

1 **Evolved increases in hemoglobin-oxygen affinity and Bohr effect**
2 **coincided with the aquatic specialization of penguins.**

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18 J.F.S. and A.V.S. designed the research; A.V.S., M.S.T., F.G.H., T.L.S. and H.M. performed the
19 research; J.F.S., A.V.S., M.S.T., F.G.H., and H.M. analyzed data; J.F.S. and A.V.S. wrote the
20 manuscript.

21

22 **This PDF file includes:**

23 Main Text
24 Figures 1 to 3

25 **Abstract**

26 Dive capacities of air-breathing vertebrates are dictated by onboard O₂ stores, suggesting that
27 physiological specializations of diving birds like penguins may have involved adaptive changes in
28 convective O₂ transport. It has been hypothesized that increased hemoglobin (Hb)-O₂ affinity
29 improves pulmonary O₂ extraction and enhance capacities for breath-hold diving. To investigate
30 evolved changes in Hb function associated with the aquatic specialization of penguins, we
31 integrated comparative measurements of whole-blood and purified native Hbs with protein
32 engineering experiments based on site-directed mutagenesis. We reconstructed and resurrected
33 ancestral Hbs representing the common ancestor of penguins and the more ancient ancestor
34 shared by penguins and their closest nondiving relatives (order Procellariiformes, which includes
35 albatrosses, shearwaters, petrels, and storm petrels). These two ancestors bracket the
36 phylogenetic interval in which penguin-specific changes in Hb function would have evolved. The
37 experiments revealed that penguins evolved a derived increase in Hb-O₂ affinity and a greatly
38 augmented Bohr effect (reduced Hb-O₂ affinity at low pH). Although an increased Hb-O₂ affinity
39 reduces the gradient for O₂ diffusion from systemic capillaries to metabolizing cells, this can be
40 compensated by a concomitant enhancement of the Bohr effect, thereby promoting O₂ unloading
41 in acidified tissues. We suggest that the evolved increase in Hb-O₂ affinity in combination with the
42 augmented Bohr effect maximizes both O₂ extraction from the lungs and O₂ unloading from the
43 blood, allowing penguins to fully utilize their onboard O₂ stores and maximize underwater foraging
44 time.

45

46

47 **Main Text**

48

49 **Introduction**

50 In air-breathing vertebrates, diving capacities are dictated by onboard O₂ stores and the efficiency
51 of O₂ use in metabolizing tissues (1). In fully aquatic taxa, selection to prolong breath-hold
52 submergence and underwater foraging time may have promoted adaptive changes in multiple
53 components of the O₂-transport pathway, including oxygenation properties of hemoglobin (Hb).
54 Vertebrate Hb is a tetrameric protein that is responsible for circulatory O₂ transport, loading O₂ at
55 pulmonary capillaries and unloading O₂ in the systemic circulation via quaternary structural shifts
56 between a high affinity (predominately oxygenated) relaxed (R-) state and a low affinity
57 (predominately deoxygenated) tense (T-) state (2). While this mechanism of respiratory gas
58 transport is conserved in all vertebrate Hbs, amino acid variation in the constituent α - and β -type
59 subunits may alter intrinsic O₂ affinity and the responsiveness to changes in temperature, red cell
60 pH, and red cell concentrations of allosteric cofactors (non-heme ligands that modulate Hb-O₂
61 affinity by preferentially binding and stabilizing the deoxy T conformation) (3, 4).

62 While the quantity of Hb is typically increased in the blood of diving birds and mammals in
63 comparison with their terrestrial relatives, there is no consensus on whether evolved changes in
64 Hb-O₂ affinity have contributed to enhanced diving capacities (1). It has been hypothesized that
65 increased Hb-O₂ affinity may improve pulmonary O₂ extraction in diving mammals, thereby
66 enhancing diving capacity (5), but more comparative data are needed to assess evidence for an
67 adaptive trend (6, 7). Experimental measurements on whole-blood suggest that the emperor
68 penguin (*Aptenodytes forsteri*) may have a higher blood-O₂ affinity relative to nondiving
69 waterbirds, a finding that has fostered the view that this is a property that characterizes penguins
70 as a group (8–10). However, blood-O₂ affinity is a highly plastic trait that is influenced by changes
71 in red cell metabolism and acid-base balance, so measurements on purified Hb are needed to
72 assess whether observed species differences in blood-O₂ affinity stem from genetically based
73 changes in the oxygenation properties of Hb. Moreover, even if species differences in Hb-O₂
74 affinity are genetically based, comparative data from extant taxa do not reveal whether observed
75 differences are attributable to a derived increase in penguins, a derived reduction in their
76 nondiving relatives, or a combination of changes in both directions.

