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Collaborative Research: Differential Expression of Oxygen-binding Proteins in Antarctic Fishes Affects Nitric Oxide-mediated Pathways of Angiogenesis and Mitochondrial Biogenesis

Bruce Sidell
Principal Investigator; University of Maine, Orono

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Principal Investigator: Sidell, Bruce D. **Award ID:** 0437887

Organization: University of Maine

Submitted By:

Sidell, Bruce - Principal Investigator

Title:

Collaborative Research: Differential Expression of Oxygen-binding Proteins in Antarctic Fishes Affects Nitric Oxide-mediated Pathways of Angiogenesis and Mitochondrial Biogenesis.

Project Participants

Senior Personnel

Name: Sidell, Bruce

Worked for more than 160 Hours: Yes

Contribution to Project:

Bruce D Sidell is the project PI and has devoted at least two summer months and two academic year months effort to this project. He recently has returned from deployment in Antarctica (April-June 2007) for field research associated with this project.

Name: Tota, Bruno

Worked for more than 160 Hours: Yes

Contribution to Project:

We are collaborating with Professor Tota's laboratory at the University of Cosenza, Italy. Professor Tota supervised work done by several members of his laboratory that has led to a manuscript that has been submitted to the journal, Nitric Oxide.

Name: Amelio, Daniela

Worked for more than 160 Hours: Yes

Contribution to Project:

Daniela Amelio executed a portion of the laboratory research performed in Professor Bruno Tota's laboratory at the University of Cosenza (Italy) on Antarctic fish tissues. This work has resulted in a manuscript currently under review by the journal, Nitric Oxide.

Name: Cerra, Maria

Worked for more than 160 Hours: No

Contribution to Project:

Maria Carmela Cerra executed a portion of the laboratory research performed in Professor Bruno Tota's laboratory at the University of Cosenza (Italy) on Antarctic fish tissues. This work has resulted in a manuscript currently under review by the journal, Nitric Oxide.

Name: Pellegrino, Daniela

Worked for more than 160 Hours: No

Contribution to Project:

DAniela Pellegrino executed a portion of the laboratory research performed in Professor Bruno Tota's laboratory at the University of Cosenza (Italy) on Antarctic fish tissues. This work has resulted in a manuscript currently under review by the journal, Nitric Oxide.

Post-doc

Name: Garofalo, Filippo

Worked for more than 160 Hours: Yes

Contribution to Project:

Filippo was a member of our field team in Antarctica during the period of April-June 2005. He is headquartered in the laboratory of Prof. Bruno Tota at the University of Cosenza, Italy. We are collaborating with Prof. Tota and his colleagues on studies involving the immunocytochemical localization of nitric oxide synthases in tissues of Antarctic fishes and the systemic effects of nitric oxide on cardiac performance in these animals.

Graduate Student

Name: Borley, Kimberly

Worked for more than 160 Hours: Yes

Contribution to Project:

Ms. Borley is a Ph.D. student whose assistantship and thesis research is supported by our award. She is focusing on the molecular biology of elements downstream of nitric oxide in the signalling pathway leading to vascular growth and proliferation. In addition to her work at the University of Maine, Ms. Borley also participated in Antarctic field work in April - June 2005 and in April - June 2007.

Name: Beers, Jody

Worked for more than 160 Hours: Yes

Contribution to Project:

Ms. Beers (formerly Wujcik) completed her M.S. thesis research. Her assistantship and thesis research have been supported by our award. She focused on quantifying vascular densities in retinal tissues of Antarctic fishes and relating this information to concentrations of circulating red blood cells and hemoglobin concentration in blood of the animals. She has now entered the Ph.D. program in Marine Biology here at the University of Maine and is conducting research supported by our award. Her present focus is on the activity, tissue distribution and molecular characteristics of nitric oxide synthases in tissues of Antarctic fishes and she is incorporating ultrastructural analyses of retinal tissues into her scope of work. In addition to her work at the University of Maine, Ms. Wujcik also participated in Antarctic field work in April - June 2005 and again in April-June 2007.

Undergraduate Student

Technician, Programmer

Name: Wang, George

Worked for more than 160 Hours: Yes

Contribution to Project:

Mr. Wang is a graduate student at the University of Washington and has considerable expertise in technical aspects of digital image analysis. He has assisted us in writing the scripted routines for analyzing digital images of the vasculature in retinas of Antarctic fishes.

Other Participant

Research Experience for Undergraduates

Organizational Partners

University of Cosenza

A scientist (Filippo Garofalo) from our collaborating laboratory at the University of Cosenza, Italy participated in field work in Antarctica during April-June 2005.

University of Rhode Island

High School teacher, Mark Harris, participated in Antarctic field work in conjunction with our project through the auspices of the ARMADA Polar Project managed by the University of Rhode Island.

University of Alaska Fairbanks Campus

This award is a collaborative research project with Dr. Kristin M. O'Brien at the University of Alaska, Fairbanks.

Other Collaborators or Contacts

Dr. Gregory Mayer, Department of Biochemistry, Microbiology and Molecular Biology, University of Maine -- Dr. Mayer is assisting us with instrumentation and technical advice for the section of our project involving Quantitative Real-Time PCR studies to assess expression of factors

in the the signalling pathway leading from nitric oxide to vascular proliferation.

