8. D. R. Rokyta *et al.*, *PLoS Genet.* **7**, e1002075 (2011).
9. M. L. Salverda *et al.*, *PLoS Genet.* **7**, e1001321 (2011).
10. D. M. Weinreich, N. F. Delaney, M. A. Depristo, D. L. Hartl, *Science* **312**, 111 (2006).
11. J. T. Bridgham, E. A. Ortlund, J. W. Thornton, *Nature* **461**, 515 (2009).
12. B. Lehner, *Trends Genet.* **27**, 323 (2011).
13. M. Lunzer, G. B. Golding, A. M. Dean, *PLoS Genet.* **6**, e1001162 (2010).
14. E. A. Ortlund, J. T. Bridgham, M. R. Redinbo, J. W. Thornton, *Science* **317**, 1544 (2007).
15. P. C. Phillips, *Nat. Rev. Genet.* **9**, 855 (2008).
16. J. F. Storz, *J. Mammal.* **88**, 24 (2007).
17. J. F. Storz, A. M. Runck, H. Moriyama, R. E. Weber, A. Fago, *J. Exp. Biol.* **213**, 2565 (2010).
18. J. F. Storz *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 14450 (2009).
19. M. A. Chappell, J. P. Hayes, L. R. G. Snyder, *Evolution* **42**, 681 (1988).
20. M. A. Chappell, L. R. G. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5484 (1984).
21. J. F. Storz, J. K. Kelly, *Genetics* **180**, 367 (2008).
22. J. F. Storz, C. Natarajan, Z. A. Cheviron, F. G. Hoffmann, J. K. Kelly, *Genetics* **190**, 203 (2012).
23. J. F. Storz *et al.*, *PLoS Genet.* **3**, e45 (2007).
24. Materials and methods are available as supplementary materials on *Science* Online.
25. N. Inoguchi *et al.*, *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **69**, 393 (2013).

Acknowledgments: Funded by grants from the NIH–National Heart, Lung, and Blood Institute (R01 HL087216 and HL087216-S1), the NSF (DEB-0614342 and IOS-0949931), and the Faculty of Science and Technology, Aarhus University. We thank S. Kachman for statistical advice, A. Bang for assistance in the lab, and M. Harms, S. Smith, and two reviewers for helpful comments. All experimental data are tabulated in the main text and in the supplementary materials.

Supplementary Materials
www.sciencemag.org/cgi/content/full/340/6138/1324/DC1
Materials and Methods
Figs. S1 to S5
Table S1
References (*26*–*41*)

21 February 2013; accepted 8 April 2013
10.1126/science.1236862

Root Effect Hemoglobin May Have Evolved to Enhance General Tissue Oxygen Delivery

Jodie L. Rummer,[1,2]* David J. McKenzie,[3] Alessio Innocenti,[4] Claudiu T. Supuran,[4] Colin J. Brauner[1]

The Root effect is a pH-dependent reduction in hemoglobin-O_2 carrying capacity. Specific to ray-finned fishes, the Root effect has been ascribed specialized roles in retinal oxygenation and swimbladder inflation. We report that when rainbow trout are exposed to elevated water carbon dioxide (CO_2), red muscle partial pressure of oxygen (PO_2) increases by 65%—evidence that Root hemoglobins enhance general tissue O_2 delivery during acidotic stress. Inhibiting carbonic anhydrase (CA) in the plasma abolished this effect. We argue that CA activity in muscle capillaries short-circuits red blood cell (RBC) pH regulation. This acidifies RBCs, unloads O_2 from hemoglobin, and elevates tissue PO_2, which could double O_2 delivery with no change in perfusion. This previously undescribed mechanism to enhance O_2 delivery during stress may represent the incipient function of Root hemoglobins in fishes.

