in erythrocyte suspensions applied for in vitro perfusion, (4) evaluation of the contribution of the Root effect for high intraretinal P02 by direct (eliminating the pseudobranch) in vitro perfusion of isolated eyes with erythrocyte suspensions possessing (trout) or lacking (human) a Root effect.

The presented data are regarded as a first step towards a closer elucidation of mechanisms involved in the elevation of ocular P02 in some teleost fish species.

Materials and methods

Experimental animals

Specimens of rainbow trout Oncorhynchus mykiss Walbaum 1792 of either sex (mass range 197–774 g; 420±130 g, mean ± s.d.; fork length 27–42 cm; 33.2±3.5 cm, mean ± s.d.; N=52) were obtained from a local hatchery (Umweltbundesamt, Berlin, Germany) and kept in large glass aquaria for at least 4 weeks before experimentation. The aquarium water was maintained between 12 and 17°C, recirculated through biological filters and supplemented with a steady flow of tapwater, dechlorinated in activated charcoal columns. The animals were fed daily on 0.5% (w/w) commercial trout pellets and kept at a fixed daily light regime of 11 h:1 h:11 h:1 h (light:transition:dark:transition). Animals were transferred to the laboratory lightly anaesthetized (25 mg l−1, 1:40000 w/v, neutralized MS222, 3-amino-benzoic acid ethyl ester methanesulphonate; Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) in aerated thermostatted water. All animal experiments were carried out in accordance with local regulations (G0294/96).

Procedures

For surgical preparations, fish were suspended on an
operating rack and the gills were irrigated with recirculated, air-equilibrated tap water thermostatted to 15°C. Deep anaesthesia was induced and maintained by addition of 60–80 mg 1−1 MS222. A tapered polyethylene catheter (PE60, o.d.=1.2 mm, Portex, Hythe, Kent, England), filled with heparinized trout Ringer solution (in mmol l−1: NaCl 150, KCl 4, CaCl2 1.3, MgCl2 1.2, D(+)-glucose 7.5, NaHCO3 5, heparin 125 i.u. ml−1), was inserted into the DA by a modified Seldinger technique, similar to the general approach of Soivio et al. (1975), Holeton et al. (1983) and Waser and Heisler (2004). Following catheter implantation, the trout were transferred to an aquarium and allowed to recover from surgery for at least 16 h before experimentation. Within the aquarium, the fish were slightly confined to submerged opaque cylinders, leaving catheters freely accessible for blood sampling.

Reference blood samples were taken from well-recovered, conscious and resting animals for determination of arterial pH and PO2 (BMS 3 Mk II, Radiometer, Copenhagen, Denmark). For experimentation, animals were then anaesthetized (MS222; 60–80·mg·l−1) and returned to the operating rack. The gills were irrigated with thermostatted, well-aerated anaesthetic-containing water throughout the course of the experiment. DA blood pressure and heart rate (HR) were continuously monitored (P23AA, Statham, Hato Rey, Puerto Rico), arterial pH and PO2 were determined repeatedly. During the experiment arterial pH was maintained essentially constant at 7.9 by changes in carbon dioxide partial pressure (the experiment arterial pH was maintained essentially constant (MS222; 60–80·mg·l−1) and returned to the operating rack. The gills were irrigated with thermostatted, well-aerated anaesthetic-containing water throughout the course of the experiment. DA blood pressure and heart rate (HR) were continuously monitored (P23AA, Statham, Hato Rey, Puerto Rico), arterial pH and PO2 were determined repeatedly. During the experiment arterial pH was maintained essentially constant at 7.9 by changes in carbon dioxide partial pressure (PCO2) of the gill irrigation water between 0.25 and 5 mmHg (0.033–0.67 kPa; Mass Flow Controllers, MKS Instruments Deutschland GmbH, München, Germany).

Retinal morphometry

In order to evaluate retinal diffusion distances, one eye ball from each of three trout were enucleated and sectioned horizontally around the limbus. After removal of lenses, the eye-cups were fixed by exposure to 2.5% glutaraldehyde in Ringer solution for several days and dehydrated for each several days in three successive baths of 80% ethanol. After embedding in paraffin, the eyes were halved by median section. Only a few slices of 6 μm each were cut from the halving interface, ensuring perpendicular cuts of the retina. Microscopic analysis of individual layer thickness as well as total retinal thickness was performed at numerous sites spanning the entire retinal arc. Tissue shrinkage due to paraffin embedding was corrected for by dividing through a factor of 0.74 (Weibel, 1979).