Mr. George Wang, University of Washington -- Mr. Wang is a graduate student who has a high level of expertise in analysis of digital images. We engaged his services to assist us in writing scripted routines for computerized digital image analysis of retinal vasculature in Antarctic fishes. This relationship evolved into a formal collaboration on our project.

Dr. Joseph Eastman, Ohio University -- Dr. Eastman collaborated with our laboratory in the initial design of experiments and analysis of data, resulting in his coauthorship of a recent publication supported by our award, which was published in the Journal of Experimental Biology (Wujcik et al. 2007).

Dr. Kristin O'Brien and Mr. Matthew Urschel, University of Alaska, Fairbanks -- Dr. O'Brien is Co-PI of our collaborative research project and Mr. Urschel is a graduate student in her laboratory who is working on our collaborative project.

Activities and Findings

Research and Education Activities: (See PDF version submitted by PI at the end of the report)

See Attached File

Findings:

Please see file submitted under 'research and education activities' above, which also contains a synopsis of findings.

Training and Development:

The thesis research of graduate students Kimberly Borley and Jody Beers has been supported by this award.

Borley has been introduced to and developed skill in a variety of molecular biological techniques that she used to obtain gene sequence for elements of the pathway that stimulates angiogenesis. She was tutored by Gregory Mayer of the University's Biochemistry Department in experimental design and use of instrumentation for Quantitative Real-Time PCR estimation of mRNA levels and used these techniques extensively in her research. She is scheduled to defend her Ph.D. thesis in April 2010.

Beers has developed considerable skills in several aspects of digital image analysis for her project, which quantifies vascular morphometry in retinal tissues of Antarctic fishes. She has also received formal training in electron microscopy, although she was unable to use these techniques for analysis of Antarctic samples from our 2005 season because of a failure in the retrograde shipment of our specimens. For her continuing research, she expanded her skill set to include more biochemical and molecular biological techniques and also incorporated additional work in electron microscopic analyses of tissues from white- and red-blooded notothenioid fishes.

Both students gained considerable field experience in Antarctica during our 2007 field season; Beers also recently participated in the 2009 field season associated with a subsequent award.

Beers successfully defended her M.S. thesis research in August 2006 and currently is scheduled to defend her Ph.D. thesis in Marine Biology here at the University of Maine in Summer 2010.

Outreach Activities:

Year 3 (1 September 2007 - present):

1. Graduate student Jody Beers provided presentations about Antarctica and USAP scientific research to 4 second-grade classes at Old Town (Maine) Elementary School (approximately 75 students) on January 23, 2008. Ms. Beers' presentation incorporated a slide show with still images and video, a display of preserved marine invertebrates and fishes from waters surrounding Antarctica and the distribution of educational booklets obtained from Office of Polar Programs.

2. Sidell was one of 6 invited plenary lecturers at the European Congress of Ichthyology held in Cavtat, Croatia in September 2007. The lecture delivered covered a summary of our work on Antarctic fishes and, as a plenary lecture, was attended by the entire population of Congress participants.

Journal Publications

Sidell, Bruce D. and O'Brien, K.M., "When bad things happen to good fish: the loss of hemoglobin and myoglobin expression in Antarctic icefishes.", Journal of Experimental Biology, p. 1791, vol. 209, (2006). Published,

Wujcik, J.M., Wang, G., Eastman, J.T. and Sidell, B.D., "Morphometry of retinal vasculature in Antarctic fishes is dependent upon the level of hemoglobin in circulation.", Journal of Experimental Biology, p. 815, vol. 210, (2007). Published, 10.1242/jeb.001867

Fenaughty, J.M., Eastman, J.T. and Sidell, B.D., "Biological implications of low condition factor "axe handle" specimens of the Antarctic toothfish, Dissostichus mawsoni, from the Ross Sea.", Antarctic Science, p. 537, vol. 20, (2008). Published, 10.1017/S095410200800

Garofalo, F., Amelio, D., Cerra, M.C., Tota, B., Sidell, B. and Pellingrino, D., "Morphological and physiological study of the cardiac NOS-NO system in the Antarctic (Hb-/Mb-) icefish, Chaenocephalus aceratus, and in the red-blooded Trematomus bernacchii.", Nitric Oxide, p. 69, vol. 20, (2009). Published, 10.1016/j.niox.2008.10.006

Beers, J.M.; Borely, K.A.; Sidell, B.D., "Relationship among circulating hemoglobin concentration, nitric oxide synthase activities and angiogenic poise in red- and white-blooded Antarctic notothenioid fishes.", Comparative Physiology and Biochemistry, p. , vol. , (2010). In manuscript. Will be submitted by Jan 2010,

Borley, K.A.; Beers, J.M.; Sidell, B.D., "Phenylhydrazine-induced anemia causes nitric oxide-mediated upregulation of the angiogenic pathway in Notothenia coriiceps.", Journal of Experimental Biology, p. , vol. , (2010). In manuscript. Will be submitted by Jan 2010,

Books or Other One-time Publications

Web/Internet Site

URL(s):

http://www.umaine.edu/marine/people/directory.php/profile/bruce_sidell

Description:

http://www.umaine.edu/marine/people/directory.php/profile/bruce_sidell: Web site of P.I.,which indicates availability of data to any interested parties.

