

METHODS & TECHNIQUES

Simultaneous high-resolution pH and spectrophotometric recordings of oxygen binding in blood microvolumes

Michael Oellermann, Hans-O. Pörtner, Felix C. Mark*

Alfred Wegener Institute for Polar and Marine Research, 27570 Bremerhaven, Germany

*Author for correspondence (e-mail: FMark@awi.de)

Key words: Diffusion chamber, oxygen equilibrium curve, hemocyanin, haemocyanin, haemoglobin, pH optode, octopus, amphipod, Antarctic fish

Words main text: 3890

1 SUMMARY

2 Oxygen equilibrium curves have been widely used to understand oxygen transport in
3 numerous organisms. A major challenge has been to monitor oxygen binding characteristics
4 and concomitant pH changes as they occur *in vivo*, in limited sample volumes. Here we report
5 a technique allowing highly resolved and simultaneous monitoring of pH and blood pigment
6 saturation in minute blood volumes. We equipped a gas diffusion chamber with a broad range
7 fibre optic spectrophotometer and a micro-pH optode and recorded changes of pigment
8 oxygenation along PO_2 and pH gradients to test the setup. Oxygen binding parameters derived
9 from measurements in only 15 μ l of haemolymph from the cephalopod *Octopus vulgaris*
10 showed low instrumental error (0.93%) and good agreement with published data. Broad range
11 spectra, each resolving 2048 data points, provided detailed insight into the complex
12 absorbance characteristics of diverse blood types. After consideration of photobleaching and
13 intrinsic fluorescence, pH optodes yielded accurate recordings and resolved a sigmoidal shift
14 of 0.03 pH units in response to changing PO_2 from 0-21 kPa. Highly resolved continuous
15 recordings along pH gradients conformed to stepwise measurements at low rates of pH
16 changes. In this study we showed that a diffusion chamber upgraded with a broad range
17 spectrophotometer and an optical pH sensor accurately characterizes oxygen binding with
18 minimal sample consumption and manipulation. We conclude that the modified diffusion
19 chamber is highly suitable for experimental biologists who demand high flexibility, detailed
20 insight into oxygen binding as well as experimental and biological accuracy combined in a
21 single set up.

22 INTRODUCTION

23 Since the first oxygen binding experiments by Paul Bert (1878) and Carl Gustav von Hüfner
24 (1890) and the pioneering work by Bohr, Hasselbalch and Krogh (1904) dating back more
25 than a century, oxygen binding experiments have served to understand human blood
26 physiology and diseases (e.g. Chanutin and Curnish, 1967; Festa and Asakura, 1979) or the
27 environmental adaptation of various organisms (e.g. Brix, 1983; Herbert et al., 2006; Meir et
28 al., 2009; Scott, 2011) and even resolved crime (Olson et al., 2010). Ever since, researchers
29 have developed and refined techniques to comprehend the complex physiology of oxygen
30 transport, leading to a variety of currently employed methods (Supplementary Table S1).

31 Such analysis commonly involves the determination of oxygen affinity (P_{50}). Accurate
32 determination of P_{50} requires to control or monitor extrinsic factors like temperature, carbon
33 dioxide (CO_2) and particularly pH. Assessing the effects of pH and the variation of pH during
34 transition from the oxygenated to the deoxygenated state, due to the oxygenation dependent
35 release or uptake of protons by the pigment (Haldane effect), requires knowledge of pH at
36 different levels of saturation for the analysis of oxygen equilibrium curves (OEC).

37 Conventionally, experimenters control pH by added buffers (e.g. Tris, HEPES, Brix et al.,
38 1994) or determine pH from sub-samples or separately conditioned samples (e.g. Seibel et al.,
39 1999; Weber et al., 2008) and rarely directly in the original sample (Pörtner, 1990; Zielinski
40 et al., 2001). While added buffers prevent pH changes and may help to address specific
41 functional characteristics of the pigment (e.g. effects of inorganic ions, Mangum and
42 Lykkeboe, 1979) they disturb the fine tuning of oxygen binding (Brix et al. 1994) and block
43 pH changes relevant as part of the oxygen transport process that need to be included for a
44 comprehensive picture of oxygen transport *in vivo*. A major challenge in monitoring pH has
45 been the relatively large sample volume required to immerse a pH electrode and its reference
46 electrode. Particularly, analysis in highly limited sample volumes or devices that employ thin
47 blood films (e.g. Diffusion chamber, HemOscan, Pwee 50, Supplementary Table S1) suffer
48 from this constraint.

49 Further, while many commercially available devices have been designed for human or
50 mammalian blood analysis (e.g. CO-Oximeter, HEMOX-Analyser) only few provide the
51 flexibility needed for the analysis of non-model organisms bloods, characterized by small
52 sample volumes, unusual spectra, extreme *in vitro* temperatures or high pH sensitivity.

53 Here we report a major step forward in the respective methodology, allowing the
54 simultaneous analyses of pH and pigment absorbance in microvolumes of blood. The
55 challenges successfully met by our technique comprise

- 56 (1) The parallel measurement of oxygenation, paired with the simultaneous monitoring of pH
57 depending on oxygenation level.
- 58 (2) The use of minimal sample volumes of 15 μ l.
- 59 (3) High resolution recordings, facilitated by continuous recordings of broad-range spectra
60 and pH.

61 We upgraded a gas diffusion chamber (Niesel and Thews, 1961; Sick and Gersonde, 1969,
62 1972; Bridges et al., 1984; Morris and Oliver, 1999; Weber et al., 2010), with an integrated
63 fibre optic micro-pH optode and a miniature broad range fibre optic spectrophotometer. The
64 experimental setup offers high flexibility to produce accurate oxygen equilibrium curves and
65 pH recordings from only minute volumes of sample.

66 RESULTS AND DISCUSSION

67 Spectrophotometric measurements

68 Using the modified diffusion chamber, we successfully performed measurements on
69 haemolymph from *Octopus vulgaris* and *Eulimnogammarus verrucosus* and whole blood of
70 *Pachycara brachycephalum* at various oxygen and carbon dioxide partial pressures and
71 temperatures. The integrated broad range spectrophotometer resolved 2048 data points per
72 spectrum from 200 to 1100 nm and yielded characteristic absorbance spectra for haemolymph
73 containing haemocyanin with an oxygenation dependent peak at 347 nm (*Octopus vulgaris*,
74 Figure 1A) and multiple responsive peaks at 540, 575, 412 and 335 nm for oxygenated and at
75 553, 427 and 366 nm for deoxygenated haemoglobin bearing blood (*Pachycara*
76 *brachycephalum*, Figure 1B). In addition, to the haemocyanin peak at 336 nm, haemolymph
77 from *Eulimnogammarus verrucosus* showed absorbance features above 400 nm that explain
78 its green coloration (Figure 1C). The detailed broad range spectra strongly facilitate and
79 enhance the analysis of solutions with complex absorbance spectra. Studies that gather only
80 snap shots of the spectrum by means of single wavelength filters (e.g. Morris et al., 1985;
81 Rasmussen et al., 2009) limit their experimental set up to a particular pigment type and may
82 miss further relevant features.