In vertebrates, hemoglobin (Hb) plays a crucial role in optimizing tissue oxygen (O_2) delivery by increasing blood O_2-carrying capacity and regulating the partial pressure (PO_2) at which O_2 is delivered. Within tissues (such as muscle), metabolically produced carbon dioxide (CO_2) reduces blood pH and thus Hb-O_2 affinity, elevating blood PO_2 and enhancing O_2 delivery, which is collectively termed the Bohr effect (*1*). In mammals, this may elicit an increase in blood PO_2 of up to 2 mmHg (*2*, *3*) in vivo, providing some 5% increase in O_2 delivery. Teleost fishes often have much more pH-sensitive Hbs, and a recent in vitro study indicates that this effect may be an order of magnitude greater (*4*). The current study confirms this in vivo. Enhanced O_2 delivery may represent an important step in the extraordinary adaptive radiation of the teleost fishes (*5*), which make up almost half of all vertebrate species.

In teleosts, a reduction in blood pH reduces both Hb-O_2 affinity and O_2 carrying capacity, which is known as the Root effect (*6*) and has a well-studied role in securing O_2 delivery to the retina and swimbladder (*7*, *8*). These tissues possess specialized acid-producing cells in conjunction with a dense counter-current capillary network (*rete*) that localizes and magnifies a large acidosis, thus promoting O_2-offloading via the Root effect (*7*, *8*). This system is effective enough to generate O_2 tensions exceeding 50 atm (~38,000 mmHg) within the gas-filled swimbladder (*7*).

[1]Department of Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, British Columbia V6T 1Z4, Canada. [2]Australian Research Council Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland 4811, Australia. [3]Equipe Diversité et Ecologie des Poissons, UMR 5119 Ecologie des Systèmes Marins Côtiers, Université Montpellier II, Place Eugène Bataillon, Case 093, F-34095 Montpellier cedex 5, France. [4]Università degli Studi di Firenze, Neurofarba Department, Sezione di Scienze Farmaceutiche, Via Ugo Schiff 6, 50019 Sesto Fiorentino (Florence), Italy.
*Corresponding author. E-mail: jodie.rummer@jcu.edu.au

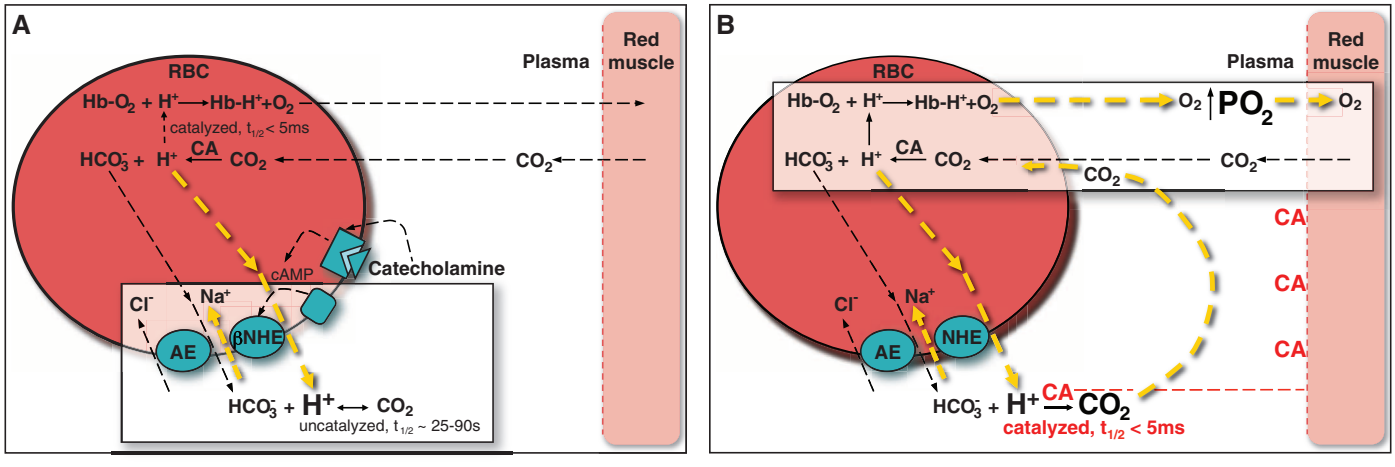

Fig. 1. (A and B) Schematic representation of a catecholamine-activated RBC pH disequilibrium (A) short-circuited by plasma-accessible CA to elevate tissue PO_2 (B). AE, anion exchange; cAMP, adenylate cyclase and 3′,5′-cyclic monophosphate.