77 To investigate evolved changes in Hb function associated with the aquatic specialization
78 of penguins, we integrated experimental measurements of whole-blood and purified native Hbs

79 with evolutionary analyses of globin sequence variation. To characterize the mechanistic basis of
80 evolved changes in Hb function in the stem lineage of penguins, we performed protein
81 engineering experiments on reconstructed and resurrected ancestral Hbs representing (*i*) the
82 common ancestor of penguins and (*ii*) the more ancient ancestor shared by penguins and their
83 closest nondiving relatives (order Procellariiformes, which includes albatrosses, shearwaters,
84 petrels, and storm petrels) (Figure 1). These two ancestors bracket the phylogenetic interval in
85 which penguin-specific changes in Hb function would have evolved.

86

87 **Results and Discussion**

88

89 **Oxygen binding properties of penguin whole-blood and purified Hbs.** Using blood samples
90 from multiple individuals of six penguin species, we measured the partial pressure of O₂ (P_{O_2}) at
91 50% saturation (P_{50}) for whole-blood and purified Hbs in the absence (stripped) and presence of
92 allosteric cofactors (+KCl +IHP [inositol hexaphosphate]) (Figure 2). Whole-blood P_{50} values were
93 similar across all penguins, averaging 33.3 ± 1.1 torr (Figure 2; Table S1), consistent with previously
94 published data for emperor, Adélie, chinstrap, and gentoo penguins (8, 9, 11). Similarly, measured
95 O₂-affinities for purified Hbs exhibited very little variation among species, both in the presence and
96 absence of allosteric cofactors (Figure 2; Table S1). Penguins express a single Hb isoform during
97 postnatal life (HbA), in contrast to the majority of other bird species that express one major and one
98 minor isoform (HbA and HbD, respectively) (12, 13). The lack of variation in Hb-O₂ affinity among
99 penguins is consistent with the low level of amino acid variation in the α - and β -chains (Figure S1).
100 The experiments revealed that penguin Hbs exhibit a remarkably large shift in the magnitude of the
101 Bohr effect (i.e. the reduction in Hb-O₂ affinity in response to reduced pH) with the addition of
102 allosteric cofactors (Table S1). The average Bohr effect of penguin Hb more than doubles with the
103 addition of allosteric cofactors, from -0.21 ± 0.03 to -0.53 ± 0.04 (Table S1).

104 Our experimental results indicate that penguins have a generally higher Hb-O₂ affinity than
105 other birds (12, 14–22), consistent with previous suggestions based on measurements on whole-
106 blood (8, 9, 23–25). Whole-blood O₂-affinities of the six examined penguin species (30.4 to 38.1
107 torr at 37°C, pH 7.40) were uniformly higher than that from a representative member of
108 Procellariformes, the southern giant petrel (*Macronectes giganteus*; 42.5 torr at 38°C, pH 7.40) (9).
109 Similarly, numerous high-altitude bird species have convergently evolved increased Hb-O₂
110 affinities (17, 18, 20, 21), which appears to be adaptive because it helps safeguard arterial O₂
111 saturation in spite of the reduced P_{O_2} of inspired air (26–28). The difference in blood P_{50} 's between
112 penguins and the southern giant petrel is generally much greater in magnitude than differences in
113 Hb P_{50} between closely related species of low- and high-altitude birds (17, 18, 20, 21). Similar to
114 the case of other diving vertebrates (29), the Bohr effect of penguin Hb also greatly exceeds typical
115 avian values.

116

117 **Ancestral protein resurrection.** In principle, the observed difference in Hb-O₂ affinity between
118 penguins and their closest non-diving relatives could be explained by a derived increase in Hb-O₂
119 affinity in the penguin lineage (the generally assumed adaptative scenario), a derived reduction in
120 the stem lineage of Procellariformes (the nondiving sister group), or a combination of changes in
121 both directions. To test these alternative hypotheses, we reconstructed the Hbs of the common
122 ancestor of penguins (AncSphen) and the more ancient common ancestor of Procellariimorphae
123 (the superorder comprising Sphenisciformes [penguins] and Procellariiformes; AncPro) (Figures
124 1, S2, S3 and S4). We then recombinantly expressed and purified the ancestral Hbs to perform *in*
125 *vitro* functional tests. Measurements of O₂-equilibrium curves revealed that the AncSphen Hb has
126 a significantly higher O₂-affinity than that of AncPro (Figure 3), indicating that penguins evolved a
127 derived increase in Hb-O₂ affinity. In the presence of allosteric cofactors, the P_{50} of AncSphen is
128 much lower (O₂-affinity is higher) compared to AncPro (11.8 vs. 20.2 torr). Much like the evolved
129 increases in Hb-O₂ affinity in high-altitude birds (18, 20–22), the increased O₂-affinity of penguin
130 Hb is attributable to an increase in intrinsic affinity rather than a reduced responsiveness to

132 allosteric cofactors, as the Hb–O₂ affinity difference between AncSphen and AncPro persists in
133 the presence and absence of Cl⁻ and IHP (Figure 3).