Other Specific Products

Contributions

Contributions within Discipline:

An extensive list of cardiovascular 'adaptations' to the loss of hemoglobin (Hb) in icefishes has been recognized for many years. Among these features are large heart size and cardiac output, large blood volumes, large bore capillaries and the potential for considerable cutaneous oxygen uptake. Most biologists have reasonably assumed that these pronounced cardiovascular characteristics have resulted from their selective retention in populations. Yet, strong selection for these compensations seems to be at odds with the fact that the original deleterious mutation of hemoglobin loss has persisted in the icefishes. And, the family itself is probably less than 10 million years old, leaving a relatively short (in evolutionary terms) time for selection of the traits.

The hypothesis that we seek to test with our project is that the mutation leading to loss of hemoglobin expression set in motion a series of homeostatic feedback mechanisms that may have led to very rapid development of the pronounced cardiovascular characteristics of the icefish family. Specifically, elimination of hemoglobin, which is the primary reactant in the degradation of constitutively produced vasoactive nitric oxide (NO) should lead to elevation of steady-state NO concentrations. NO is known to be a triggering compound in signaling pathways leading to angiogenesis (growth and proliferation of blood vessels), increases in lumenal diameter of capillaries and hypertrophy of cardiac muscle ? all hallmark characteristics of icefishes compared to their red-blooded relatives. The results of our experiments, thus, will help us understand the mechanisms leading to many of the cardiovascular characteristics of notothenioid fishes that appear compensatory to lowering

of hematocrits in red-blooded species and loss of expression of oxygen-binding proteins in white-blooded species.

Results of our experiments also have broader application to vertebrate animals other than polar fishes and even provide unique opportunities to understand processes of clinical interest in human medicine (see below).

Contributions to Other Disciplines:

All vertebrate animals probably share the mechanisms that drive or regulate angiogenesis. It is likely that nitric oxide plays a role in this process in all vertebrate lineages. Understanding angiogenesis is of intense interest to clinical medicine because of the potential for clinical intervention to prevent proliferation of vascular blood supply to growing tumors. By studying this process in closely related families of Antarctic fishes, some of whom lack oxygen-binding proteins that are pivotal in chemical degradation of NO, we have an unique opportunity. The hemoglobin-lacking icefishes and the subset of this family that also lacks myoglobin in their hearts represent naturally occurring genetic knockouts for expression of these proteins. Unlike laboratory-produced genetic knockouts, Antarctic fishes have survived the challenges of real world biology. The hemoglobin 'knockout' is particularly unique because any attempt to produce such a mutation in the laboratory in mammalian systems would fail because of its lethality. We thus have the unusual opportunity to ask questions of 'what would happen if one or more of the pathways for degradation of NO were shut off?'

Contributions to Human Resource Development:

One component of our project is the focus of Ph.D. thesis research of Ms. Kimberly Borley at the University of Maine. She is scheduled to defend her Ph.D. thesis in April 2010.

One component of our project has been the focus of M.S. thesis research of Ms. Jody Beers at the University of Maine. Ms. Beers defended her M.S. thesis successfully in August 2006 and is now a Ph.D. candidate in Marine Biology here at UM. Her Ph.D. thesis research likewise focuses on aspects of our project with Antarctic fishes. She is scheduled to defend her Ph.D. thesis in Summer 2010.

Mr. George Wang, a graduate student at the University of Washington, has helped us develop scripted subroutines for computer analysis of digital images of retinal structure in Antarctic fishes. Although this work is outside the bounds of his specific graduate research, his contribution will earn him co-authorship of the scientific paper that we are preparing to report results of these studies.

Contributions to Resources for Research and Education:

1. Our NSF-sponsored work on Antarctic fishes also was the subject of an invited Plenary Lecture that Sidell presented at the European Congress of Ichthyology in Cavtat, Croatia in September 2007.

2. Sidell presented an invited talk in the University of Delaware's named William S. Carlson lecture series in celebration of the International Polar Year. This talk presented a synopsis of our NSF-sponsored research on Antarctic fishes and was entitled: 'Fish tales from the Southern Ocean: the curious consequences of life in the icebox.' The talk was presented on 20 November 2008.

3. Sidell presented an invited named lecture, the Williams Lecture in Biology, at the University of Akron, Akron, OH in March of 2009. This talk also presented a synopsis of our NSF-sponsored research on Antarctic fishes. During the 3-day visit to Akron, Sidell also gave an informal talk to the Biology Department about research activities and logistic support of United States Antarctic Program.

Contributions Beyond Science and Engineering:

Please see section above on Contributions to Other Disciplines for possible relevance of our work to human clinical medicine.

Conference Proceedings

Categories for which nothing is reported:

Any Book

Any Product

Any Conference

Final Project Report Update
Award: ANT 04- 37887
Principal Investigator: Sidell, B.D.

This description of activities and findings is intended to update and build upon, but not duplicate, those submitted during annual reports for years 1-3 of our project.