The Root effect could, however, be a liability because it could severely limit O_2 uptake at the gills if systemic blood becomes too acidic. Many teleosts release stress hormones (noradrenaline and adrenaline) that stimulate RBC Na^+/H^+ exchange (βNHE), which removes H^+ in exchange for Na^+, creating a pH disequilibrium across the RBC membrane (Fig. 1A) (*9*). This increases RBC pH and ensures O_2 loading at the gill during acidotic stress associated with exhaustive exercise, hypoxia, or hypercarbia (Fig. 1A) (*10*, *11*). Carbonic anhydrase (CA), which catalyzes the reversible conversion of HCO_3^- and H^+ to CO_2 for diffusive excretion, would rapidly short-circuit this process (Fig. 1B) (*4*, *12*). Consequently, there is no plasma-accessible CA in the gills of teleosts (*13*), and this is understood to ensure O_2-loading during stressful conditions. However, there is evidence for plasma-accessible CA in muscle capillaries (*14*, *15*). This would short-circuit βNHE, reduce RBC pH and Hb-O_2 affinity, and enhance O_2-unloading and tissue delivery (Fig. 1B). A recent in vitro study provided proof of principle that this can occur; exposure of acidified and adrenergically stimulated rainbow trout blood to CA elevated PO_2 by over 30 mmHg (*4*, *12*). Thus, plasma-accessible CA may allow Root effect Hbs to enhance O_2 delivery to aerobic tissues during acidotic stress (*4*, *12*). The present study was designed specifically to determine whether plasma-accessible CA and the associated elimination of RBC pH disequilibrium states in capillary beds could markedly increase tissue PO_2 and the driving force for O_2 delivery in vivo.

We implanted fiber optic O_2 sensors into rainbow trout red muscle (RM) (fig. S1) to monitor RM PO_2 in real-time. We then monitored changes in RM PO_2 during a mild acidosis elicited by hypercarbia (elevated water CO_2), which should enhance the RBC pH disequilibrium at the gills. We hypothesized that if present in the RM capillary endothelium, plasma-accessible CA would abolish the pH disequilibrium and increase RM PO_2. To provide evidence for a role of plasma-accessible CA in this phenomenon, a new membrane-impermeant CA inhibitor, C18 [compound 18, laboratory-synthesized according to (*16*)] (fig. S2), was injected into the bloodstream so as to abolish the hypercarbic increase in RM PO_2.

After anesthesia, dorsal aorta (DA) cannulation, and RM O_2 sensor implantation (fig. S1) (*17*), rainbow trout recovered overnight in a black Perspex chamber while RM PO_2 was continuously monitored. In the morning, blood was sampled, and fish were lightly anaesthetized and treated with a skeletal muscle relaxant (tubocurarine) so as to prevent movement of the fish and damage to the O_2 sensor during hypercarbia. All blood variables recovered by 90 ± 21 min after tubocurarine, which represented "baseline" conditions against which subsequent responses were compared (Fig. 2 and fig. S3).

Fish were force-ventilated to ensure that arterial PO_2 (P_aO_2; 111.1 ± 14.9 mmHg), Hb-O_2 saturation (86.8 to 99.0%), and O_2 content (7.1 to 7.7 ml O_2 100 ml $blood^{-1}$) remained constant (Fig. 2 and fig. S3). Plasma catecholamines (noradrenaline, NA; adrenaline, AD) were not significantly elevated over published resting values (≤12 and ≤9 nM, respectively) (fig. S4) (*18*).