Oxygen microelectrodes: construction, calibration and characteristics

Polarographic O2 microelectrodes for determination of intraocular PO2 were constructed following the general approach of Whalen et al. (1967) and Linsenmeier and Yancey (1987). Single-barrelled borosilicate capillaries (GC100-10, Clark Electrochemical, Pangbourne, Reading, UK) were pulled to fine tip diameters (<5 μm; Model P-97, Sutter Instruments, Novato, CA/USA). A thin bar of low-melting alloy (47.2°C; Whalen et al., 1967) was inserted into the pulled capillary and gently heated until completely molten. The fluid alloy was pushed towards the tip of the electrode under microscopic control, taking care to leave a metal-free recess in the tip of the glass capillary. The recess provides a diffusional resistance, greatly reducing O2 consumption and diminishing stirring-induced spurious current signals (Schneiderman and Goldstick, 1978). The recessed metal electrode was connected by a soldered-on copper wire (fixed in the rear capillary aperture by quick bond resin) to a power supply for electrolytic plating. A thin layer of gold was electro-plated to the metal surface in the capillary recess after filling the tip completely with the plating solution (200 mmol l−1 ammonium citrate with 5% K[Au(CN)2], pH 6.3) by application of 1.5 V for about 10–30 min between electrode lead and a secondary platinum electrode in the plating solution. After plating, electrodes were soaked in deionized water for at least 24 h and stored dry; before use each electrode was checked microscopically and some arbitrarily selected specimens from each production batch were tested electronically. The resulting PO2 microelectrodes were characterized physically as having tip diameters of less than 5 μm and an average recess of 77±20 μm (mean ± s.d., N=42).

After establishing an individual polarogram (current vs voltage), the microelectrodes were polarized in the plateau range of the relationship (usually at about –800 mV). The very low current signals (in the fA to pA range) were converted into voltage signals using special head stages, incorporating customised electronic circuitry on the basis of low bias-current operational amplifiers (OPA128JM, Burr Brown, Darmstadt, Germany). Teflon-coated silver wires (Gi 1106, 0.37/0.45 mm; Advent ResearchMaterials, Eysham Oxon, England, UK), chlorinated at the exposed tip, served as reference electrodes. The electrode chains were calibrated at the experimental temperature (15°C) in isotonic saline solution (0.9% NaCl) or trout Ringer solution, equilibrated with gases of known PO2 values ranging from 0 to 760 mmHg (101 kPa; gases provided by precision gas mixing pumps; Type 1 M 303/a-F, Wösthoff GmbH, Bochum, Germany).

Calibration of zero intersect and sensitivity was performed for each individual electrode immediately before experimentation. Sensitivity of the gold-plated sensor averaged 173±82 fA mmHg−1 (mean ± s.d., N=78). The linearity in the range of PO2 from 0 to 760 mmHg (0–101 kPa) was 0.99987 (s.d.=0.00023, N=15) in terms of the average correlation coefficient. Following repeated exposure to tissues, the electrodes showed a slight decrease in sensitivity, probably due to masking part of the catalytic metal surface by contamination with proteins or nucleotides. Linearity and zero intersect current, however, remained essentially unaffected during any one experiment. There was no analytical quality degradation during long-term operation; the lifetime of PO2 microelectrodes was limited only by physical destruction of the tip during experimentation.

The PO2 sensor was insensitive to metabolic and respiratory changes in pH from 5.8 to 8.8. As expected, the current signal
was sensitive to temperature changes, rising by about 1% per °C (range 10–35°C), although not quite as much as reported for other polarographic O₂ sensors (Gnaiger and Forstner, 1983). Since ionic strength is a modulator of electrode sensitivity (determined as –0.05 mmHg per 1 mmol l⁻¹ of ionic strength, range 75–1200 mmol·l⁻¹) the electrodes were exclusively calibrated in solutions resembling extracellular fluid of trout. Calibrations and checks were generally conducted at the experimental temperature of 15°C.

**Intraocular hydrostatic pressure**

Introduction of electrodes into the eye for the purpose of \( P_{O_2} \) measurement may well disrupt the intraocular pressure (IOP) regime, effecting local changes in perfusion and thus \( P_{O_2} \). This possibility was checked out by direct measurement of IOP during determination of intraocular \( P_{O_2} \). After induction of anaesthesia as described above, the anterior chamber of the eye was punctured with a 0.4 mm hypodermic needle. IOP was recorded by means of a pressure transducer (P230b, Statham, Hato Rey, Puerto Rico) connected to the hypodermic via PE-tubing (Portex, Hythe, Kent, England, UK), taking care to completely fill the pressure pathway with physiological fluid. After reading the IOP for 15 min, further preparations required for determination of intraocular \( P_{O_2} \) (see below) were conducted in order to directly correlate eventual changes in ocular \( P_{O_2} \) with impacts on IOP and vice versa.

**Intraretinal \( P_{O_2} \): in vivo determination**

After induction of anaesthesia as described above, cornea and iris were punctured ventro-laterally just inside the limbus, using a 1.5 mm diameter hypodermic needle. The needle was replaced by a guide for the \( P_{O_2} \) electrode made of 1.5 mm diameter stainless steel tube, which remained in place throughout the experiment. \( P_{O_2} \) microelectrodes were threaded through the guiding tube and advanced with the tip close to the retina, with visual check of the position through a binocular operating microscope (Carl Zeiss, Jena, Germany) in combination with an ophthalmoscopic lens (Super Pupil XL, 132 dpt, Biomicroscopy lens JH0987, Volk Optical Inc., Mentor, Ohio, USA). The electrodes were advanced towards and into the retina in reproducibly positioned (0.1 μm), using a step motor-driven 3-axis micromanipulator (HS 6, Märzhäuser, Wetzlar, Germany) in combination with a digital programmable electronic driving unit (N. Heisler and H. Slama, unpublished). A chlorinated silver wire (see above) inserted into the dorsal muscle behind the head served as a reference for \( P_{O_2} \) microelectrodes.