83 The modified diffusion chamber yielded reproducible and accurate results employing both
84 conventional stepwise measurements along a PO_2 gradient (Figure 2) as well as
85 measurements along a pH gradient (Figure 3), designed for pH sensitive pigments like
86 cephalopod haemocyanins (Pörtner, 1990). Oxygen equilibrium curves of *Octopus vulgaris*
87 haemolymph constructed from five replicated experiments with discrete oxygenation steps at
88 constant carbon dioxide partial pressure (PCO_2 ; 1 kPa) showed low variability among OECs
89 and the derived parameters (Figure 2, Table 1, Supplementary Figure S4) with a relative error
90 of 0.93% for oxygen affinity P_{50} and of 2.74% for the Hill coefficient n_{50} . This low
91 instrumental error and the good agreement of P_{50} and n_{50} with published data on *Octopus*
92 *vulgaris* haemolymph recorded at the same temperature and pH (Table 1, Figure 4), underline
93 the accuracy of the setup. The Bohr coefficient recorded at 15°C was higher than reported in
94 Brix et al. (1989) but closely matched values reported for *Octopus vulgaris* by Houlihan et al.
95 (1982) and Bridges (1994) *Octopus dofleini* (-1.7 (pH 7.0-8.3), Miller, 1985) or *Octopus*
96 *macropus* (-1.99 (pH 7.3-7.5), Lykkeboe and Johansen, 1982) (Figure 4, Table 1). The
97 difference in Bohr coefficients towards the study by Brix et al. (1989) relates to the pH ranges

98 used to determine the Bohr coefficient. In octopods, P_{50} increases linearly with pH and levels
99 off at lower pH (~7.0, Figure 4, Miller, 1985) as oxygen binding becomes pH insensitive (see
100 13 kPa OEC, Figure 3). Brix et al. (1989) included pH values below 7.0 (6.85-7.40), which
101 consequently led to reduced regression slopes and underestimated Bohr coefficients. Thus,
102 agreement with studies using pH ranges above 7.0 confirms the accuracy of Bohr coefficients
103 determined with the modified diffusion chamber.

104 Both pigment absorbance and haemolymph pH responded to changes of gas composition
105 within 30 seconds (Figure 5). The recording of one oxygen equilibrium curve, including
106 calibration at 100% and 0% oxygen saturation, lasted on average 3.5 hours (± 0.23) for
107 measurements ($n = 5$) with eight discrete oxygenation steps and 5.2 hours (± 0.21) for
108 measurements ($n = 4$) with eight discrete pH steps. While the maximum absorbance signal at
109 347 nm (haemocyanin oxygenation peak) drifted by -3.5% ($\pm 0.6%$, $n = 5$) per hour, minimal
110 absorbance remained nearly constant ($0.27\% \pm 0.21%$, $n = 5$). The protein peak drifted less
111 and varied more among experiments ($2.3\% \pm 2.7%$, $n = 5$, Figure 5B). Negative drift observed
112 for the maximum oxygenation signal was reported previously and explained by autoxidation
113 of the blood pigment (Wells and Weber, 1989). Consequently, each measurement needs to
114 comprise calibration steps with pure oxygen at the beginning and end to determine and
115 include the apparent and constant drift in the calculation of pigment oxygenation by
116 readjusting maximum absorbance at each consecutive oxygenation step (Wells and Weber,
117 1989). The less pronounced positive drift of the protein peak (Figure 5B) indicates a low
118 degree of sample drying and no apparent dilution by condensation or denaturation of the
119 haemolymph sample.

120 Interestingly, the height of the protein peak neither remained stable but increased/decreased
121 upon oxygenation/deoxygenation (Figure 5, Supplementary Figure S5). This unexpected
122 response of the protein peak to oxygenation/deoxygenation of the pigment (Figure 5,
123 Supplementary Figure S5) cannot be ascribed to irreversible protein denaturation as the
124 protein absorbance increased again upon re-oxygenation (Figure 5). Consequently,
125 oxygenation status may affect protein absorbance spectra and thus measurements of protein
126 concentration in haemolymph or blood. Conformational changes depending on the degree of
127 oxygenation may affect the absorbance features of the aromatic tryptophan, tyrosine and
128 phenylalanine residues that account for the absorbance at 270-295 nm (Alexander and Ingram,
129 1980). Although the protein peak may not always vary with oxygen content (Bolton et al.,
130 2009), it would be advisable to test a given blood type for such oxygenation dependency.

131

132 **pH recordings in blood microvolumes**

133 Simultaneous monitoring of pH, using a pH micro-optode in the same 15 μ l sample, yielded
134 stable recordings within the calibrated range between pH 6.5-8.2. In response to changing
135 oxygen partial pressure (PO_2 ; 0-21 kPa), these recordings were sufficiently precise to resolve
136 a sigmoidal shift by 0.03 pH units, running reverse directional to the OEC (Figure 2). This
137 shift denotes oxygenation dependent, lowered affinity of the pigment for protons (Haldane
138 effect) and agrees well with other studies (e.g. *Carcinus maenas* Δ pH = 0.02 (Truchot, 1976).
139 A sigmoidal rather than linear change of pH (Lapennas et al., 1981) may corresponds to the
140 linked sigmoidal trajectory of oxygen binding. The continuous recording of pH further
141 revealed pronounced but reversible decreases of \sim 0.1 pH units upon initial oxygenation,
142 which indicates a high affinity state for protons in fully deoxygenated haemocyanins (Figure
143 5).

144 P_{50} derived from OECs recorded along pH gradients matched those recorded along PO_2
145 gradients and together showed a linear interdependence of pH and P_{50} in the pH range
146 between 7.1 – 7.7 (Figure 4). The OEC recorded by a continuous decrease of pH was highly
147 resolved (\sim 500 data points, Figure 6) and fully equilibrated at slow PCO_2 changes of 0.015
148 kPa min^{-1} , as confirmed by the close match with the stepwise OEC (Figure 3). This further
149 underlines the validity of this alternative methodology, designed for pH sensitive pigments.
150 Simultaneous pH recordings here allow to construct highly resolved, continuous OECs from
151 which blood parameters may be directly derived without curve modelling (Figure 3). At faster
152 rates (0.045 kPa min^{-1}) the OEC shifted left as saturation required longer to stabilise than pH
153 (Figure 3). A shift of continuous OEC at higher rates of PCO_2 or PO_2 changes has been
154 explained by diffusion related, dislike durations between sensor and sample to fully
155 equilibrate with the surrounding gas ('dynamic error', Lapennas et al., 1981). However, as the
156 pH optode was immersed in the sample, gas diffusion rates between sample and sensor were
157 likely similar, suggesting some delay in oxygenation of octopus haemocyanin in response to
158 pH changes.

159 The signal of the pH optode drifted by -0.016 pH units per 100 recordings (\pm 0.004) and was
160 corrected accordingly (Figure 5A). This drift was higher than stated by the manufacturer (-
161 0.0035 pH units per 100 recordings, Manufacturer manual, Presens, 2004, 2012), probably
162 due to incomplete protection from the light beam of the UV-VIS light source and resulting
163 photobleaching of the optode's fluorescent dyes. Thus, pH optodes may be re-calibrated prior

164 to each measurement and the pH signal corrected for instrumental signal drift. Light exposure
165 and therefore pH signal drift can be reduced by a software-controlled shutter in the light path
166 that opens only during measurements or by using optically isolated sensor tips. The pH signal
167 was also corrected for autofluorescence emitted by the sample, which decreased the pH signal
168 in haemolymph by 0.06 units. Optical isolation but also calibration in the analysed medium
169 can reduce or prevent the effects by intrinsic fluorescence between 530 – 660 nm, caused by
170 e.g. porphyrine structures, which affect phase and amplitude of the pH raw signal (PreSens,
171 personal communication, Sept 10, 2013).