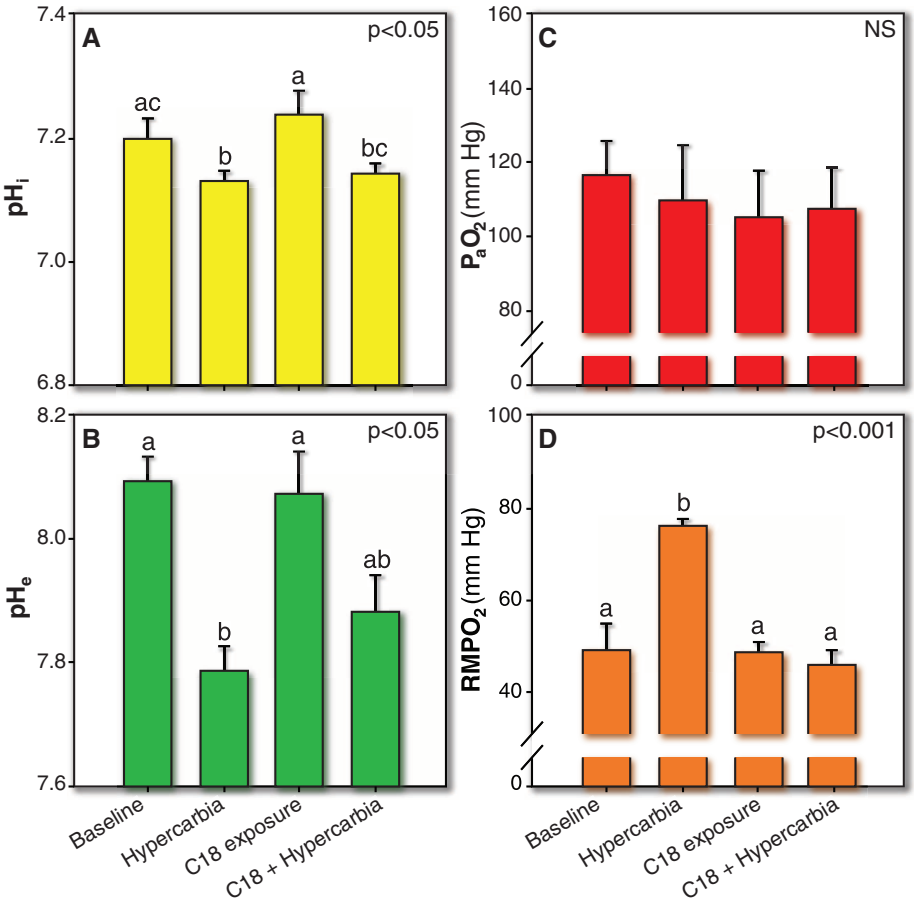

Fig. 2. The effect of hypercarbia, membrane-impermeant carbonic anhydrase inhibitor (C18) and combined hypercarbia and C18 on (A) pH_i, (B) pH_e, (C) P_aO_2, and (D) RM PO_2. Different lowercase letters demarcate significant differences between treatment groups within a panel. All data are presented as means ± SEM, n = 7 to 11 fish per treatment per treatment. Statistical significance (repeated measures analysis of variance) is indicated with P values (nonsignificance is indicated "NS") and assessed using $\alpha < 0.05$.