Profiles of \( P_{O_2} \) were recorded in the range of the posterior pole of the eyeball, slightly anterior to the optic disc. After inserting the electrode into the eye to just above the retina (at 800 μm s⁻¹), the electrode was gradually inserted into the retina in preprogrammed steps of 25 to 100 μm (at 3200 μm s⁻¹; the step magnitude depended on the extent of the preceding change in \( P_{O_2} \)), each time awaiting stable readings of electrode current until \( P_{O_2} \) readings levelled off during further advancement. It was assumed that the tip had then reached or passed Bruch’s membrane. The electrode was then gradually withdrawn, applying the reverse of the advancement profile, and the return \( P_{O_2} \) profile was recorded.

**Intraretinal \( P_{O_2} \): in vitro experiments**

**Enucleation of eyes**

After establishment of normal pH and \( P_{O_2} \) in appropriately anaesthetized specimens (see above), the conjunctiva were cut and removed from the eye. Covering bones and muscle mass from ventral and temporal sectors of the orbita as well as the eye muscles were severed and completely removed. After carefully exposing optic nerve as well as ophthalmic artery and vein by removal of the suspending adipose tissue, two ligatures for later use were threaded under the ophthalmic artery, proximal at the entry into the orbita and distal directly at the eye cup.

After inserting a custom-made suspending holder under the eye cup, a preformed catheter attached to the holder was quickly inserted into the ophthalmic artery to supply the eye and the ligatures were tightened around catheter/artery as well as around the cut-off artery at the orbital entry point. Perfusion of the eye with trout erythrocytes (red blood cells, RBC) suspension started immediately after, limiting ischaemia of the eye to less than 60 s. After sectioning optic nerve and ophthalmic vein the eye was removed from the orbita. During perfusion, the eye surface was kept hydrated by irrigation with water thermostatted to 15°C.

**Perfusion**

Isolated eyes were perfused with erythrocyte suspensions (see below) rather than full blood in order to avoid any direct or indirect effects of chatecholamines and other humoral factors on the release of \( O_2 \) from the carrier (Hb). Suspensions were supplied to the ophthalmic artery by a peristaltic pump (Type IP-4, Ismatec, Wertheim-Mondfeld, Germany) at the flow rate of 180 μl min⁻¹, previously determined in vivo in the afferent pseudobranchial artery (Waser and Heisler, 2004). A miniature bubble trap in the inflow path immediately before the eye served for elimination of gas bubbles from the perfusate. Vascular occlusions in eye capillaries by micro-clots and cell aggregations were prevented by passing the perfusate through a 40 μm mesh filter (Polyester 07-40/25, Bückmann, Millipore, Eschborn, Germany). Perfusion pressure was monitored by a transducer T-connected to the catheter leading into the ophthalmic artery (P23AA, Statham, Hato Rey, Puerto Rico).

**Preparation of erythrocyte suspensions**

Trout blood pooled from several individuals and human blood (human transfusion blood supplied by Charité, Berlin, Germany) was centrifuged, plasma and white blood cells removed and the RBCs were three times washed in trout Ringer solution (in mmol l⁻¹: NaCl 146.6, KCl 4, CaCl₂ 1.3, MgCl₂ 1.2, d(+)-glucose 7.5, NaHCO₃ 5.4, sodium pyruvate 3,
polyvinylpyrrolidone 0.5% (w/v), heparin 50 i.u. ml⁻¹), before being resuspended and stored overnight at 4°C. The washing procedure (3×) was repeated next morning before resuspension to the nominal haematocrit (Hct) used during experimentation (0.20). The resulting suspensions were conditioned for the experiment by at least 45 min equilibration at 15°C in rotating 100 ml round bottom glass flasks (Farhi, 1965) with the experimental gas of 0.27% CO₂ in air [PCO₂: 2.0 mmHg (0.27 kPa), PO₂: 156.2 mmHg (20.8 kPa)] prepared by Wösthoff gas mixing pumps. Glucose transfer into the cells was facilitated by addition of 10 u l⁻¹ insulin (Insuman Rapid, Hoechst Marion Roussel, Bad Soden, Germany; Pelster et al., 1989).

Perfusates
For an evaluation of the role of the Root effect for complete O₂ supply of the trout retina, eyes were perfused with two different species of red blood cells: trout cells, having a pronounced Root effect and thus capable of massive O₂ release into physical solubility upon acidification, and human erythrocytes lacking any effect of O₂ release upon acidification at high PO₂. The following perfusates were utilized. ‘Tr’, trout RBCs in trout Ringer, pH about 7.48 (start of the steep range of the Root effect curve), Hb saturation high (~91%, Root effect less than 15%; ‘H’, human RBCs in trout Ringer, pH about 7.16, Hb saturation high (100%), no Root effect.

As a reference for full Hb oxygenation (100% saturation, 0% Root effect), a control suspension of trout RBCs in trout Ringer (‘TrC’, pH>8) was prepared and concomitantly equilibrated.