172 **Advantages and disadvantages**

173 The modified diffusion chamber benefits the analysis of oxygen binding in several ways.
174 Simultaneous recording of pigment oxygenation and pH allows to characterise oxygen
175 binding and intrinsic pH responses under close to *in vivo* conditions. Minute sample volumes
176 facilitate the analysis of blood from small organisms or less invasive and repeated sampling
177 from the same individual. Small sample volumes reduce the need to pool blood, facilitate
178 replicate measurements and shorten measurement time due to accelerated gas equilibration.
179 Highly resolved broad-range spectra capture detailed spectral properties of the pigment and
180 promote the analysis of diverse blood types. Thin optical microsensors (for pH or PO_2)
181 deliver stable and rapid recordings down to 0°C (own observation) and allow to record
182 continuous and highly resolved OECs (Figure 3). The gain of details improves the accuracy of
183 biophysical oxygen binding models, particularly if OECs do not follow a simple sigmoidal
184 shape (Wells and Weber, 1989). The additional flexibility to operate at a large range of
185 experimental temperatures and gas compositions makes this device not only highly suitable
186 for standard applications but particularly for the functional analysis of blood from non-model
187 organism.

188 The use of optical microsensors and thin layers of blood also require specific care. The
189 fluorescent dyes of the pH optodes are prone to photobleaching, which can be overcome by
190 optical isolation or determination and correction for signal drift. Effects by intrinsic
191 fluorescence of the sample are resolved by optical isolation, calibration in the same type of
192 sample or by an initial cross validation of pH with a pH electrode. The non-linear dynamic
193 range of pH optodes restricts accurate recordings to pH values between 5.5-8.5, which
194 however, suffices in most of the blood-physiological experiments. Optical pH sensors that
195 cover the extreme and even the full pH range may remove this limitation soon (Safavi and
196 Bagheri, 2003). Further, the 150 μm sensor tip breaks easily and requires careful handling.

197 Like pH electrodes, pH optodes require calibration with buffers of similar ionic strength as
198 the sample analysed and at the same experimental temperature (Manufacturer manual,
199 Presens, 2004). Some blood types may clog on the sensor tip, which can be avoided by
200 bathing the pH optode in a heparin solution (1000 units ml⁻¹). Lastly, thin layers of blood are
201 at higher risk to desiccate (Reeves, 1980), particularly at higher temperatures. Measures
202 include decreased gas flow or covering of the blood sample with a gas permeable Teflon
203 membrane (Reeves, 1980; Lapennas and Lutz, 1982; Clark et al., 2008).

204 **CONCLUSION**

205 In this study we showed that a diffusion chamber upgraded with a broad range
206 spectrophotometer and a fibre optical pH sensor allows for parallel measurement of pH and
207 pigment saturation in microvolumes of blood samples. The set up yields reproducible and
208 accurate results and offers high flexibility regarding the type of samples and experimental
209 settings. The availability of optical *PO*₂ or *PCO*₂ probes and the rapid development of other
210 optical sensor types (e.g. nitrogen oxide) suggest a broad array of future implementations that
211 will help to address novel biological questions.

212 MATERIAL AND METHODS

213 Experimental set up and modifications

214 A gas diffusion chamber (Eschweiler Co., Kiel, Germany) designed and described in detail
215 by (Niesel and Thews, 1961; Sick and Gersonde, 1969, 1972) has been used to determine
216 OECs by recording absorbance of a thin layer of a haemoglobin or haemocyanin bearing
217 solution during continuous or stepwise changes of PO_2 (Wells and Weber, 1989). The original
218 principle, characterised by full pigment deoxygenation with nitrogen gas followed by the time
219 dependent diffusion of oxygenated gas into the chamber (Niesel and Thews, 1961; Sick and
220 Gersonde, 1969), was essentially abolished in subsequent studies that continuously perfused
221 the chamber with defined gas mixtures (e.g. Bridges et al., 1984; Morris and Oliver, 1999;
222 Weber et al., 2010). We adopted this amendment and further modified the diffusion chamber
223 as follows. (1) A broad range (200 to 1100 nm) fibre optic spectrophotometer (USB2000+,
224 Ocean Optics, USA) was connected via two fibre optic cables fitted to the central cylinder of
225 the diffusion chamber to direct the light beam via collimating lenses from the deuterium
226 halogen light source (DT-Mini-2-GS, Ocean Optics, USA) through the sample glass plate
227 back to the 2048-element CCD-array detector of the spectrophotometer (Figure 7,
228 Supplementary Table S2). (2) The plastic slide that holds the sample glass plate in the light
229 tunnel was modified to fit a fibre optic micro-pH optode (NTH-HP5-L5-NS*25/0.8-OIW,
230 PreSens, Germany), housed in a syringe and connected to a phase detection device (μ PDD
231 3470, PreSens, Germany, Figure 7, Figure 8). The needle of the syringe was then inserted
232 through a silicone ring to prevent the leakage of gas (Figure 8). In contrast to pH electrodes,
233 pH optodes exhibit very small sensor tips ($<150\ \mu\text{m}$) and determine pH from the intensity
234 ratio between two pH sensitive fluorescent dyes (Presens, 2004).

235 The water reservoir of the diffusion chamber was filled with a 20% ethylene glycol solution
236 (anti-freeze agent, AppliChem, Germany) and the temperature monitored and controlled by
237 means of a supplied temperature sensor (PreSens, Germany) and a connected circulating
238 thermostatted water bath (LAUDA Ecoline Staredition RE 104, Germany). A gas mixing
239 pump (Wösthoff, Germany) supplied an adjustable mixture of nitrogen, oxygen and carbon
240 dioxide gas, humidified by an integrated scrubber to prevent desiccation of the sample (Figure
241 8).

242 Prior to each experiment, we performed a six point calibration (pH 6.7, 7.0, 7.2, 7.4, 7.7, 8.1)
243 of the pH optode, in MOPS-buffered ($40\ \text{mmol L}^{-1}$, 3-(N-Morpholino)-propanesulfonic acid),

244 filtered artificial sea water (35 psu) at the corresponding experimental temperature. pH of
245 buffers was checked with a pH glass electrode (InLab Routine Pt1100, Mettler Toledo,
246 Germany) and a pH meter (pH 330i, WTW, Germany), calibrated with low ionic strength pH
247 standards (AppliChem, Germany, DIN19266) and corrected to the Free Scale pH with Tris-
248 buffered seawater standard (Dickson, CO₂ QCLab, batch 4 2010, USA, Riebesell et al., 2010)
249 equilibrated at the same temperature. Aliquots of 18 µl of haemolymph (octopus/amphipod)
250 were thawed on ice, shortly spun down to collect content (5 sec at 1000 g) and 0.35 µl of 0.2
251 mmol L⁻¹ NaOH (4.6 µmol L⁻¹ final concentration) added to raise haemolymph pH above 8.0
252 to ensure full pigment oxygenation. To avoid haemolysis and the formation of
253 methaemoglobin by freezing, we used freshly sampled whole blood from *Pachycara*
254 *brachycephalum* and diluted the sample with one volume of blood plasma to improve light
255 transmission during the measurement. The pH of haemolymph/whole blood was not stabilized
256 with extrinsic buffers such as Tris or HEPES as they disturb the effects by ligands and
257 temperature on pigment oxygenation (Brix et al., 1994). We then spread out 15 µl of
258 haemolymph/whole blood on the glass plate without contacting the sealing ring. The pH
259 optode needle was inserted through the sealing ring and the sensor tip moved into the edge of
260 the droplet to reduce bleaching of the dye by the light beam passing through the centre of the
261 glass plate (Figure 8). Both the glass plate holder and the fitted pH optode were then inserted
262 and fixed in the diffusion chamber (Figure 7). The spectrophotometer required the recording
263 of light and dark spectra without blood sample before each measurement and was set to 15
264 milliseconds integration time, 100 scans to average and 30 seconds measurement intervals.
265 pH drift of the pH optode was evaluated by measuring the pH difference of a MOPS buffered
266 sea water pH standard (pH = 7.2) at 15°C before and after each experiment. Effects by
267 intrinsic fluorescence were assessed by comparing pH recordings of the pH optode and the pH
268 electrode in octopus haemolymph at 15°C.