134 In addition to the derived increase in Hb–O₂ affinity, comparisons between AncSphen and
135 AncPro also revealed that the Hb of penguins evolved an enhanced responsiveness to pH (Bohr
136 effect). Under stripped conditions, the Bohr effect of AncSphen and AncPro (-0.30±0.09 and -
137 0.27±0.1, respectively) were highly similar to one another and were similar to values measured for
138 native penguin Hbs under the same conditions (Figure 3E; Table S1). However, in the presence of
139 allosteric cofactors the Bohr effect of AncSphen increases more than two-fold (similar to that of
140 native penguin Hbs), whereas that of AncPro shows little change (Figure 3E), demonstrating that
141 penguins evolved an increased cofactor-linked Bohr effect following divergence from their non-
142 diving relatives. An increased Hb–O₂ affinity is expected to reduce the gradient for O₂ diffusion from
143 systemic capillaries to the cells of metabolizing tissues, and an increased Bohr effect can
144 compensate for this by reducing Hb–O₂ affinity at low pH, thereby promoting O₂ unloading in
145 acidified tissues. A similar augmentation of the Bohr effect was recently documented in the Hb of
146 high-altitude Tibetan canids (30). In summary, the Hbs of penguins evolved an increase in O₂-
147 affinity and enhanced Bohr effect in association with other physiological and morphological
148 specializations for a more fully aquatic existence.

149
150 **Tests of positive selection.** Given that joint increases in the O₂-affinity and Bohr effect of penguin
151 Hb represent derived character states, we performed a molecular evolution analysis to test for
152 evidence of positive selection in the α - and β -globin genes. Specifically, we tested for an
153 accelerated rate of amino acid substitution in the stem lineage of penguins (the branch connecting
154 Anc Procellariimorphae [AncPro] to the common ancestor of penguins [AncSphen]) using the
155 branch-sites test. This test revealed no evidence for an accelerated rate of amino acid substitution
156 in the stem lineage of penguins (Table S2), and a clade test revealed no significant variation in
157 substitution rate among different penguin lineages (Table S3). Thus, if the increased Hb–O₂ affinity
158 of penguins represents an adaptation that evolved via positive selection, the nature of the causative
159 changes did not produce a detectable statistical signature in the α - and β -type globin genes.

160
161 **Molecular modelling.** We used molecular modelling to identify which specific amino acid
162 substitutions may be responsible for the increased Hb–O₂ affinity of AncSphen relative to AncPro.
163 Of the 17 amino acid substitutions that distinguish AncSphen and AncPro, our analyses identified
164 four substitutions that could potentially alter O₂-binding properties. The substitution Thr β 119Ser in
165 the branch leading to AncSphen affects the stabilization of R-state (oxygenated) Hb. Specifically,
166 the hydroxyl group of β 119Ser in helix G is oriented toward the subunit interface by forming a
167 hydrogen-bond with β 120Lys, which permits an intersubunit contact with α 111Ile (Figure 3A,B).
168 This bond between β 119Ser and α 111Ile stabilizes the R-state conformation by clamping the
169 intersubunit motions, which is predicted to increase Hb–O₂ affinity by raising the free energy of the
170 oxygenation-linked allosteric R→T transition in quaternary structure. Additionally, our model
171 identified three other amino acid substitutions, α A138S, β A51S and β I55L, that create intersubunit
172 contacts and further stabilize the R-state conformation.

173
174 **Testing causative substitutions.** To test model-based predictions about the specific substitutions
175 that are responsible for the increased O₂-affinity of penguin Hb, we used site-directed mutagenesis
176 to introduce combinations of mutations at four candidate sites on the AncPro background. We first
177 tested the effect of a single mutation whereby β 119Thr was replaced with Ser (AncPro β T119S).
178 We then tested the net effect of mutations at all 4 sites on the AncPro background (AncPro+4;
179 α A138S, β A51S, β I55L, and β T119S). The protein engineering experiments revealed that β T119S
180 produced a negligible individual effect on Hb–O₂ affinity when introduced on the AncPro
181 background, but it produced an appreciable increase in the Bohr effect (Figure 3D, E). The 4
182 mutations in combination produced a modest increase in Hb–O₂ affinity and a more pronounced
183 increase in the Bohr effect, but they did not fully recapitulate observed differences between AncPro
184 and AncSphen in either of these properties (Figure 3). These data suggest the evolved functional

185 changes in penguin Hb must be attributable to the net effect of multiple amino acid substitutions at
186 structurally disparate sites.