Determination of intraocular PO₂
Initial preparations for determination of intraocular PO₂ were identical to those described above (in vivo conditions). For the isolated eyes, however, the metal suspending eye holder served as a reference for the PO₂ microelectrode. Reference readings of PO₂ were acquired always during perfusion with trout RBC suspensions (Tr: pH about 7.48; see above). PO₂ microelectrodes were advanced into the retina until PO₂ attained a maximal level. The electrode was then left in position for the remainder of the experiment, during which the response in PO₂ was recorded during alternating perfusion with trout and human RBC suspensions. In a few occasions, eyes were also flushed with trout Ringer solution.

Determination of the Root effect in vitro
The relationship between changes in extracellular pH and the release of Hb-associated O₂ into physical dissolution was determined in erythrocyte suspensions identical to those used for perfusion experiments. Individual samples from the same preparation of erythrocyte suspension were adjusted to pH over the range 6.0–8.5, either by changes in PCO₂ of the equilibrating gas, in plasma [HCO₃⁻], or by addition of each 200 μl of HCl of the required concentration. Gases with specified PCO₂ (0.033% to 7.13%) were produced by mixing air with CO₂ by Wösthoff gas mixing pumps.
Results

Morphometry of the trout retina

Trout retina reflects the typical vertebrate pattern of layer arrangement (Fig. 1), but lacks any vascular supply. The thickness of individual layers are presented in Fig. 1. The overall thickness of the retina depends on the location: thickness increases from the anterior margin (~200 μm) to the posterior regions in the range of the optic nerve (~500 μm, Fig. 2), with an average of 407 μm (Fig. 1). The local variability is somewhat related to the density of photoreceptors, reflected in the thickness of the neuronal layers PRL and GCL+NFL (Fig. 2B,C), with the NFL appearing particularly extended close to the optic nerve. In contrast, the nuclear and plexiform layers were found more uniform throughout the retina. The whole retina completely lacks blood vessels, and the inner surface is not supplemented by hyaloid vessels often found in species without intraretinal vascularization. This pattern is in vast contrast to the European eel Anguilla anguilla, for example, with its clear intraretinal vascularization (Fig. 1, insert).

Oxygen profiles were measured in the posterior part of the trout retina. In order not to affect correct estimate of the average representative for this site by values of largely different thickness from other areas of the retina, the thinner anterior parts (25% of all data) and six exceptionally high values obtained close to the optic nerve with an extremely thick NFL layer were not included into the data pool for averaging. The posterior retina thickness was accordingly estimated to be 436±75 μm (N=95 measurement points, in 3 eyes of three fishes).

Experiments in vivo

Variables of homeostasis

In conscious, resting trout, arterial pH and $P_{O_2}$ averaged 7.89±0.11 (N=41) and 102±19 mmHg (13.6±2.5 kPa; N=39), respectively. During anaesthesia and artificial gill irrigation, arterial $P_{O_2}$ was hardly affected (99±21 mmHg, 13.2±2.8 kPa; N=38, respectively). Arterial plasma pH (8.02±0.12, N=41) was slightly but significantly higher ($P<0.001$) than in non-anaesthetized controls. DA blood pressure and HR of anaesthetized trout averaged 28±6.8 mmHg (3.7±0.9 kPa; N=52) and 73±9.6 beats min$^{-1}$ (N=51), respectively.

Intraocular hydrostatic pressure (IOP)

IOP measured in the anterior eye chamber was 4.9±0.56 mmHg (0.65±0.07 kPa; N=8). After puncturing the eye, inserting the guiding tube and $P_{O_2}$ electrode, IOP was not significantly different (4.6±1.06 mmHg, 0.61±0.14 kPa; N=8; P=0.58, paired Student’s t-test). Evidently, insertion of the guiding tube with electrode into the eye effectively sealed the corneal puncture, thus supporting maintenance of constant IOP. The magnitude of IOP obtained by this study is identical to the value reported by Hoffert (1966), whereas other species...
Root effect causes high \( P_O_2 \) in fish eyes

Intraretinal \( P_O_2 \)

Intraretinal \( P_O_2 \) was minimal at the interface between vitreous humor and retina (ILM, see Fig. 1), averaging 10±21 mmHg (1.33±2.8 kPa; \( N=23 \); Fig. 3). Traversing the retina, \( P_O_2 \) rose to 382±143 mmHg (50.9±19.1 kPa; \( N=23 \)) in the region of Bruch’s membrane (Fig. 3). \( P_O_2 \) was significantly different among ILM, arterial blood, and at Bruch’s membrane (\( P<0.05 \); Kruskall–Wallis one-way ANOVA, pairwise multiple comparison procedure: Dunn’s method). The electrode path driven between minimal and maximal \( P_O_2 \) averaged 433±106 \( \mu m \) (\( N=23 \); Fig. 3).

In vitro experiments

Root effect in trout erythrocyte suspensions

The maximum \( T_O_2 \) (100% Hb saturation, 0% ‘Root on’) of alkaline (pH 8.0–8.5) trout RBC suspension (Hct=0.20) was 3.31 mmol l\(^{-1}\) (Fig. 4). Minimum \( T_O_2 \) (100% ‘Root on’) at acidic pH (6.0–6.8) was 1.37 mmol l\(^{-1}\), 41% of the maximum \( T_O_2 \). A sigmoidal curve fit on the raw data, characterized by a curve inflection at pH 7.36 and \( T_O_2 \) of 2.34 mmol l\(^{-1}\) (at 50% ‘Root on’), resulted in a correlation coefficient \( r=0.962 \). According to the curve fit, Root effect was 10% activated at pH 7.67 and 90% activated at pH 7.03.