Hypercarbia caused a significant increase in blood P_{CO_2} from 1.7 ± 0.1 to 4.0 ± 0.3 mmHg [$F(5, 10) = 8.20$, $P < 0.001$] but no significant change in RBC or plasma total CO_2 (T_{CO_2}) (table S1). The elevated P_{CO_2} elicited a significant decrease in intracellular RBC pH (pH_i) (7.23 ± 0.01 to 7.13 ±0.02) and extracellular (plasma) pH (pH_e) (8.09 ± 0.04 to 7.79 ± 0.04) (Fig. 2 and fig. S3). There was a 65% increase in RM PO_2 from 47.1 ± 6.4 to 75.2 ± 2.2 mmHg, a ΔPO_2 of 30.9 ±7.6 mmHg (Figs. 2 and 3 and figs. S3 and S4). Upon return to normocarbia, all physiological variables returned to baseline (Fig. 2, figs. S3 and S4, and table S1). Intra-arterial C18 injection during normocarbia decreased [Hb] and Hct with no significant effects on blood, plasma, or RM variables (Fig. 2, figs. S3 and S5, and table S1). Upon reexposure to hypercarbia (C18+hypercarbia), blood P_{CO_2} [$F(5, 10) = 8.20$, $P < 0.001$] and RBC T_{CO_2} [$F(5, 10) = 3.88$, $P < 0.001$] both increased, with plasma T_{CO_2} unaffected (table S1). pH_i decreased to 7.14 ± 0.02 [$F(5, 8) = 4.62$, $P < 0.01$], but no changes in pH_e were observed (Fig. 2 and fig. S3). After C18 treatment, however, hypercarbia did not elicit the significant change in RM PO_2 that had previously been observed [ΔPO_2 = 0.01 ± 2.96 mmHg; $t(15) = 3.96$, P = 0.001] (Fig. 2 and figs. S3 and S5).

Our findings support the hypothesis that in a teleost fish possessing a Root effect Hb and plasma-accessible CA, O_2 delivery to tissues other than the retina and swimbladder—specifically, RM—can be greatly enhanced during acidotic stress. In RM, the proposed mechanism is a cascade by which plasma-accessible CA at the tissue eliminates an arterial RBC pH disequilibrium state to acidify RBCs. This causes Hb-O_2 unloading and elevates RM PO_2 (Fig. 1B). The level of hypercarbia we used caused only a moderate acidosis but was associated with a profound increase in RM PO_2 over baseline. This effect on RM PO_2 was abolished by the membrane-impermeant CA inhibitor (C18), highlighting the