187
188 **Adaptive significance of increased Hb-O₂ affinity.** The key to extending dive times for aquatic
189 vertebrates is to increase O₂ carrying capacity while keeping metabolic O₂ demands as low as
190 possible during breath-hold submergence. Submergence induces intense bradycardia and
191 peripheral vasoconstriction, which conserves finite O₂ stores for tissues that are intolerant to
192 hypoxia (i.e. the central nervous system and heart) (31). O₂ stores are typically increased in diving
193 vertebrates via increased blood volume, increased blood Hb concentration, increased myoglobin
194 concentration in skeletal muscle, increased muscle mass and, occasionally, increased diving lung
195 volume (1). As deep diving cetaceans and pinnipeds exhale before submergence, their lungs
196 account for less than 10% of total O₂ stores (1, 32). This reduction in diving lung volume reduces
197 gaseous N₂ and O₂, which presumably limits decompression sickness. Conversely, as penguins
198 inhale at the onset of a dive, their diving lung volume accounts for a much larger percentage of
199 total O₂ stores (19% and 45% for the emperor and Adélie penguins, respectively) (1, 33). Indeed,
200 in diving emperor penguins, O₂ extraction from pulmonary stores is continuous during
201 submergence (34, 35). An elevated Hb-O₂ affinity (such as that found in penguins) can maximize
202 O₂ extraction from pulmonary stores, as greater blood-O₂ saturation can be achieved at any given
203 parabronchial P_{O_2} . However, while increased Hb-O₂ affinity may confer more complete transfer of
204 O₂ from the lungs to the blood, it can inhibit subsequent O₂ transfer from the blood to the tissues.
205 Despite this, emperor penguins almost completely deplete their circulatory stores during extended
206 dives, as their end-of-dive venous P_{O_2} can be as low as 1-6 torr (34). The enhanced Bohr effect of
207 penguin Hb should improve O₂ delivery to working (acidic) tissues, allowing more complete O₂
208 unloading of the blood. We suggest this modification works in tandem with increased Hb-O₂ affinity
209 to maximize both O₂ extraction from the lungs and O₂ unloading from the blood, allowing penguins
210 to fully utilize their onboard O₂ stores and maximize underwater foraging time.

211

212

213 Materials and Methods

214

215 **Blood collection.** We collected blood from 18 individual penguins representing six species:
216 *Aptenodytes forsteri*, *A. patagonicus*, *Pygoscelis adeliae*, *P. papua*, *P. antarcticus*, and
217 *Spheniscus magellanicus* ($n=3$ individuals per species). All birds were sampled during routine
218 health checks at SeaWorld of California (San Diego, California). Blood was collected by
219 venipuncture of the jugular vein using Vacutainer® Safety-Lok™ blood collection set (Becton
220 Dickinson, Franklin Lakes, NJ) with 21 G x $\frac{3}{4}$ " (0.8 x 19 mm) needle attached to a heparin blood
221 collection tube (Becton Dickinson). A subsample of whole-blood (200 μ l) was set aside for oxygen
222 equilibrium curves (see below) and the remaining blood was centrifuged at 5000xg for 15
223 minutes. Plasma, buffy coat, and hematocrit fractions from the centrifuged samples were
224 immediately placed in separate tubes and flash frozen at -80°C for future analyses.

225

226 **Sequencing of penguin globin genes.** RNA was extracted from ~100 μ l of flash frozen
227 erythrocytes using an RNeasy Universal Plus Mini Kit (Qiagen). cDNA was synthesized from
228 freshly prepared RNA using Superscript IV Reverse transcriptase (Invitrogen). Gene specific
229 primers used to amplify the α - and β -type globin transcripts were designed from the 5' and 3'
230 flanking regions of all publicly available penguin globin genes. PCR reactions were conducted
231 using 1 ml of cDNA template in 0.2 ml tubes containing 25 μ l of reaction mixture (0.5 μ l of each
232 dNTP (2.5 mM), 2.5 μ l of 10x Reaction Buffer (Invitrogen), 0.75 μ l of 50 mM MgCl₂, 1.25 μ l of
233 each primer (10 pmol/ μ l), 1 μ l of Taq polymerase (Invitrogen) and 16.75 μ l of ddH₂O), using an
234 Eppendorf Mastercycler® Gradient thermocycler. Following a 5-min denaturation period at 94°C,
235 the desired products were amplified using a cycling profile of 94°C for 30 sec; 53-65°C for 30 sec;
236 72°C for 45 sec for 30 cycles followed by a final extension period of 5 min at 72°C. Amplified
237 products were run on a 1.5% agarose gel and bands of the correct size were subsequently
238 excised and purified using Zymoclean Gel DNA recovery columns (Zymo Research). Gel-purified