\( P_O_2 \) upon anaerobic acidification of RBC suspensions

Under anaerobic conditions, graded acidification of trout RBC suspensions resulted in an enhancement of \( P_O_2 \) from 156 mmHg (equilibration \( P_O_2 \)) at pH 8.1 to a maximum of 449 mmHg (59.9 kPa) at pH 6.4. Similar to the \( O_2 \) content series, the data could be well fitted by a sigmoidal curve (\( r=0.949 \); Fig. 4B). Identical treatment of human RBC suspension did not result in any significant change in \( P_O_2 \) (blue symbols, Fig. 4B). The correlation coefficient of the linear regression fitted to the measured values was \( r=0.055 \) (Fig. 4B).

Parameters of perfusates

Physiological parameters determined during perfusion in the RBC suspensions are listed in Table 1. The pH of human RBC suspensions was significantly lower than the pH of trout RBC suspension (\( P=0.0026 \)), although both suspensions had been prepared and treated identically. Also, human RBC suspensions exhibited a significantly higher \( T_O_2 \) than trout RBC suspension (\( P=0.0029 \), cf. Fig. 4A), due to the higher cell Hb concentration of human erythrocytes. \( T_O_2 \) of trout RBC suspension ‘Tr’ (pH=7.48±0.04, \( N=3 \)) was 0.91±0.079 (\( N=3 \)) as compared to the concomitantly equilibrated control trout RBC suspensions (‘TrC’) at a more alkaline pH (>8.0).

Intraretinal \( P_O_2 \) upon perfusion with trout RBC vs human RBC

Hydrostatic perfusion pressure at the entry to the ophthalmic artery catheter was the same (\( P=0.9031 \)) for trout and human RBC suspensions (Hct 0.20), but was much lower for pure Ringer solution (cf. Table 1). A large part of the hydrostatic pressure measured at that site was related to the pressure drop according to the flow resistance of the catheter itself (trout
RBC, 60 mmHg/8.0 kPa; human RBC, 67 mmHg/8.9 kPa; Ringer, 42 mmHg/5.6 kPa). Thus, net tissue perfusion pressure (equivalent to the vascular resistance) was only 43 mmHg (5.7 kPa) for trout RBC, 37 mmHg (4.9 kPa) for human RBC, and 16 mmHg (2.1 kPa) for Ringer, respectively. When in vitro perfusion was switched from trout RBC suspension (Root effect) to human RBC suspension (lacking a Root effect), intraretinal $P_{O_2}$ was largely and significantly reduced by a factor of 3.3 (Fig. 5; $P=0.003$) and fell even further during perfusion with Ringer solution (Table 1).

**Discussion**

Experimentation in order to elucidate the mechanisms involved in elevating intraocular $P_{O_2}$ above atmospheric values is most complicated due to limited physical access to the site of mechanisms. This is related to the anatomical conditions with blood supply of the eye provided from the DA only through the rudimentary hemibranch designated as pseudobranch, but is further complicated by a vascular connection (the ‘commissura’) between the two collateral flow...
Root effect causes high \( P_O2 \) in fish eyes

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paths (Waser and Heisler, 2004). Literature studies on elevated \( P_O2 \) in the eye of teleosts have always dealt with the combined serial arrangement of pseudobranch and eye, rendering differentiation of contributing mechanisms in those two organs difficult if not impossible. Severe disturbances of homoiostasis with respect to pH, \([HCO_3^-]\) or \( P_O2 \) in the arterial supply, as often prevailing in literature studies (Fairbanks et al., 1969, 1974) may have further compromised elucidation of the complex pattern.

In order to avoid any influence of non-physiological conditions, large efforts were taken in the course of the present study to maintain normal blood homoiostasis. The average values measured (\( P_O2 \) 99 mmHg, 13.2 kPa; DA blood pressure 28 mmHg, 3.73 kPa; HR 74 beats min\(^{-1}\)) are well within the reported range of normal values of conscious, free swimming trout (Tetens and Christensen, 1987; Playle et al., 1990; Wood et al., 1996; Bernier and Perry, 1999; Perry et al., 1999), despite the necessity for long-term anaesthesia imposed by the experimental approach and the related loss of respiratory activity. The gill surface had to be irrigated for respiratory gas exchange, which evidently did allow sufficient transfer of \( O_2 \), but at the same time resulted in some hypocapnia that could only incompletely be compensated by addition of \( CO_2 \) to the inspired water. This is probably related to heterogeneous distribution of blood and water flow at the gas exchange surface, which can hardly be avoided with present techniques. However, the induced marginal alkaline shift in pH is considered much less disturbing to animal homoiostasis than excessive acid shifts in pH resulting from anaerobiosis induced by extremely low arterial \( P_O2 \) values, as often found in earlier studies on high intraocular \( P_O2 \) values (e.g. Fairbanks et al., 1969, 1974; Hoffert and Ubels, 1979; see also below).