As indicated in the "Contributions within Discipline" section of this report, the overarching hypothesis that we seek to test with our project is that the mutation leading to loss of hemoglobin (Hb) expression in Antarctic icefishes set in motion a series of homeostatic feedback mechanisms that may have led to very rapid development of the pronounced cardiovascular characteristics of the icefish family. Specifically, elimination of Hb, which is the primary reactant in the degradation of constitutively produced nitric oxide (NO) should lead to elevation of steady-state NO concentrations. NO is known to be a triggering compound in signaling pathways leading to angiogenesis (growth and proliferation of blood vessels), increases in luminal diameter of capillaries and hypertrophy of cardiac muscle, all hallmark characteristics of icefishes compared to their red-blooded relatives. To test this hypothesis, we have broken down our approach into the following questions:

1. ***Does the absence of circulating Hb lead to elevation of NO levels?*** One of the central elements of our hypothesis is that steady-state levels of NO should be higher in –Hb icefish than +Hb notothenioids, leading to elevations in angiogenic poise and mitochondrial biogenesis in the former group. Consequently, estimation of the levels of circulating NO in representative species of these groups would provide valuable information. NO, however, is notoriously difficult to quantify directly because of its extremely short half-life and because of requirements for specialized analytical apparatus. Many investigators, however, have relied upon quantifying the combined levels of circulating NO_2 and NO_3 (nitrite and nitrate) as a proxy for [NO], given that >95% of these compounds in circulation are derived directly from the breakdown of NO. Estimation of circulating $NO_2 + NO_3$ can be executed with relative ease using the Griess Reagent after enzymatically reducing the latter compound to NO_2 *in vitro*.

Conclusions: Apparent NO levels (as indicated by nitrite + nitrate) are higher in –Hb animals (Figure 1). Higher steady-state levels of NO thus accumulate from constitutive rates of NO synthesis when the primary participant in the degradation pathway for the compound (*i.e.* Hb) is removed..

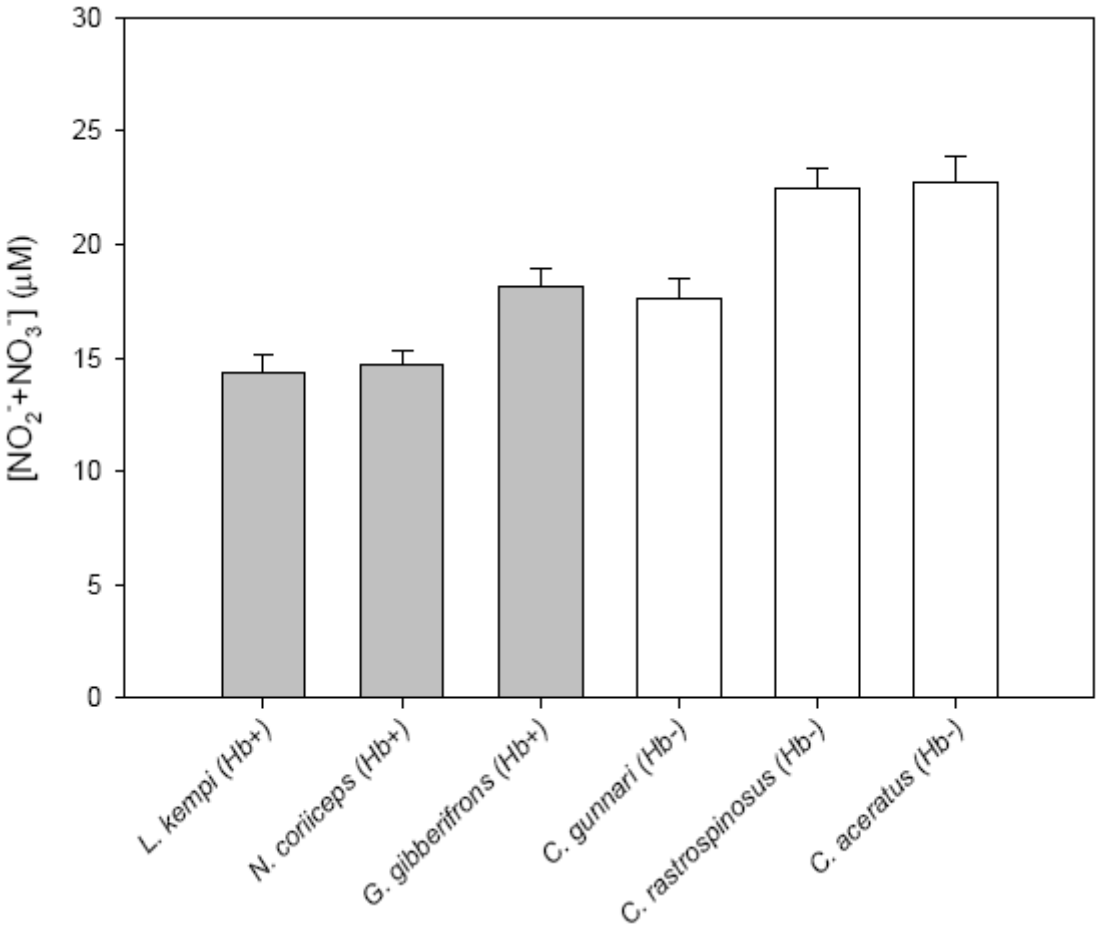

Figure 1. *Plasma [NO_2^- + NO_3^-] of red-blooded and white-blooded Antarctic notothenioids. Concentrations of nitrite and nitrate were measured using the Griess Assay. Parenthetical insertions (Hb+ and Hb-) indicate presence or absence of hemoglobin (Hb). Values are presented as means ± s.e.m.; N=8 for all species. Different lowercase letters denote significance differences between species (P≤0.05).*

2. ***Given that the concentration of endogenous NO is the result of both its rate of breakdown and rate of synthesis, do the activities of nitric oxide synthetase correlate with any observed differences in apparent NO concentrations among species?*** Nitric oxide is endogenously synthesized from arginine by a family of enzymes, the nitric oxide synthetases (NOS). We have adapted a radiochemical assay that measures the production of radioactive citrulline from ^{14}C-arginine substrate. This assay measures the total aggregate NOS activity of the tissues without discriminating between isoforms of the enzyme.