239 PCR products were ligated into pCR™4-TOPO® vectors using a TOPO™ TA Cloning™ Kit and
240 were then transformed into One Shot™ TOP10 Chemically Competent *E. coli* (Thermo Fisher
241 Scientific). Three to six transformed colonies were cultured in 5 ml of LB medium and plasmids
242 were subsequently purified with a GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific).
243 Purified plasmids were sequenced by Eurofins Genomics.
244

245 **Sequence analyses.** Genomic sequences containing the complete α - and β -globin gene clusters
246 for the emperor penguin (*A. forsteri*), Adélie penguin (*P. adeliae*), northern fulmar (*Fulmarus*
247 *glacialis*), band-rumped storm-petrel (*Hydrobates castro*), southern giant petrel (*Macronectes*
248 *giganteus*), flightless cormorant (*Nannopterum harrisi*), crested ibis (*Nipponia nippon*), and the
249 little egret (*Egretta garzetta*) were obtained from GenBank. The α - and β -globin gene clusters
250 from the remaining 19 extant penguin species were obtained from GigaDB (36). Coding
251 sequences of α - and β -globin genes extracted from these genomic sequences were combined
252 with the newly generated cDNA sequences mentioned above (Figure S2). Sequences were
253 aligned using MUSCLE (37) and were then used to estimate phylogenetic trees. The best fitting
254 codon substitution model and initial tree search were estimated using IQ-TREE with the options -
255 st CODON, -m TESTNEW, -allnni, and -bnni (38, 39). Initial trees were then subjected to 1000
256 ultrafast bootstrap replicates (40). Bootstrap consensus trees (Figure S3) were used to estimate
257 ancestral globin sequences using IQ-TREE with the option -asr (Figures S2 and S4).
258

259 **Selection analyses.** We tested for selection in the evolution of the penguins' α - and β -globin
260 genes in a maximum likelihood framework with the codon-based models implemented in the
261 codeml program from the PAML v4.9 suite (41), using the phylogenetic trees described above
262 (see "Sequence analyses"). We used the branch-site and clade models to examine variation in ω ,
263 the ratio of the rate of nonsynonymous substitutions per nonsynonymous site, dN, to the rate of
264 synonymous substitutions per synonymous site, dS. We used branch-site model A (42, 43) to test
265 for positive selection in the branch connecting AncPro to AncSphen (the stem lineage of
266 penguins) (Table S2), and we used clade C model (44) to test for selection in the penguin clade
267 using M2a_rel from Weadick and Chang (45) as the null model (Table S3).
268

269 **Molecular modelling.** Structural modeling was performed on the SWISS MODEL server
270 (46) using graylag goose hemoglobin in oxy form (PDB 1faw). AncPro and AncSphen Hbs had
271 QMEAN values of -0.61 and -0.65, respectively. The root mean square distance of the main
272 chain between template and model (RMSD) values $< 0.09\text{\AA}$ were considered usable (47).
273 Structural mining and preparation of graphics were performed using the PyMOL Molecular
274 Graphics System, version 2.3.2 (Schrödinger, LLC, New York, NY, USA). Hydrogen bond listing
275 was performed using a PyMol script list_hb.py (Robert L. Campbell, Biomedical and Molecular
276 Sciences, Queen's University, Canada). The interface binding energy was calculated by the
277 ePISA server (48).
278

279 **Construction of Hb expression vectors.** Reconstructed ancestral globins were synthesized by
280 GeneArt Gene Synthesis (Thermo Fisher Scientific) after optimizing the nucleotide sequences in
281 accordance with *E. coli* codon preferences. The synthesized globin gene cassette was cloned
282 into a custom pGM vector system along with the methionine aminopeptidase (MAP) gene, as
283 described previously (49). We engineered the Thr β 119Ser substitution by whole plasmid
284 amplification using mutagenic primers and Phusion High-Fidelity DNA Polymerase (New England
285 BioLabs), phosphorylation with T4 Polynucleotide Kinase (New England BioLabs), and
286 circularization with an NEB Quick Ligation Kit (New England BioLabs). All site-directed
287 mutagenesis steps were performed using the manufacturer's recommended protocol. Each
288 plasmid was verified with DNA sequencing by Eurofins genomics.
289

290 **Expression and purification of recombinant Hbs.** Recombinant Hb expression was carried out
291 in the *E. coli* JM109 (DE3) strain as described previously (15, 49, 50). Bacterial cell lysates were
292 loaded onto a HiTrap SP HP anion exchange column (GE Healthcare) and were then equilibrated

293 with 50 mM HEPES/0.5 mM EDTA (pH 7.0) and eluted with a linear gradient of 0 - 0.25 M NaCl.
294 Hb-containing fractions were then loaded on to a HiTrap Q HP cation exchange column (GE
295 Healthcare) equilibrated with 20 mM Tris-HCl/0.5mM EDTA (pH 8.6) and eluted with a linear pH
296 gradient 0 - 0.25 M NaCl. Eluted Hb fractions were concentrated using Amicon Ultra-4 Centrifugal
297 Filter Units (Millipore).