269 Using *Octopus vulgaris* haemolymph we tested the setup via two previously employed
270 methodologies. OECs were obtained 1) by stepwise changes of discrete PO_2 (1, 2, 4, 9, 13,
271 17, 21 kPa) at constant PCO_2 (e.g. Wells and Weber, 1989) or 2) by stepwise as well as
272 continuous decreases of pH by means of increasing CO_2 concentrations (0–20 kPa) and
273 constant PO_2 (e.g. Pörtner, 1990). While stepwise measurements allow the sample to fully
274 equilibrate at several successive but discrete PO_2 or pH steps (Lapennas et al., 1981; Pörtner,
275 1990), continuous measurements characterize a constant change and monitoring of PO_2 or pH
276 (Wells and Weber, 1989). Each experiment involved the calibration with pure nitrogen as
277 well as pure oxygen at the beginning and at the end to determine the drift of the maximum

278 absorbance signal. The exemplary analysis of whole blood of *Pachycara brachycephalum*
279 was performed from 21-0 kPa PO_2 and pH 8.2-7.1 at 0°C and of thawed haemolymph of
280 *Eulimnogammarus verrucosus* at a constant PO_2 of 21 kPa and pH 7.7-6.9 at 6°C.

281 **Animals**

282 One major incentive to advance this method was to enhance the investigation of non-model
283 organisms, which often suffer from instrumental restrictions (e.g. sample volume, wavelength
284 filters, temperature setting) by devices optimised for human or rodent blood. We thus chose
285 three non-model organisms with diverse experimental demands to test the flexibility and
286 accuracy of the modified diffusion chamber. The cephalopod *Octopus vulgaris* (Lamarck,
287 1798) lives between 11 and 18°C and evolved a closed circulatory system containing blue
288 haemolymph with the extracellular, pH-sensitive respiratory pigment haemocyanin at high
289 concentrations ($54.3 \pm 6.9 \text{ g L}^{-1}$, Wells and Smith, 1987; Brix et al., 1989), which evolved
290 independently from arthropod haemocyanin (van Holde et al., 2001). Published data on
291 *Octopus vulgaris* blood physiology allowed us to test the accuracy of the modified diffusion
292 chamber. The Antarctic eelpout *Pachycara brachycephalum* (Pappenheim 1912) lives at
293 freezing temperatures, yields only little blood and circulates intracellular haemoglobin at - for
294 teleosts - low concentrations (37.4 g L^{-1}) in a closed system. The Baikal amphipod
295 *Eulimnogammarus verrucosus* (Gerstfeld, 1858) lives at 5-6°C and yields small amounts of
296 green coloured haemolymph that transports oxygen via extracellular haemocyanin (45.3 ± 7.9
297 g L^{-1}) in an open vascular system (Wirkner and Richter, 2013).

298 *Octopus vulgaris* specimens were hand-caught by snorkelling at Banuyls sur Mer, France at
299 16°C. Animals were anaesthetised in 3% EtOH (Ikeda et al., 2009), and after withdrawing
300 haemolymph from the cephalic vein, were finally killed by a cut through the brain following
301 sampling. *Pachycara brachycephalum* was caught on Polarstern cruise ANTXXV/4 near
302 Maxwell Bay at King George Island, Antarctica in May 2009 using fish traps, transported to
303 the Alfred Wegener Institute, Bremerhaven, Germany and kept in aerated tanks connected to a
304 re-circulating aquaculture system at 0°C. The animal was anaesthetised with 0.3 g L^{-1} tricaine
305 methano-sulphonate (MS222), blood withdrawn using a heparinised syringe and finally killed
306 by a spinal cut (Animal research permit no. 522-27-11/02-00(93), Freie Hansestadt Bremen,
307 Germany). *Eulimnogammarus verrucosus* (Gerstfeld, 1858) was collected in Bolshie Koty,
308 Lake Baikal, Russia during summer 2012, transported to Irkutsk, Russia and kept in aerated
309 2.5 l tanks at 6°C. Haemolymph was withdrawn using a dorsally inserted capillary. All

310 haemolymph samples were centrifuged at 15.000 *g* for 15 min at 0°C to remove cell debris
311 and stored at -20°C.

312 **Data analysis**

313 The processing, time-matching and analysis of data from both the spectrophotometer and the
314 pH meter were performed using the “R” statistical language (R Core Team, 2013) (R script
315 see supplementary material S3).

316 An integrated five parameter logistic model (Equation 1, R “drc” add-on package (Ritz and
317 Streibig, 2005) was applied to fit sigmoidal curves to stepwise OECs.

$$318 \quad f(x, (b, c, d, e, f)) = c + \frac{(d-c)}{\left(1 + \exp\left\{b\left(\log\left(\frac{x}{e}\right)\right)\right\}\right)^f} \quad (1)$$

319 The parameters *c* and *d* denote the upper and lower asymptotes and *f* the asymmetry of the
320 curve. The parameters *b* and *e* correspond to the slope and inflection point of a four parameter
321 logistic model if the parameter *f* equals 1. Note that this equation represents an empirical
322 curve fit that does not describe the functional properties of the haemocyanin sub-units
323 according to mechanistic insight.

324 Oxygen affinity (P_{50}) was interpolated from fitted OECs at half saturation and cooperativity
325 (Hill’s coefficient, n_{50}) determined via Hill plots by regressing $\log_{10}(Y/(1-Y))$ versus $\log_{10}PO_2$
326 in the linear mid-range (~20-80% saturation), with *Y* denoting the fractional saturation. The
327 Bohr coefficient was calculated from the regression slope ($\Delta\log_{10}P_{50}$ versus ΔpH) between pH
328 7.1-7.7. In pH/saturation diagrams, P_{50} denotes the \log_{10} of the oxygen isobar and the pH of
329 the isobar at half saturation (pH_{50} , Pörtner, 1990). Data were expressed as means \pm 95%
330 confidence intervals if not stated otherwise.

331 **LIST OF SYMBOLS AND ABBREVIATIONS**

OEC	Oxygen equilibrium curve
PO_2	Oxygen partial pressure
PCO_2	Carbon dioxide partial pressure
P_{50}	Oxygen affinity
n_{50}	Hill coefficient

332

333 **ACKNOWLEDGEMENTS**

334 We would like to thank Dr. Jan Strugnell (La Trobe University, Bundoora) for providing lab
335 space and her extensive support, Michael Imsic and all other staff and technicians of the La
336 Trobe University for their kind support during the initial tests of the modified diffusion
337 chamber; Erich Dunker, Matthias Littmann and Dirk Wethje (scientific workshop, Alfred
338 Wegener Institute, Bremerhaven) for the technical modifications and drawings of the
339 diffusion chamber, Lena Jakob, for providing haemolymph samples from *Eulimnogammarus*
340 *verrucosus*; Marian Y Hu for his help to catch *Octopus vulgaris*; the logistics department of
341 the Alfred Wegener Institute and last of all my wife and young son for their enriching
342 company. We further would like to thank the two anonymous reviewers for their many
343 helpful remarks and suggestions on an earlier version of this manuscript. We dedicate this
344 article to the memory of Steve Morris and want to express our special thanks to Maria Morris
345 and Elizabeth Morgan (University of Bristol) who saved and donated the diffusion chamber to
346 our department.