Conclusions: Elevated NO levels in –Hb icefishes appear to be the result solely of reduction in the rate of NO degradation, resulting from the absence of Hb, and not due to elevation in rate of synthesis of the compound (Table 1). Indeed, NOAS activities are generally lower in icefishes than red-blooded notothenioids, implying the presence of a negative feedback pathway where accumulation of NO to elevated levels throttles back the constitutive rate of synthesis of the compound.

Table 1. Measurement of nitric oxide synthase acitivity in tissues of Antarctic fishes

	Enzyme activity [pmol • min-1 • g wet wt.-1]			
	C. aceratus (Hb-)	*C. gunnari* (Hb-)	*G. gibberifrons* (Hb+)	*N. coriiceps* (Hb+)
Brain	95.7 ± 10.1^{a}	126.6 ± 9.8^{a}	216.2 ± 39.3^{b}	$159.3 \pm 10.9^{a,b}$
Heart	0.5 ± 0.2	0.6 ± 0.3	1.1 ± 0.6	2.9 ± 1.4
Retina	ND	1.7 ± 1.0^{a}	26.3 ± 10.8^{b}	1.8 ± 1.1^{a}
Pectoral muscle	ND	ND	1.0 ± 0.5	0.3 ± 0.2

Values are means ± s.e.m.; N=4 for all species.
ND, not detectable.
Different superscript letters denote significant differences between species ($P \leq 0.05$).

3. ***Are any differences observed in circulating NO levels correlated with angiogenic poise of the tissues, as indicated by gene expression of factors from the NO-mediated angiogenic pathway?*** To address this question, we employed Quantitative Realtime Polymerase Chain Reaction to assess the mRNA levels of the following factors: prolylhyrdroxilase 2 (PHD2), hypoxia-induced factor 1-alpha (HIF 1-α) and vascular endothelial growth factor (VEGF). The major trigger of angiogenesis among these factors is VEGF, which is known to be upregulated via NO-mediated signaling. Upregulation of VEGF can be induced by NO in concert with HIF-1α, a transcription factor that binds to hypoxia responsive elements (HREs) to stimulate expression of a suite of responses to decreases in oxygen supply. Elevated PHD2 expression is one of the most sensitive and rapid indicators of hypoxic insult. This enzyme catalyzes the targeting of HIF-1α for breakdown, but only in the presence of oxygen. Thus, elevation of its expression immediate upon hypoxic exposure primes the system for breakdown of its HIF-1α target immediately upon reoxygenation of the tissue.

Conclusions: We observed no significant difference in expression of factors in the pathway of NO-mediated angiogenesis between –Hb icefishes and red-blooded notothenioid species (Figure 2). This finding superficially seems at odds with the markedly higher vascular densities that we have reported in icefishes compared to their red-blooded relatives (see Wujcik *et al.*, 2007). However, we have recognized that the stable vascular structures of these fully differentiated animals may not require differences in angiogenic tone for their maintenance. Our observations do not rule out a NO-mediated elevation in angiogenic poise of icefishes during their early developmental stages, which ultimately leads to the markedly greater vascular densities that are stabilized in the adult form. It is still reasonable to poltulate a

causative relationship between the absence of Hb and elevation of NO that, at least in early ontogenetic development of icefishes, may induce vascular proliferation.