**Intraretinal \( P_O2 \) in vivo**

Intraretinal \( P_O2 \) (\( P_{retO2} \)) measured in anaesthetized trout with well preserved homoiostatic conditions were almost four times as high as simultaneously recorded arterial \( P_O2 \) (\( P_{aO2} \); \( P_{retO2} \), 382 mmHg, 50.9 kPa, average of highest recordings/path profile, vs \( P_{aO2} \), 99 mmHg, 13.2 kPa). This partial pressure is sufficient to completely satisfy the demand of the thick and avascular retina by passive diffusion, as demonstrated by comparative considerations on the \( O_2 \) supply in the well-vascularized human retina with maximal diffusion distances of 142 mm (Chase, 1982). Since \( P_O2 \) at Bruch’s membrane is four times higher than human blood \( P_O2 \) (380 vs 90 mmHg, 50.6 vs 12 kPa) and the lower temperature \( T \) in trout can be expected to reduce metabolic rate by another factor of at least 4 (\( Q_{10} \) approx. 20°C, \( (Q_{10})^2 \approx 4–16 \)), the diffusion path deduced from the conditions in humans (142 \( \mu \text{m} \times \sqrt{16} \)) provides a safe margin of 568 \( \mu \text{m} \) depth of \( O_2 \) entry.

**Table 1. Parameters of the media used for perfusion of isolated eyes**

<table>
<thead>
<tr>
<th></th>
<th>Trout RBC suspension</th>
<th>Human RBC suspension</th>
<th>Ringer solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematocrit</td>
<td>0.20±0.01</td>
<td>0.20±0.01</td>
<td>–</td>
</tr>
<tr>
<td>( P_{CO2eq} ) (mmHg)</td>
<td>2.0</td>
<td>2.0</td>
<td>ND</td>
</tr>
<tr>
<td>pH</td>
<td>7.48±0.04</td>
<td>7.16±0.02</td>
<td>approx. 7.5</td>
</tr>
<tr>
<td>([HCO_3^-]) (mmol l(^{-1}))</td>
<td>2.6±0.16</td>
<td>1.1±0.10</td>
<td>5.4</td>
</tr>
<tr>
<td>([O_2]_{Hb}) (mmol l(^{-1}))</td>
<td>2.8±0.16</td>
<td>3.9±0.23</td>
<td>–</td>
</tr>
<tr>
<td>( P_{perf} ) (mmHg)</td>
<td>103±10.9</td>
<td>104±7.5</td>
<td>58±2.0</td>
</tr>
<tr>
<td>Net tissue ( P_{perf} ) (mmHg)</td>
<td>43</td>
<td>37</td>
<td>16</td>
</tr>
<tr>
<td>Intraretinal ( P_O2 ) (mmHg)</td>
<td>99±1.6</td>
<td>30±3.5</td>
<td>20±10.7</td>
</tr>
</tbody>
</table>

Values are means ± s.d. (N=3). ND, not determined. \( P_{CO2eq} \), equilibration \( CO_2 \) partial pressure; \( P_{perf} \), perfusion pressure.

**Fig. 5. Time course of selected in vitro determination of intraocular \( P_O2 \).** Periods of perfusion with different RBC suspensions (Tr, trout; H, human; R, Ringer solution) are indicated by grey bars above the graph. Initial increase in \( P_O2 \) was due to the advance of the microelectrode into the retina.
The magnitude of the present intraretinal $P_O_2$ data is in good agreement with relevant literature data (Fairbanks et al., 1969, 1974; Hoffert and Ubels, 1979; Pratt and Hoffert, 1982; Desrochers et al., 1985), although in previous experiments little attention was paid to maintaining the general physiological conditions of the animals. Evidently, the regulatory process for $P_O_2$ in the eye is capable of compensating for even largely non-physiological border conditions of the homoiostatic system as expressed by extremely low arterial $P_O_2$ and pH values ($P_O_2$: 20 mmHg, Fairbanks et al., 1969; 13 mmHg, Hoffert and Ubels, 1979; pH: 7.22–7.62, Hoffert and Ubels, 1979).

Undoubtedly, a low pH of the blood before entry into the eye will lower the amount of $O_2$ available by activation of the Root effect, providing only 25% at pH 7.2 and about 84% at 7.6 of the full Root capacity at pH 8 (cf. Fig. 4). However, even more than by the cited low pH values the amount of $O_2$ available for release through activation of the Root effect will be reduced by extremely low arterial $P_O_2$ values (20 and 13 mmHg, 2.7 and 1.7 kPa, respectively; Fairbanks et al., 1969; Hoffert and Ubels, 1979), allowing for only minor oxygenation (20% and 12%, respectively) of trout Hb (Randall, 1970). As a matter of speculation, the interposed pseudobranch, already attributed ‘a role for vision’ by Müller (1839), may be capable of ‘altering the blood chemistry’ in order to adjust the threshold for the onset of the Root effect (Bridges et al., 1998), a notion recently supported by experiments on isolated pseudobranchial cells showing an acidifying effect (Kern et al., 2002). But even if the pseudobranch was actually capable of alkalinizing the blood during passage before entry into the eye, for the lack of additional $O_2$ on the flow path to the eye the correction of the $O_2$ binding characteristics by the pseudobranch would not result in an enhanced amount of Hb-bound $O_2$ to be released by activation of the Root effect in the retina.