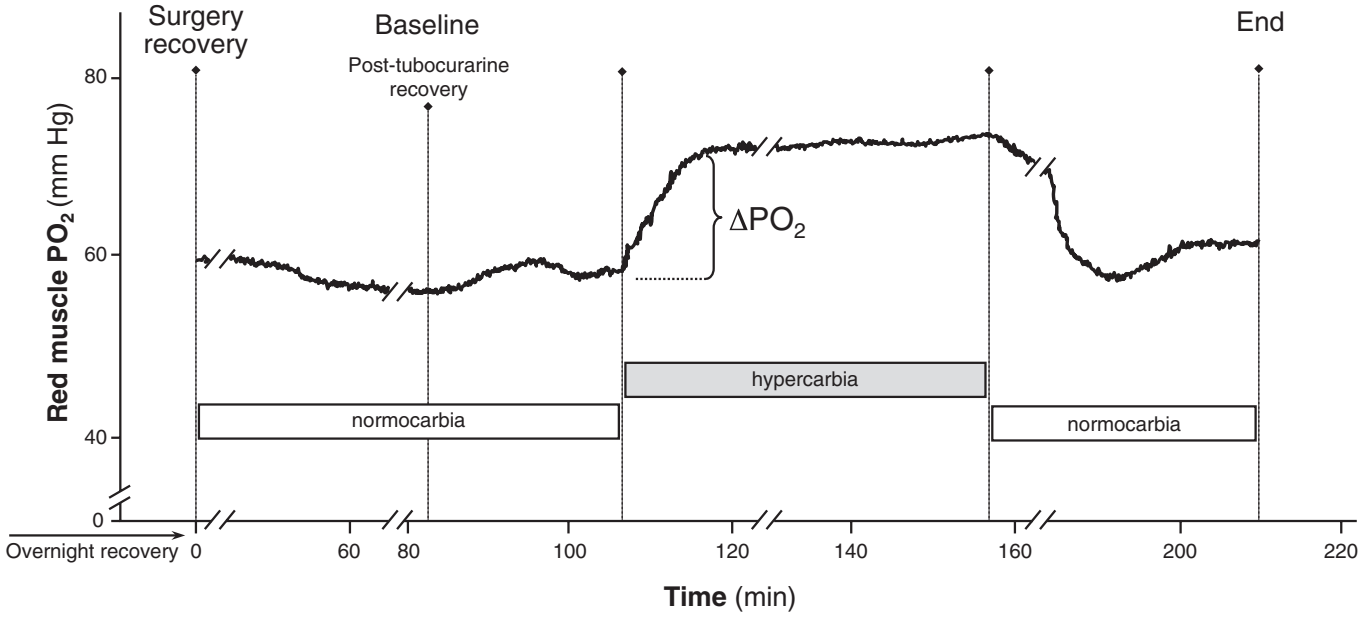

Fig. 3. Representative trace illustrating on-line changes in rainbow trout RM P_{O_2} (ΔP_{O_2}) upon exposure to hypercarbia. The *y* axis represents RM P_{O_2} (mmHg), and the *x* axis indicates elapsed time (min). Vertical lines indicate experimental treatments.

importance of plasma-accessible CA in the response. We estimate that a ΔP_{O_2} of this magnitude could almost double tissue O_2 delivery with no change in perfusion (supplementary text and fig. S8). Exposure to this level of hypercarbia did not significantly elevate catecholamine levels, indicating that the treatment was not overly stressful, and activation of RBC βNHE may not have been the prime source of the pH disequilibrium (*4*); other stimuli may activate the βNHE (*19*, *20*), or additional RBC NHE isoforms may exist. Activation of the RBC βNHE under more stressful conditions, such as exercise or hypoxaemia, may have an even greater influence on tissue oxygenation. This may be particularly important to increase aerobic metabolism during environmental or exercise stress or to speed up recovery after an intense bout of exercise—for example, after a predator-prey encounter or during long, upstream migrations, as exhibited in Pacific salmon.

It has been suggested that the Bohr effect evolved independently three times in vertebrates, but only once was this associated with the Root effect (*21*, *22*). According to the mechanism we propose [both here and in vitro (*4*)], the larger Bohr associated with the Root effect could actually promote O_2 delivery in teleosts. Thus, in response to previous arguments (*23*), a large Bohr coefficient could have been selected for without compromising general O_2 delivery. For this to be the case, however, three components are required: (i) pH-sensitive Hbs, such as Root effect Hbs; (ii) plasma-accessible CA in the respective capillary beds but not at the gill (*4*); and (iii) RBC βNHE or NHE activity to generate a pH disequilibrium in the absence of CA. All of these traits may depend on phylogeny, life history, and/or lifestyle of the species. With these components in place, however, a Root effect Hb can greatly enhance tissue O_2 delivery during a relatively mild acidosis. Further studies are required to investigate the degree to which this occurs in other metabolically active tissues, other species, and ultimately, the role that this mechanism of enhanced oxygen delivery may have played in the extraordinary success of teleosts.

Root effect Hbs evolved in ray-finned fishes ~400 million years ago, at least 150 million years before the appearance of the choroid *rete* at the eye or *rete mirabile* at the swimbladder, the structures that are generally associated with this exceptional O_2 delivery system (*21*, *22*). Here, we provide in vivo evidence supporting the hypothesis that Root effect Hbs, in the presence of plasma-accessible CA, can greatly enhance systemic tissue O_2 delivery during periods of acidotic stress. We estimate that the 65% elevation in RM P_{O_2} would almost double tissue O_2 delivery with no change in perfusion. The role of Root effect Hbs in the eye and swimbladder may, therefore, be an exaptation, as proposed by Berenbrink *et al*. (*22*). That is, an incipient function of Root Hbs—general O_2 delivery—may have been co-opted to give rise to the complex physiological system at the eye and swimbladder 150 million to 270 million years later.