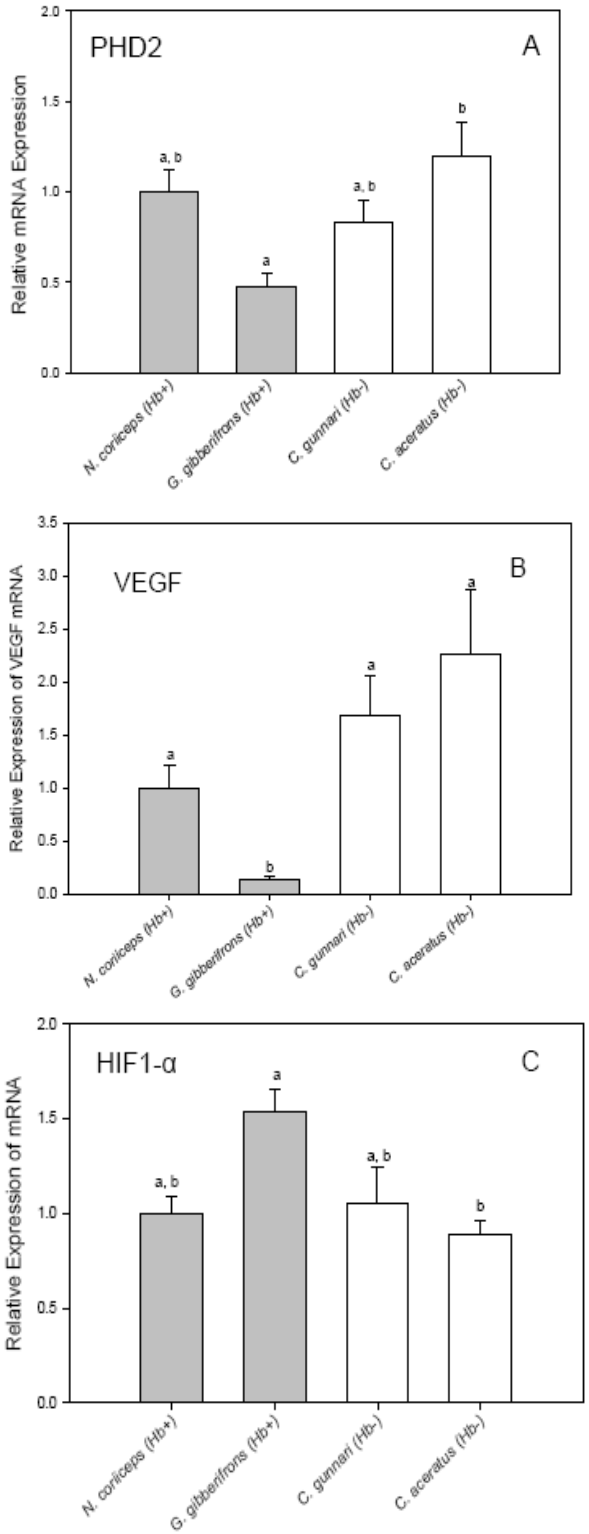

Figure 2. *mRNA expression of genes involved in angiogenesis in the retinae of red- and white-blooded Antarctic notothenioids. Expression for PHD2 (A), VEGF (B) and HIF1-α (C) is normalized to total RNA and reported relative to N. coriiceps. Values are means ± s.e.m.; N= 4 for all species. Statistical significance between species denoted by different lowercase letters (P≤0.05).*

The results summarized in Questions 1-3 above have been described in a manuscript by J.M. Beers, K.A. Borley and B.D. Sidell, which will be submitted to *Comparative Physiology and Biochemistry* by January 2010.

The results described above lead to recognition that maintenance of stable high vascular densities in icefishes may not require elevated tone of angiogenic factors and that induction of angiogenesis may, in fact, be a transitory process seen only in early developmental stages of –Hb icefishes. It is extraordinarily difficult to examine this process in early life history stages of icefishes due to inability to collect or culture these developmental stages of the species. We, therefore, sought to develop an experimental system that would permit us to explore the relationship between circulating Hb concentration and NO levels and ascertain whether an NO-mediated pathway of angiogenesis is present in Antarctic notothenioid fishes. We treated red-blooded *Notothenia coriiceps* with the hemolytic compound, phenylhydrazine (PHZ), which induces profound anemia of the animals. We then examined the effect of this reduction in level of circulating Hb upon apparent NO concentration and the level of gene expression of factors associated with the NO-mediated pathway of angiogenesis, as described above.

Although most previous studies that have used PHZ to induce anemia in fishes have used a single intraperitoneal injection , we wished to expose our experimental animals to sustained delivery of the drug

4. ***Does induction of anemia in red-blooded notothenioid fishes lead to an elevation in circulating NO?*** As described earlier, we utilized an experimental approach that measures the aggregate concentration of nitrite + nitrate in circulation as proxy for assessing NO levels in control *N. coriiceps* and specimens of this species that were made anemic by treatment with the hemolytic agent, phenylhydrazine (PHZ), which lyses red blood cells, leading to the degradation and clearance of the hemoglobin that they contain. In addition to an initial intraperitoneal injection of PHZ that is typically used in such experiments, we also surgically implanted an Alzet osmotic pump into the peritoneal cavity of experimental animals. This pump was filled with a PHZ solution and delivered the solution continuously over a 10 day period to the animals. We were both surprised and pleased to encounter 100% post-surgical survival of experimental animals for the entire 10-day course of the experiment. This treatment resulted in a dramatic reduction in hematocrit (Hct) and Hb concentration (Figure 3), with several animals attaining an Hct level that was below 1%, thus approaching the condition of –Hb icefishes by the end of the experimental period.

Conclusions: Induction of experimental anemia for a period of 10 days in *N. coriiceps* resulted in a significant elevation of apparent NO levels, as indicated by aggregate concentration of circulating nitrite + nitrate, approaching that observed in the –Hb icefish (Figure 4). Thus, reduction in the primary degradation agent for NO, circulating Hb, results in an elevation of apparent steady-state level of NO, as predicted by our hypothesis.