347 **FUNDING**

348 This study was supported by a Journal of Experimental Biology travelling fellowship, a PhD
349 scholarship by the German Academic Exchange Service (DAAD, D/11/43882) to MO, the
350 Deutsche Forschungsgemeinschaft (MA4271/1-2 to FCM) and the Alfred Wegener Institute
351 for Polar and Marine Research.

352 **AUTHOR CONTRIBUTIONS**

353 MO and FCM developed the technical modifications, compiled the manuscript and interpreted
354 results. MO performed the experiments and analysis. HOP initiated the discussion and
355 contributed to data interpretation and manuscript editing.

356 **FIGURE LEGENDS**

357 Figure 1: **(A)** Broad range spectra of haemolymph from *Octopus vulgaris* (15°C), measured at
358 4 kPa PO_2 from high to low pH (ca. 8.2-6.8). Exemplary, broad range spectral recordings of
359 whole blood from **(B)** the Antarctic eelpout *Pachycara brachycephalum* (0°C) and **(C)** of
360 haemolymph from the Lake Baikal amphipod *Eulimnogammarus verrucosus* (6°C) reveal
361 complex absorbance features. Spectral zones responding to oxygenation were marked by
362 boxes and zoomed in. Spectra were coloured according to the visual appearance of the
363 respective haemolymph/blood type. (Animal photos with permission by: Vladimír Motyčka,
364 Christoph Held and Lena Jakob).

365 Figure 2: Replicated ($n = 5$) oxygen equilibrium curves of haemolymph from one *Octopus*
366 *vulgaris* specimen obtained by stepwise changes to discrete PO_2 at constant PCO_2 (1 kPa).
367 Five parameter logistic curves were fitted and individually plotted for each measurement to
368 illustrate instrumental variability. Haemolymph pH (means \pm S.E.M., blue), recorded by an
369 optical pH microsensor immersed in the same sample droplet, changed sigmoidal and reverse
370 directional to pigment saturation (black). The pH at half saturation (mean 7.23, \pm S.E.M.
371 0.018) can be derived from the intersection between P_{50} (dashed line) and the fitted pH line.

372 Figure 3: Stepwise oxygen equilibrium curves (OEC) along a pH gradient (Pörtner, 1990),
373 each derived from 15 μ l haemolymph of *Octopus vulgaris* and measured by means of the
374 modified diffusion chamber at 15°C and decreasing pH and various constant PO_2 (1, 4, 13, 21
375 kPa). The continuous OEC recorded at slow rates of PCO_2 changes (0.015 kPa min^{-1} , blue
376 points) was highly resolved and closely matched the stepwise curves while at faster rates the
377 OEC shifted left (dashed grey curve).

378 Figure 4: Bohr plot illustrating the pH dependence of oxygen affinity (P_{50}) of *Octopus*
379 *vulgaris* haemolymph measured at 15°C. P_{50} from experiments with stepwise changes of PO_2
380 (filled circles) and pH (open circles) agreed with literature data (Brix *et al.* 1989, blue triangle
381 and Bridges 1994, green square). The data point at 13 kPa was excluded from the linear
382 regression fit as P_{50} leveled off at low pH (\sim 7.0).

383 Figure 5: Response of **(A)** pH (uncorrected – grey trace, corrected by instrumental pH drift –
384 blue trace) and **(B)** absorbance of the oxygenation dependent peak (347 nm) and the protein
385 peak of *Octopus vulgaris* haemolymph to stepwise changes of PO_2 recorded at 15°C.
386 Numbers above the absorbance trace indicate PO_2 of each oxygenation step. Horizontal

387 dashed lines indicate the change of maximal and minimum absorbance (at 347 nm) over time
388 and vertical dashed lines the sudden but reversible pH changes upon initial oxygenation.

389 Figure 6: Number of data points (means \pm 95% C.I.) for single oxygen equilibrium curves
390 (OEC) compared between different methods (for references see supplementary table S7). The
391 modified diffusion chamber method refers to continuous OEC measurements along a pH
392 gradient.

393 Figure 7: (A) 3D model of the modified diffusion chamber and (B) a detailed cross section
394 through the central cylinder of the gas diffusion chamber illustrating the embedded fibre optic
395 micro-pH optode and an upper and lower custom made tube containing collimating lenses and
396 fittings for in- and outgoing fibre optic cables of the light source and the broad range
397 spectrophotometer. Illustrations were drawn with 3D CAD software (SolidWorks, version
398 12.0, files in supplementary material S6).

399 Figure 8: Detailed illustration of the pH optode housed in a syringe, mounted with a screw
400 and fitted to a plastic holder, which is moved into the gas tight room centred in the diffusion
401 chamber. The bended syringe needle is inserted through a silicon ring, which prevents gas
402 leakage, and the sensor tip moved into the edge of the sample droplet located on a silica glass
403 plate.

404 Table 1: Comparison of blood physiological parameters of *Octopus vulgaris* haemolymph
 405 with literature data. Bold values denote parameters recorded under the same conditions.

406

407 Table 1:

P_{50} (kPa)	n_{50}	Bohr coefficient	pH	Temp (°C)	Source
0.72	1.56		7.68		
1.72	1.59		7.45		
4.72 (0.07)	1.75 (0.07)	-1.79 (pH 7.1-7.7)	7.23 (0.046)	15	This study
7.94	1.61		7.08		
2.20 [†]	-		7.4		
2.45	1.5	-1.34 (pH 6.85-7.4)	7.4	15	
4.41	1.6	-1.10 (pH 6.85-7.4)	7.4	25	Brix <i>et al.</i> 1989
3.20	2.6*		7.588		
4.09	2.9*	-1.58 / -1.73	7.520		
4.80	3.5*	(pH 7.2-7.6 / 7.3-7.7)	7.415	22	Houlihan <i>et al.</i> 1982
6.76	2.9*		7.327		
1.91	-	-1.86	7.4	10	
2.61	-	-1.83	7.4	15	Bridges 1994