298
299 **Sample preparation for O₂-equilibrium curves.** Fresh whole-blood was diluted 1:15 with each
300 individual's own plasma and oxygen-equilibrium curves were measured immediately after
301 sampling. To obtain stripped hemolysate, 100 μ l centrifuged red blood cells were added to a 5x
302 volume of 0.01 M HEPES/0.5 mM EDTA buffer (pH 7.4) and incubated on ice for 30 min to lyse
303 the red blood cells. NaCl was added to a final concentration of 0.2 M and samples were
304 centrifuged at 20,000 x g for 10 min to remove cell debris. Hemolysate supernatants and purified
305 recombinant hemoglobins were similarly desalting by passing through a PD-10 desalting column
306 (GE Healthcare) equilibrated with 25 ml of 0.01 M HEPES/0.5mM EDTA (pH 7.4). Eluates were
307 concentrated using Amicon Ultra-4 Centrifugal Filter Units (Millipore). From these concentrated
308 samples, Hb solutions (0.1 mM hemoglobin in 0.1 M HEPES/0.05 M EDTA buffer) were prepared
309 in the absence (stripped) and the presence of 0.1 M KCl and 0.2 mM inositol hexaphosphate
310 (+KCl +IHP). Stripped and +KCl +IHP treatments were prepared at three different pHs (for a total
311 of 6 treatments per Hb sample), where working solutions was adjusted with NaOH to as near 7.2,
312 7.4, or 7.6 as possible, then pH was precisely measured with an Orion Star A211 pH Meter and
313 Orion™ PerpHecT™ ROSS™ Combination pH Micro Electrode.

314
315 **Measuring O₂-binding properties.** O₂-equilibrium curves were measured using a Blood Oxygen
316 Binding System (Loligo Systems) at 37°C. The pH of whole-blood samples was set by measuring
317 curves in the presence of 45 torr CO₂, whereas the pH of Hb solutions was set with HEPES buffer
318 (see above). Each whole-blood sample and Hb solution were sequentially equilibrated with an
319 array of oxygen tensions (P_{O_2}) while the sample absorbance was continually monitored at 430 nm
320 (deoxy peak) and 421 nm (oxy/deoxy isobestic point). Each equilibration step was considered
321 complete when the absorbance at 430 nm had stabilized (2 - 4 minutes). Only oxygen tensions
322 yielding 30 - 70% Hb–O₂ saturation were used in subsequent analyses. Hill plots (log[fractional
323 saturation/[1-fractional saturation]] vs. log P_{O_2}) were constructed from these measurements. A
324 linear regression was fit to these plots and was used to determine the P_{O_2} at half-saturation (P_{50})
325 and the cooperativity coefficient (n_{50}), where the X-intercept and slope of the regression line
326 represent the P_{50} and n_{50} , respectively. Whole-blood samples (n=3) are presented as mean \pm SE.
327 For Hb solutions, a linear regression was fit to plots of log P_{50} vs. pH, and the resulting equation
328 was used to estimate P_{50} values at pH 7.40 (\pm SE of the regression estimate).