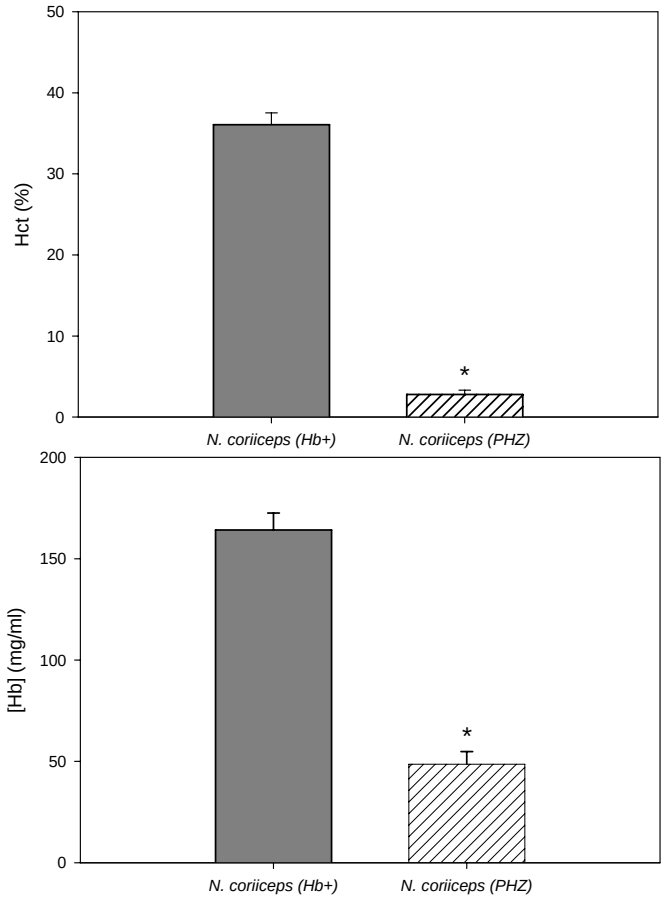

Figure 3. Hematocrit and hemoglobin concentration in blood of *N. coriiceps* treated with Phenylhydrazine. *N. coriiceps* were treated for ten days with the hemolytic agent, phenylhydrazine (PHZ). Asterisk denotes significant difference between control and PHZ-treated *N. coriiceps* ($P \leq 0.05$). Values are mean ± s.e.m; $N=8$ for both control and treatment groups.

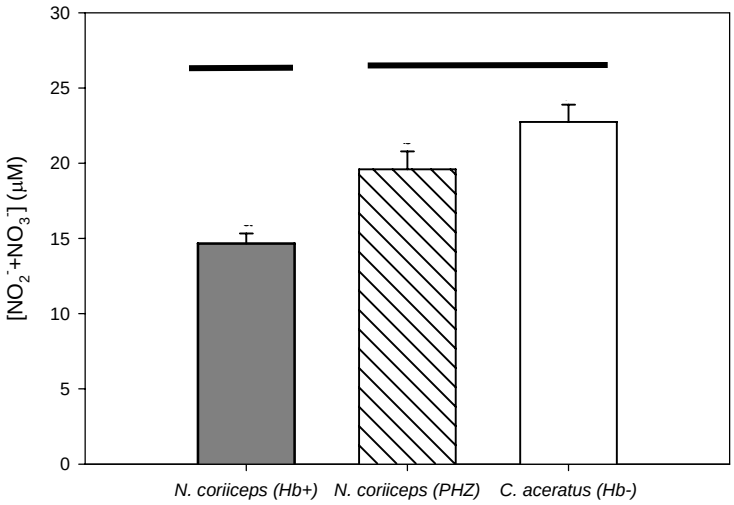

Figure 4. Plasma concentration of nitrate and nitrite is increased in +Hb *N. coriiceps* treated with phenylhydrazine (PHZ). *C. aceratus* is a species that lacks Hb. Values are means ± s.e.m.; N=8 for each species. Bars indicate lack of significant difference between groups at $P \leq 0.05$.

5. ***Is elevation in circulating levels of NO in response to anemia due solely to removal of its degradative agent, Hb, or is there also a contribution of elevated NOS activity?*** To address this question, we compared the catalytic activity of aggregate NOS in tissues of control and experimentally anemic *N. coriiceps*, using the radiochemical assay described in Section 2 above (Figure 5).

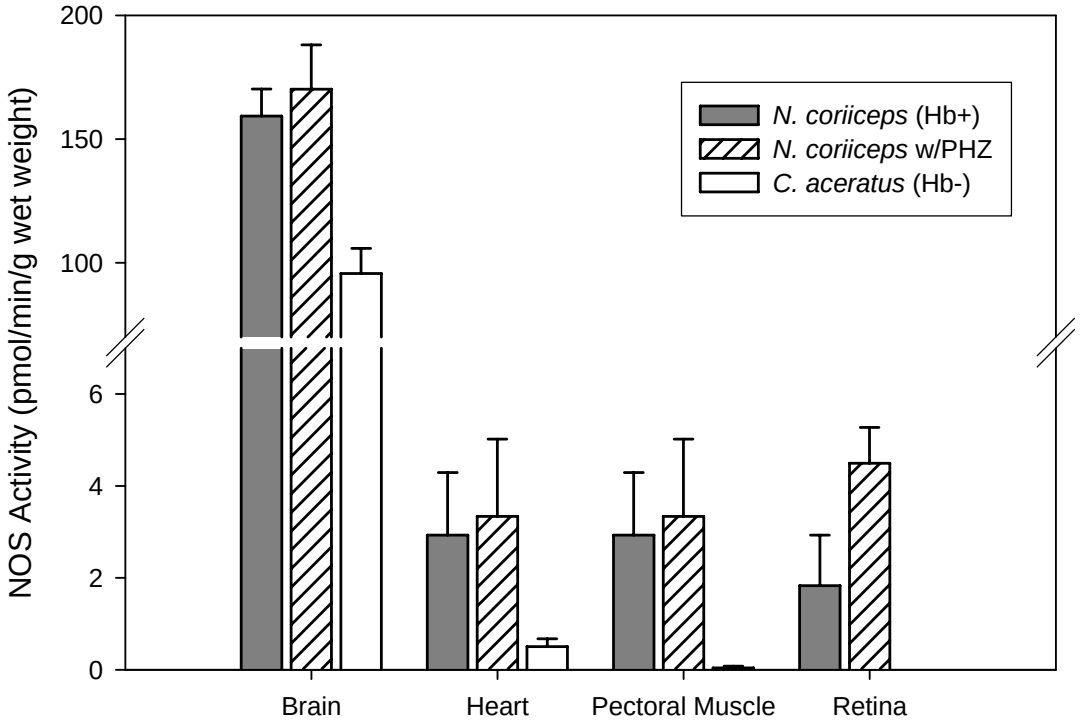

Figure 5. Nitric oxide synthase (NOS) activity in tissues of control *N. coriiceps*, experimentally anemic *N. coriiceps* and –Hb *C. aceratus*. Anemia in *N. coriiceps* was induced by treatment with phenylhydrazine (PHZ). Values are means ± s.e.m.; N=4 for all groups. NOS activity in all tissues of *C. aceratus* is significantly different from control and PHZ-treated *N. coriiceps* ($P \leq 0.05$).