408 *Hill coefficients were recalculated from Houlihan *et al.* 1982

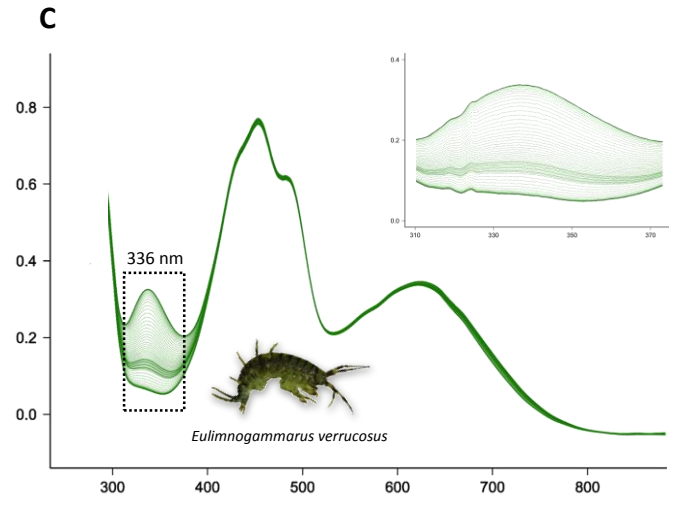
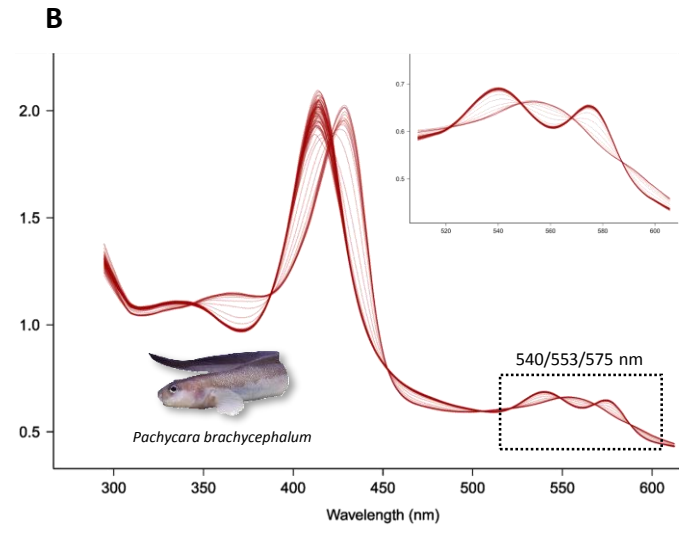
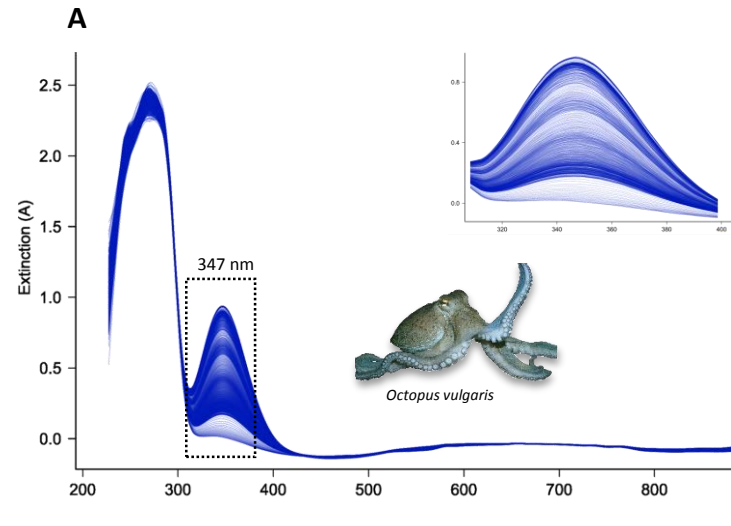
409 [†] P_{50} extrapolated from linear regression line of Bohr plot at pH 7.4 (Figure 4)