References and Notes

1. C. Bohr, K. Hasselbalch, A. Krogh, *Skand. Arch. Physiol.* **16**, 402 (1904).
2. B. J. Behnke, C. A. Kindig, T. I. Musch, S. Koga, D. C. Poole, *Respir. Physiol.* **126**, 53 (2001).
3. J. Hutter *et al*., *J. Appl. Physiol.* **86**, 860 (1999).
4. J. L. Rummer, C. J. Brauner, *J. Exp. Biol.* **214**, 2319 (2011).
5. G. S. Helfman, B. B. Collette, D. E. Facey, *The Diversity of Fishes* (Blackwell, Oxford, 1997).
6. R. W. Root, *Biol. Bull.* **61**, 427 (1931).
7. P. F. Scholander, L. Van Dam, *Biol. Bull.* **107**, 247 (1954).
8. J. B. Wittenberg, B. A. Wittenberg, *Biol. Bull.* **146**, 116 (1974).
9. A. Baroin, F. Garcia-Romeu, T. Lamarre, R. Motais, *J. Physiol.* **356**, 21 (1984).
10. S. F. Perry, R. Kinkead, *Respir. Physiol.* **77**, 365 (1989).
11. D. R. Primmett, D. J. Randall, M. Mazeaud, R. G. Boutilier, *J. Exp. Biol.* **122**, 139 (1986).
12. J. L. Rummer, thesis, University of British Columbia, British Columbia, Canada (2010).
13. S. M. Rahim, J. P. Delaunoy, P. Laurent, *Histochem. Cell Biol.* **89**, 451 (1988).
14. R. P. Henry, E. R. Swenson, *Respir. Physiol.* **121**, 1 (2000).
15. Y. Wang, R. P. Henry, P. M. Wright, G. J. F. Heigenhauser, C. M. Wood, *Am. J. Physiol.* **275**, R1766 (1998).
16. C. T. Supuran, *Natl. Rev.* **7**, 168 (2008).
17. D. J. McKenzie *et al*., *J. Exp. Biol.* **207**, 3629 (2004).
18. M. Nikinmaa, *Physiol. Rev.* **72**, 301 (1992).
19. Y. R. Weaver, K. Kiessling, A. R. Cossins, *J. Exp. Biol.* **202**, 21 (1999).
20. M. G. Romero, H. Guizouarn, B. Pellissier, F. Garcia-Romeu, R. Motais, *J. Exp. Biol.* **199**, 415 (1996).
21. M. Berenbrink, *J. Exp. Biol.* **210**, 1641 (2007).
22. M. Berenbrink, P. Koldkjaer, O. Kepp, A. R. Cossins, *Science* **307**, 1752 (2005).
23. G. N. Lapennas, *Respir. Physiol.* **54**, 161 (1983).

Acknowledgments: This project was funded by a Natural Sciences and Engineering Research Council of Canada Discovery grant to C.J.B. and a University of British Columbia Graduate Fellowship and Australian Research Council grant to J.L.R. The authors wish to thank S. F. Perry for catecholamine analysis, W. K. Milsom for tubocurarine advice, and D. J. Randall, M. Regan, D. W. Baker, C. Ciuhandu, A. P. Farrell, K. Gilmour, A. Morash, P. Allen, N. Farrell, C. Fu, L. Hanson, K. Kamo, M. Roshan-Moniri, K. Suvadzjic, and the University of British Columbia Zoology workshop for interesting discussions and editorial and technical assistance. Additional information and all supporting data can be found in the supplementary materials.

Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6138/1327/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S8
Table S1
References (*24*–*77*)

5 December 2012; accepted 4 April 2013
10.1126/science.1233692

Root Effect Hemoglobin May Have Evolved to Enhance General Tissue Oxygen Delivery

Jodie L. Rummer *et al.*

Science **340**, 1327 (2013);
DOI: 10.1126/science.1233692