On the basis of present knowledge, the above adverse homoiostatic conditions could only be offset if the choroid rete mirabile actually supported elevation of $P_O_2$ by counter-current multiplication. This mechanism is suitable for largely reducing the amount of $O_2$/unit of time required to maintain high $P_O_2$ values in the retina and thus render blood border conditions less important. Although the contribution of any of the mentioned mechanisms to the regulation of retinal $P_O_2$ cannot be quantified to date, it becomes quite clear in face of the above data how robust and insensitive to fluctuations of arterial parameters this regulatory chain must be.

**Root effect in vitro**

Although generally determined as a function of the extracellular (plasma) pH, the Root effect is clearly a function of pH directly at the Hb substrate. Intracellular pH of trout red blood cells, however, is not a direct function of extracellular pH, but is affected by catecholamines (cf. Nikinmaa and Salama, 1998) and possibly other humoral factors. In order to avoid such effects on pH, and also complications with vasopressive substances carried by plasma, the present study has utilized red blood cell suspensions rather than full blood for the *in vitro* perfusion experiments. This decision has made necessary the determination of the Root effect for the special RBC preparations used.

The Root effect of RBC suspensions (Hct 0.20) equilibrated with high $P_O_2$ at various pH is characterized by the expected sigmoidal relationship between $T_O_2$ and pH, with the maximal release by full activation of the Root effect of about 60% of the total $O_2$ capacity (Fig. 4A). Total release of the difference between maximal (3.31) and minimal (1.37 mmol l$^{-1}$) $O_2$ by full Root activation will accordingly transfer 1.94 mmol $O_2$ l$^{-1}$ blood into physical dissolution, equivalent to an additional $P_O_2$ of 1093 mmHg (145.7 kPa) (on the basis of $\alpha_O_2$=1.7745 μmol l$^{-1}$ mmHg$^{-1}$ for human plasma at 15°C; Boutilier et al., 1984). Complete activation of the Root effect will accordingly result in supersaturation of the blood, which may persist *in vivo* for a limited vascular range with laminar flow and lack of condensation points, also because the formation of gas bubbles is counteracted by the extremely high bubble pressures at small radii (LaPlace’s Law).

During determination of $P_O_2$ upon closed system acidification *in vitro* (cf. Fig. 4B), supersaturation occurring after activation of the Root effect is not maintained at atmospheric pressure, due to the vigorous shaking applied and thus turbulent flow and cavitation within the samples. The higher than atmospheric sum of partial pressures then leads to the establishment of a gas phase and a new distribution of gases between aqueous and gaseous compartments. With such an *in vitro* system, full activation of the Root effect will theoretically produce a $P_O_2$ of 442 mmHg (58.9 kPa), which is in very good agreement with the measured $P_O_2$ of acidified trout RBC suspension (449 mmHg, 59.9 kPa).

Full activation of the Root effect actually may not be required for $O_2$ supply to the retina *in vivo*. The measured retinal $P_O_2$ values average only about one third (382 mmHg, 50.9 kPa) of the theoretical $P_O_2$ upon maximal Root activation, and with the additional enhancement of retinal $P_O_2$ by counter-current multiplication the required fraction of Root activation will be even smaller. Nevertheless, teleost fish may live close to the limit with respect to gas embolism. To date, nothing is known as to the correlation between water pressure on fish and retinal $P_O_2$.

In *vitro* eye perfusion: the Root effect as a crucial factor for elevated retinal $P_O_2$

*In vitro* perfusion of the trout eye was chosen as an experimental model in order to completely eliminate external factors such as the pseudobranch activity from the process of $P_O_2$ enhancement in the retina. This approach allowed for extensive and immediate change of perfusate quality and direct identification of factors responsible for observed effects. Aside from these important experimental advantages the preparation certainly carries a number of problems generally involved in extracorporeal perfusion studies.

Perfusion of the organ at an adequate rate is one of the most important prerequisites for maintenance of tissue function. In
the course of the present study the eyes were perfused at the rate determined in a unilateral pseudobranchial artery (Waser and Heisler, 2004). Blood supply in vivo is from the DA through the pseudobranch to the ophthalmic artery, with no significant arterial vessels arising from this path to other tissues, except the bilateral connection of the ‘commissura’ (cf. Waser and Heisler, 2004). During steady state conditions, significant flow through the ‘commissura’ cannot be expected because of same pressure conditions on both sides of the visual blood supply system, but part of the pseudobranchial inflow may have been diverted from the eye path into the secondary circulatory system of the pseudobranch, being directly returned to the sinus venosus. However, on the basis of typical flow rates for the secondary circulatory system (cf. Ishimatsu et al., 1988, Iwama et al., 1993, Heisler, 1993), any possible misestimate for ocular blood flow has to be considered small.

The perfusion pressure as a second prominent characteristic of tissue blood supply was maintained essentially constant during the experiment, in particular between perfusion with trout RBC and human RBC suspensions, and was accordingly not correlated with establishment of different retinal \( P_{O_2} \) values. Perfusion pressure was also rather constant as a function of time, indicating good stability of the preparation. The absolute values of perfusion pressure were higher as compared to normal arterial blood pressure in trout [net tissue perfusion pressure of 43 mmHg (5.7 kPa) for trout RBC and 37 mmHg (4.9 kPa) for human RBC suspensions vs 28 mmHg (3.7 kPa) average blood pressure in the DA; see above]. The normal hydrostatic pressure in vivo in the ophthalmic artery has to be expected to be even smaller than in the DA, due to the flow resistance of the pseudobranch connected in series in the blood supply path.