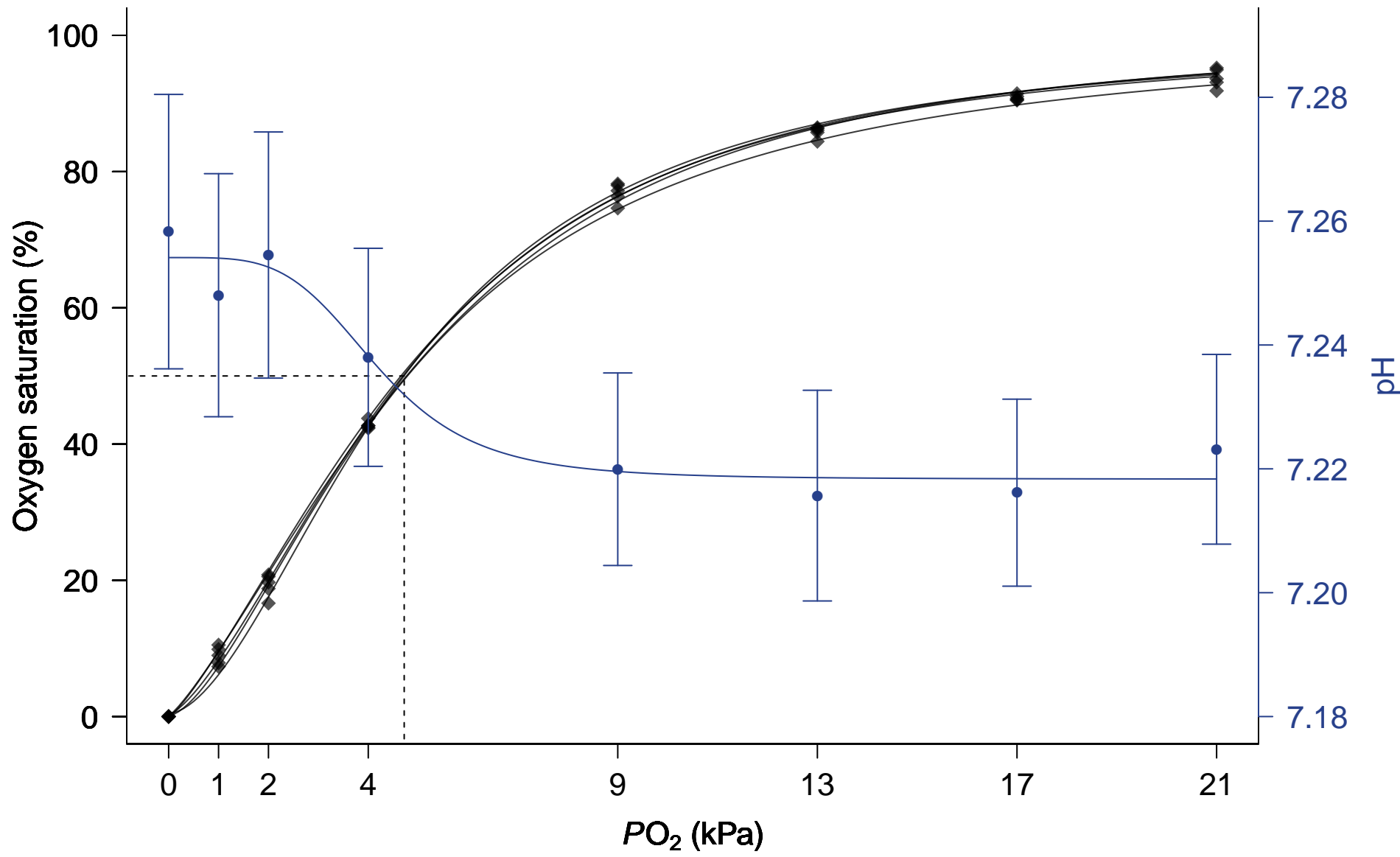
410 REFERENCES

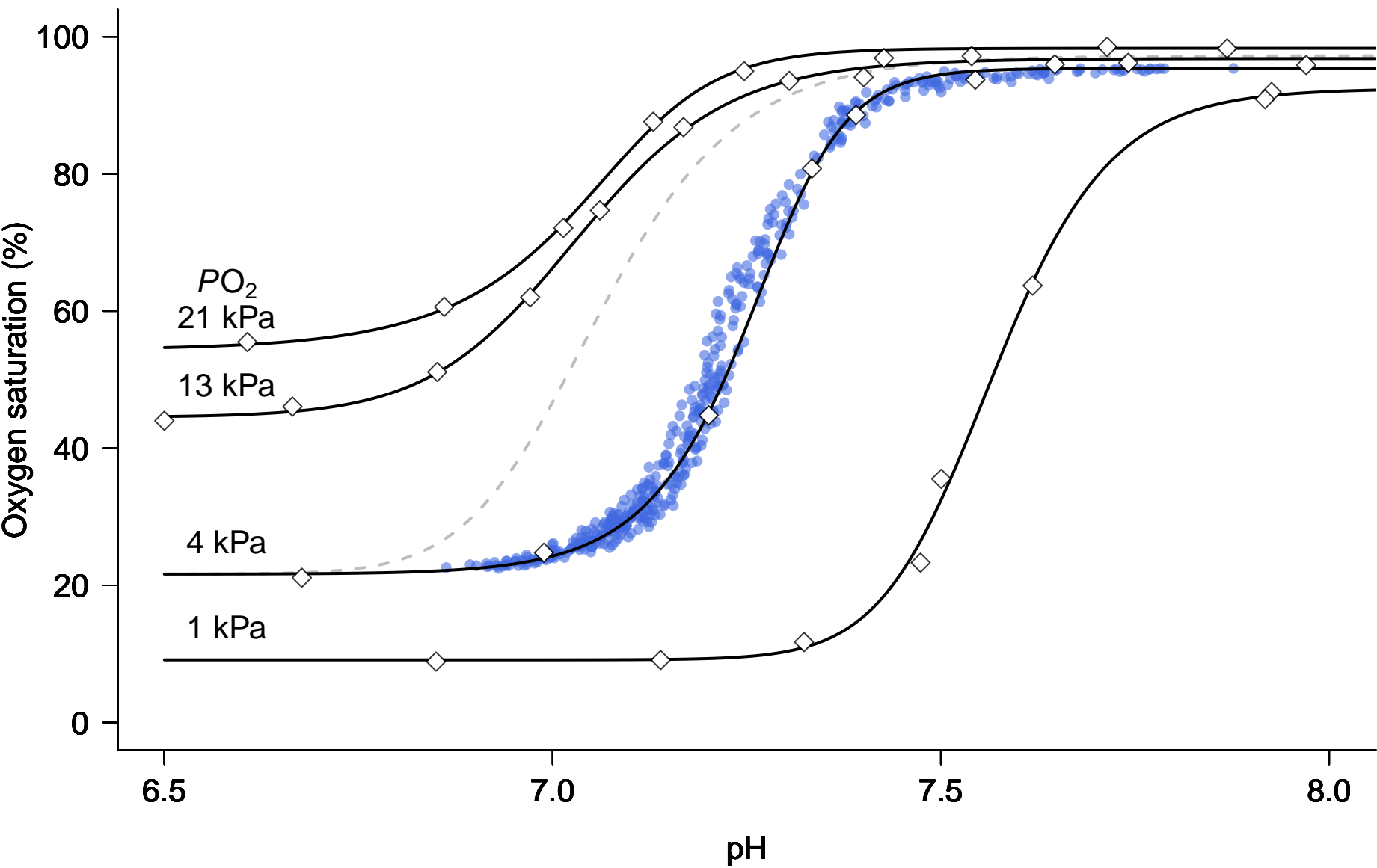
- 411 **Alexander, J. B. and Ingram, G. A.** (1980). A comparison of five of the methods commonly
412 used to measure protein concentrations in fish sera. *J. Fish Biol.* **16**, 115-122.
- 413 **Bert, P.** (1878). La pression barométrique: recherches de physiologie expérimentale. Paris: G.
414 Masson.
- 415 **Bohr, C., Hasselbalch, K. and Krogh, A.** (1904). Über einen in biologischer Beziehung
416 wichtigen Einfluss, den die Kohlensäurespannung des Blutes auf dessen
417 Sauerstoffbindung übt. *Skand. Arch. Physiol.* **16**, 402-412.
- 418 **Bolton, J. C., Collins, S., Smith, R., Perkins, B., Bushway, R., Bayer, R. and Vetelino, J.**
419 (2009). Spectroscopic analysis of hemolymph from the American lobster (*Homarus*
420 *americanus*). *J. Shellfish Res.* **28**, 905-912.
- 421 **Bridges, C., Morris, S. and Grieshaber, M.** (1984). Modulation of haemocyanin oxygen
422 affinity in the intertidal prawn *Palaemon elegans* (Rathke). *Resp. Physiol.* **57**, 189-
423 200.
- 424 **Bridges, C. R.** (1994). Bohr and root effects in cephalopod haemocyanins □ paradox or
425 pressure in *Sepia officinalis*? *Mar. Freshw. Behav. Physiol.* **25**, 121-130.
- 426 **Brix, O.** (1983). Giant squids may die when exposed to warm water currents. *Nature* **303**,
427 422-423.
- 428 **Brix, O., Colosimo, A. and Giardina, B.** (1994). Temperature dependence of oxygen
429 binding to cephalopod haemocyanins: ecological implications. *Mar. Freshw. Behav.*
430 *Physiol.* **25**, 149-162.
- 431 **Brix, O., Bårdgard, A., Cau, A., Colosimo, A., Condo, S. and Giardina, B.** (1989).
432 Oxygen-binding properties of cephalopod blood with special reference to
433 environmental temperatures and ecological distribution. *J. Exp. Zool.* **252**, 34-42.
- 434 **Chanutin, A. and Curnish, R. R.** (1967). Effect of organic and inorganic phosphates on the
435 oxygen equilibrium of human erythrocytes. *Arch. Biochem. Biophys.* **121**, 96-102.
- 436 **Clark, T. D., Seymour, R. S., Wells, R. M. G. and Frappell, P. B.** (2008). Thermal effects
437 on the blood respiratory properties of southern bluefin tuna, *Thunnus maccoyii*. *Comp.*
438 *Biochem. Physiol. Part A Mol. Integr. Physiol.* **150**, 239-246.
- 439 **Festa, R. S. and Asakura, T.** (1979). Oxygen dissociation curves in children with anemia
440 and malignant disease. *Am. J. Hematol.* **7**, 233-244.
- 441 **Herbert, Neill A., Skov, Peter V., Wells, Rufus M. G. and Steffensen, John F.** (2006).
442 Whole blood–oxygen binding properties of four cold □ temperate marine fishes: Blood
443 affinity is independent of pH-dependent binding, routine swimming performance, and
444 environmental hypoxia. *Physiol. Biochem. Zool.* **79**, 909-918.
- 445 **Houlihan, D., Innes, A., Wells, M. and Wells, J.** (1982). Oxygen consumption and blood
446 gases of *Octopus vulgaris* in hypoxic conditions. *J. Comp. Physiol. B Biochem. Syst.*
447 *Environ. Physiol.* **148**, 35-40.
- 448 **Hüfner, G.** (1890). Über das Gesetz der Dissociation des Oxyhaemoglobins und über einige
449 daran sich knüpfende wichtige Fragen aus der Biologie. *Arch. Anat. Physiol.* **1**, 1-27.
- 450 **Ikeda, Y., Sugimoto, C., Yonamine, H. and Oshima, Y.** (2009). Method of ethanol
451 anaesthesia and individual marking for oval squid (*Sepioteuthis lessoniana* Férussac,
452 1831 in Lesson 1830–1831). *Aquac. Res.* **41**, 157-160.
- 453 **Lapennas, G. N. and Lutz, P. L.** (1982). Oxygen affinity of sea turtle blood. *Resp. Physiol.*
454 **48**, 59-74.
- 455 **Lapennas, G. N., Colacino, J. M. and Bonaventura, J.** (1981). Thin-layer methods for
456 determination of oxygen binding curves of hemoglobin solutions and blood. In
457 *Methods Enzymol.*, vol. Volume 76 (ed. L. R.-B. E. C. Eraldo Antonini), pp. 449-470:
458 Academic Press.