329
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331
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341
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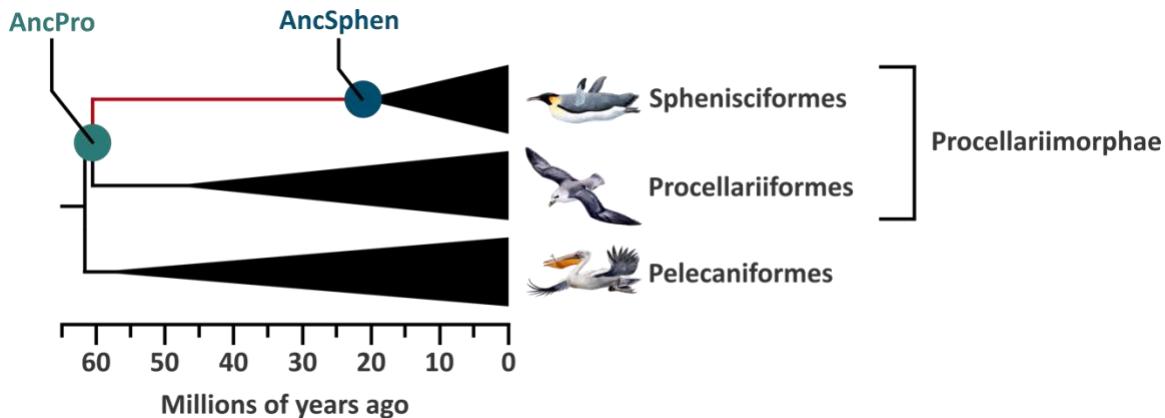
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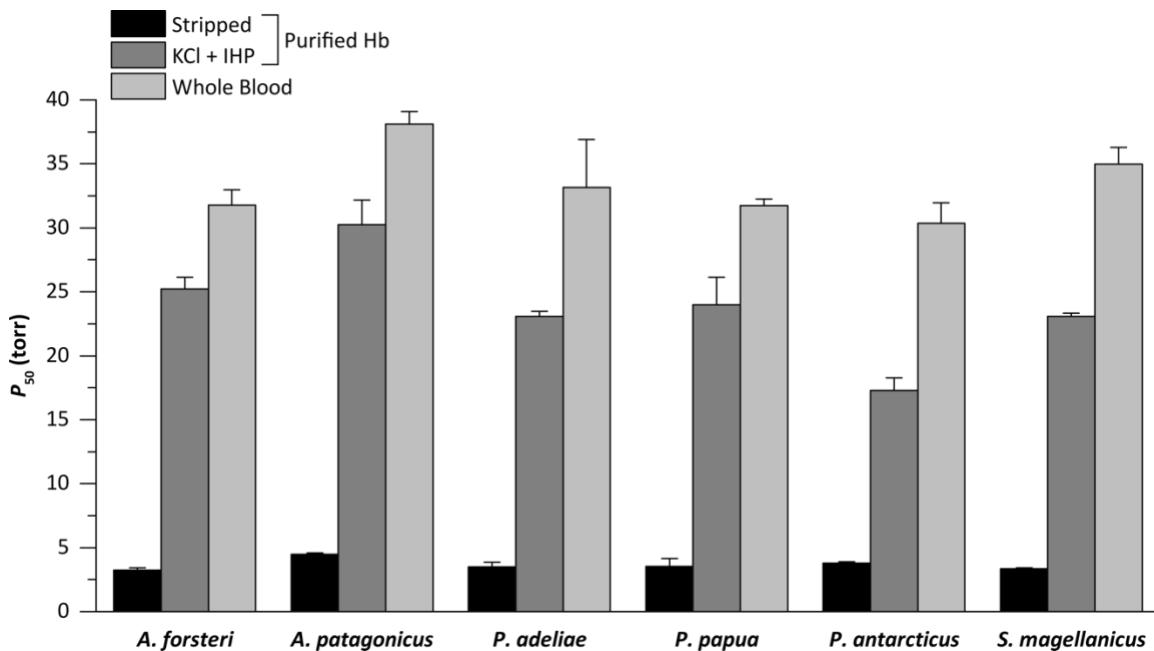
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453 **Figures and Tables**



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Figure 2. Oxygen tensions at half saturation (P_{50}) for penguin whole-blood and purified hemoglobins at 37°C, in the absence (Stripped) and presence of 100 mM KCl and 0.2 mM inositol hexaphosphate (+KCl +IHP). The higher the P_{50} , the lower the Hb-O₂ affinity. Whole-blood P_{50} values are presented as mean±S.E (n=3). Purified Hb P_{50} values are derived from plots of $\log P_{50}$ vs. pH, where a linear regression was fit to estimate P_{50} at exactly pH 7.40 (± S.E. of the regression estimate).