Conclusions: Elevation in [NO] of anemic *N.coriiceps* is due solely to reduction in circulating levels of Hb and not due to an induction in activity of NOS.

6. ***Does the elevation in circulating levels of NO caused by experimental anemia induce an increase in expression of genes associated with the NO-mediated pathway of angiogenesis?*** If a patent NO-mediated pathway of angiogenesis is conserved within notothenioid fishes, we would anticipate that genes for factors downstream of NO in this pathway, notably that for VEGF, would be upregulated in response to anemia-induced elevation in NO levels. To test this, we used an experimental approach of Quantitative Real-Time Polymerase Chain Reaction (QPCR) as described in Section 3 above. Gene expression of VEGF and the

hypoxia-induced factors PHD2 and HIF-1α, as indicated by elevated mRNA levels, was increased dramatically by anemia in retinal tissue (Figure 6).

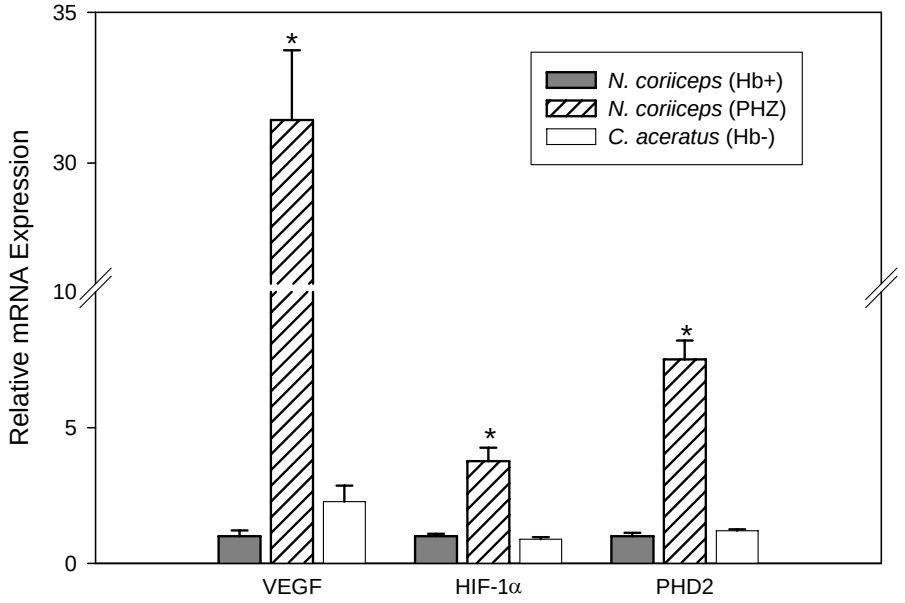

Figure 6. Experimental anemia induces upregulation of the NO-mediated angiogenic pathway in retinal tissue. mRNA expression for each gene was normalized to total RNA and is shown as relative to control *N. coriiceps.* Values are shown as mean ± s.e.m. (N= 4 per group). Asterisks signify significant difference from values for control *N. coriiceps* $P \leq 0.05$.

Conclusions: Angiogenesis is stimulated (as indicated by a 25-fold increase in mRNA encoding VEGF) in red-blooded *N. coriiceps* that have been made experimentally anemic by treatment with PHZ. Because the induction of VEGF is correlated with an elevation in circulating levels of NO, the pathway of NO-mediated angiogensis appears to be conserved in Antarctic notothenioids. Concomitant induction in expression of the hypoxia-induced factors PHD2 and HIF-1α, strongly suggests that hypoxia acts in concert with elevation in NO as proximate causes for the induction of vascular proliferation.

The overall conclusions of the above experiments are: a) the NO-mediated pathway of angiogensis remains patent in Antarctic notothenioids. b) an inverse correlation exists between circulating levels of Hb and NO in these fishes. c) a similar pathway may be turned on during early development of icefishes, leading to proliferation of vasculature that is stabilized in adult animals. Results described in Sections 4 through 6 above are described in a manuscript by K.A. Borley, J.M. Beers and B.D. Sidell that will be submitted to *Journal of Experimental Biology* by no later than January 2010.

The results described above provide a potential explanation of the underlying mechanisms responsible for evolution of the marked cardiovascular adaptations observed in Antarctic icefishes. If our interpretation is correct, inherent physiological homeostatic mechanisms were present in the ancestral progenitor of the icefish lineage that may have led rapidly to development of many of the hallmark cardiovascular characteristics of this group, immediately upon the loss of Hb expression via spontaneously arising mutation. The presence of such compensatory mechanisms further would help explain partially why the precipitous loss of expression of this normally critical oxygen-binding protein was not lethal to early icefish.