The perfusion pressure elevated in comparison with in vivo blood pressure may be related to release of vasopressory activity due to haemolysis in the tonometer, the perfusion pump and other constituents of the perfusion system, or to the lack of vasodilators in the normal in vivo blood supply, but may also reflect microembolism of the vascular bed. Filtering of the perfusate through 40 \( \mu \)m mesh width may not have been sufficient to prevent occlusion of capillary vessels with smaller emboli. In particular, small clots may have been produced during the short time (1 min) of ischaemia before perfusion of the organ was initiated through the catheter inserted into the ophthalmic artery. Another possibility may be related to immunological differences. Although no direct incompatibility has been observed, and naturally the plasma factors were eliminated during preparation of perfusates, membrane proteins may interact with the endothelium of capillary vessels. However, the tendency to lower perfusion pressure with human erythrocytes renders this factor unlikely.

Regardless of the mechanism, partial occlusion of the vascular bed may have led to the relatively low intraretinal \( P_{O_2} \) values registered during in vitro perfusion (99 mmHg, 13.2 kPa vs 382 mmHg, 50.9 kPa in vivo). \( P_{O_2} \) enhancement may have been hampered by a reduced overall area of \( P_{O_2} \) enhancement just outside the retina or by a generally reduced \( O_2 \) supply as compared to normal conditions in vivo. Also an overall reduction of retinal \( O_2 \) consumption (due to the lack of central neural connection or by damage of neural retinal cells during ischaemia) and thus less demand for high \( P_{O_2} \) at the entry of the diffusion path cannot be excluded at present.

Independent of the reduced level of absolute \( P_{O_2} \) values, the immediate and direct response to perfusion with trout vs human RBC suspensions clearly indicates the crucial role of the Root effect for retinal \( O_2 \) supply. High \( P_{O_2} \) (about 100 mmHg, 13.3 kPa) during perfusion with trout RBCs was promptly reduced by a factor of 3.3 upon perfusion with human RBCs (to about 30 mmHg, 4 kPa) and was as promptly returned to the high initial value, when perfusion was switched back to trout RBCs with Root effect (cf. Fig. 5). This response was achieved only on the basis of \( O_2 \) release by the Root effect, regardless of the absolute amount of \( O_2 \) bound to Hb in the suspension (2.8 mmol l\(^{-1}\) in trout RBCs vs 3.9 mmol l\(^{-1}\) in human RBCs). \( O_2 \)-loaded but Root-effect-lacking human RBCs actually present little advantage over pure Ringer solution with respect to the produced intraocular \( P_{O_2} \) (cf. Fig. 5). These data accordingly represent the first direct demonstration of the involvement of the Root effect for the enhancement of \( P_{O_2} \) in the teleost eye.

Conclusions

1. Estimates of the depth of \( O_2 \) entry on the basis of measured retinal diffusion pathways indicate that elevated ocular \( P_{O_2} \) values are indispensable for the transfer of \( O_2 \) by simple diffusion across the thick avascular retina.

2. The in vitro Root capacity of trout RBC suspensions is capable of providing enough \( O_2 \) for the establishment of the retinal tissue \( P_{O_2} \) gradients and does not necessarily require counter-current enhancement.

3. Direct in-tissue-tests utilizing Root-effect-containing trout erythrocytes contrasted with Root-effect-lacking human erythrocytes clearly demonstrated that the Root effect is directly involved and an indispensable prerequisite for any enhancement of \( P_{O_2} \) in the retina of the teleost eye.

List of symbols and abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA</td>
<td>dorsal aorta</td>
</tr>
<tr>
<td>H</td>
<td>human RBC suspension used for perfusion</td>
</tr>
<tr>
<td>Hb</td>
<td>haemoglobin</td>
</tr>
<tr>
<td>[Hb(_d)]</td>
<td>Hb tetramer concentration</td>
</tr>
<tr>
<td>Hct</td>
<td>haematocrit</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>IOP</td>
<td>intraocular pressure</td>
</tr>
<tr>
<td>[O(<em>2)](</em>{Hb})</td>
<td>Hb-bound ( O_2 )</td>
</tr>
<tr>
<td>pHa</td>
<td>arterial pH</td>
</tr>
<tr>
<td>pH(_e)</td>
<td>extracelluar (plasma) pH</td>
</tr>
<tr>
<td>pH(_i)</td>
<td>intracellular pH</td>
</tr>
<tr>
<td>( P_{O_2} )</td>
<td>oxygen partial pressure</td>
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<tr>
<td>( P_{retO_2} )</td>
<td>retinal ( P_{O_2} )</td>
</tr>
<tr>
<td>( P_{aO_2} )</td>
<td>arterial ( P_{O_2} )</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
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</table>
We would like to thank Dr Heikki Tuuralla of University of Helsinki for preparation of trout retina sections, and Brigitte Geue for her invaluable help during the experiments.

References
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