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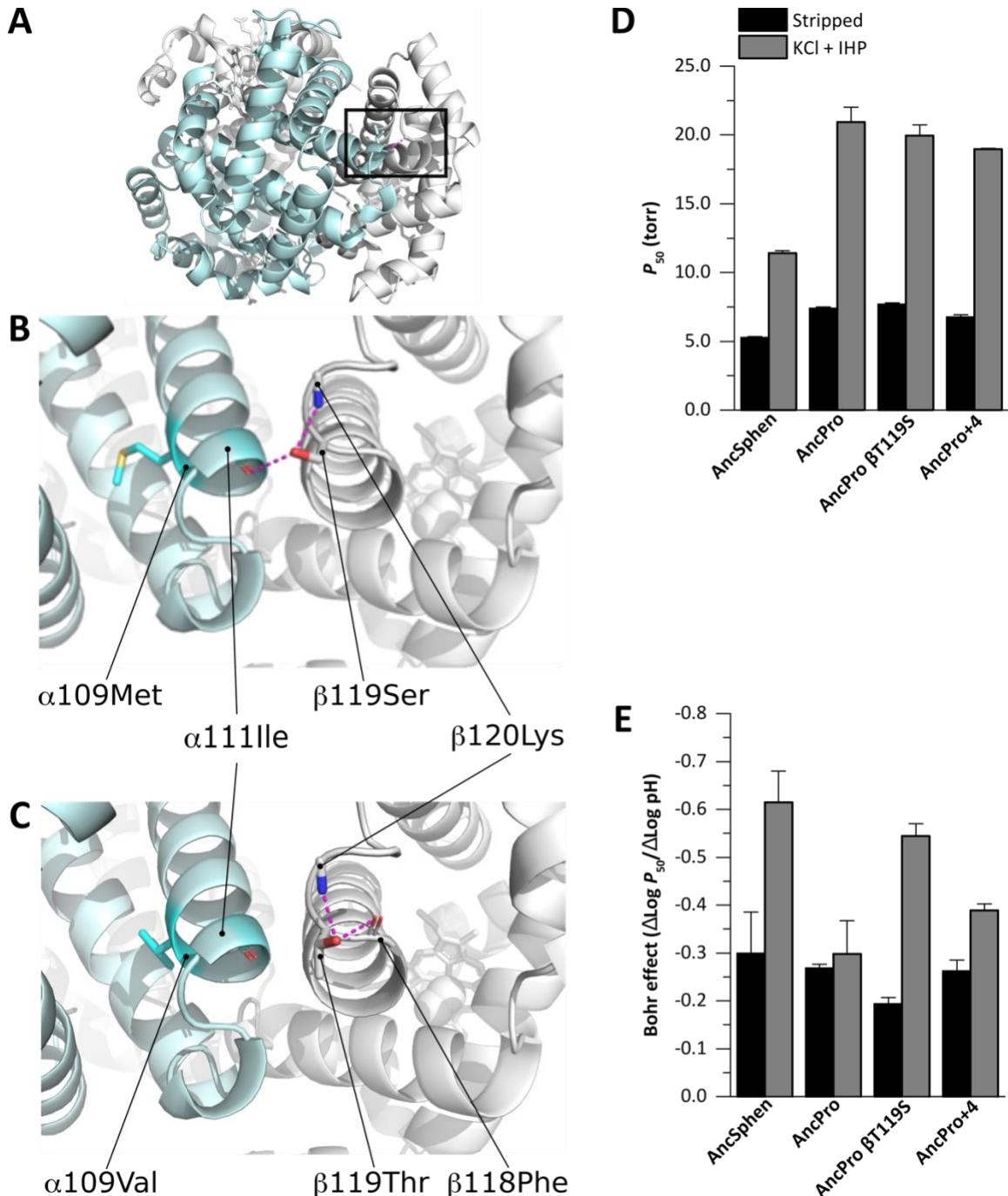
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474 **Figure 3.** Structural (A–C) and physiological effects (D, E) of amino acid substitutions in the
475 reconstructed Hb proteins of the penguin ancestor (AncSphen) and the last common ancestor
476 penguins shared with Procellariiformes (AncPro). A) Molecular model of the AncSphen Hb
477 tetramer where the black box indicates the regions highlighted in panels B and C. B) Molecular model of AncSphen Hb showing inter-subunit stabilizing H-bonds (pink) between β119Ser and
478 both α111Ile and β120Lys. C) Molecular model of AncPro Hb showing that replacement of
479 β119Ser with Thr removes the inter-subunit stabilizing H-bonds. D) Hb-O₂ affinity (as measured
480 by P₅₀, the O₂ tension at half saturation) of AncSphen, AncPro, and two mutant rHbs with
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482 penguin-specific amino acid replacements introduced on the AncPro background
483 (AncPro β 119Ser and AncPro+4). See text for explanation regarding the choice of candidate sites
484 for mutagenesis experiments. Measurements were performed on Hb solutions (0.1 mM Hb in 0.1
485 M HEPES/0.5 mM EDTA) at 37°C in the absence (stripped) and presence of 0.1 M KCl and 0.2
486 mM inositol hexaphosphate (+KCl +IHP). P_{50} values are derived from plots of $\log P_{50}$ vs. pH,
487 where a linear regression was fit to estimate P_{50} at exactly pH 7.40 (\pm S.E. of the regression
488 estimate). E) Bohr coefficients ($\Delta \text{Log } P_{50}/\Delta \text{Log pH}$) were estimated from plots of $\log P_{50}$ vs. pH,
489 where the Bohr effect is represented by the slope of a linear regression (\pm S.E. of the slope
490 estimate).