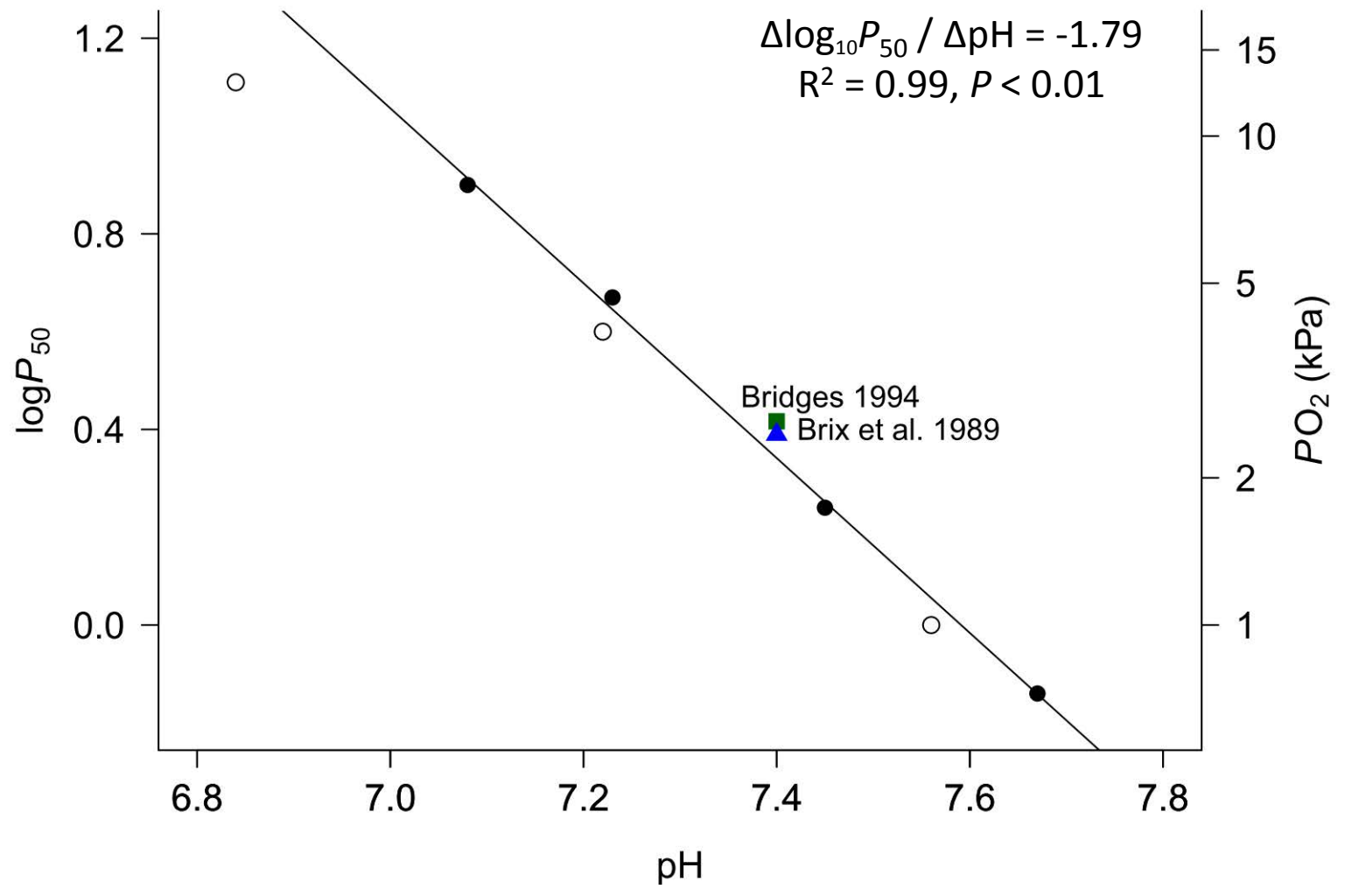
- 459 **Lykkeboe, G. and Johansen, K.** (1982). A Cephalopod Approach to Rethinking about the
460 Importance of the Bohr and Haldane Effects. *Pac. Sci.* **36**, 305-313.
- 461 **Mangum, C. P. and Lykkeboe, G.** (1979). The influence of inorganic ions and pH on
462 oxygenation properties of the blood in the gastropod mollusc *Busycon canaliculatum*.
463 *J. Exp. Zool.* **207**, 417-430.
- 464 **Meir, J. U., Champagne, C. D., Costa, D. P., Williams, C. L. and Ponganis, P. J.** (2009).
465 Extreme hypoxemic tolerance and blood oxygen depletion in diving elephant seals.
466 *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **297**, R927-R939.
- 467 **Miller, K. I.** (1985). Oxygen equilibria of *Octopus dofleini* hemocyanin. *Biochemistry* **24**,
468 4582-4586.
- 469 **Morris, S. and Oliver, S.** (1999). Respiratory gas transport, haemocyanin function and acid-
470 base balance in *Jasus edwardsii* during emersion and chilling: simulation studies of
471 commercial shipping methods. *Comp. Biochem. Physiol. A* **122**, 309-321.
- 472 **Morris, S., Taylor, A. C., Bridges, C. R. and Grieshaber, M. K.** (1985). Respiratory
473 properties of the haemolymph of the intertidal prawn *Palaemon elegans* (Rathke). *J.*
474 *Exp. Zool.* **233**, 175-186.
- 475 **Niesel, W. and Thews, G.** (1961). Ein neues Verfahren zur schnellen und genauen Aufnahme
476 der Sauerstoffbindungskurve des Blutes und konzentrierter Hämoproteidlösungen.
477 *Pflügers Archiv* **273**, 380-395.
- 478 **Olson, K. N., Hillyer, M. A., Kloss, J. S., Geiselhart, R. J. and Apple, F. S.** (2010).
479 Accident or arson: Is CO-Oximetry reliable for carboxyhemoglobin measurement
480 postmortem? *Clin. Chem.* **56**, 515-519.
- 481 **Pörtner, H. O.** (1990). An analysis of the effects of pH on oxygen binding by squid (*Illex*
482 *illecebrosus*, *Loligo pealei*) haemocyanin. *J. Exp. Biol.* **150**, 407.
- 483 **Presens.** (2004). Instruction manual pH-4 mini, pp. 51. Regensburg, Germany: Precision
484 Sensing GmbH
- 485 **Presens.** (2012). Product sheet for pH microsensors, pp. 4. Regensburg, Germany: Precision
486 Sensing GmbH
- 487 **Rasmussen, J. R., Wells, R. M. G., Henty, K., Clark, T. D. and Brittain, T.** (2009).
488 Characterization of the hemoglobins of the Australian lungfish *Neoceratodus forsteri*
489 (Kreff). *Comp. Biochem. Physiol., A: Comp. Physiol.* **152**, 162-167.
- 490 **Reeves, R. B.** (1980). A rapid micro method for obtaining oxygen equilibrium curves on
491 whole blood. *Resp. Physiol.* **42**, 299-315.
- 492 **Riebesell, U., Fabry, V. J., Hansson, L. and Gattuso, J.-P.** (2010). Guide to best practices
493 for ocean acidification research and data reporting: Publications Office of the
494 European Union Luxembourg.
- 495 **Ritz, C. and Streibig, J. C.** (2005). Bioassay analysis using R. *J. Stat. Softw.* **12**, 1-22.
- 496 **Safavi, A. and Bagheri, M.** (2003). Novel optical pH sensor for high and low pH values.
497 *Sensor Actuat. B-Chem.* **90**, 143-150.
- 498 **Scott, G. R.** (2011). Elevated performance: the unique physiology of birds that fly at high
499 altitudes. *J. Exp. Biol.* **214**, 2455-2462.
- 500 **Seibel, B., Chausson, F., Lallier, F., Zal, F. and Childress, J.** (1999). Vampire blood:
501 respiratory physiology of the vampire squid (Cephalopoda: Vampyromorpha) in
502 relation to the oxygen minimum layer. *Exp Biol Online* **4**, 1-10.
- 503 **Sick, H. and Gersonde, K.** (1969). Method of continuous registration of oxygen binding
504 curves of hemoproteins by means of a diffusion chamber. *Anal. Biochem.* **32**, 362-
505 376.
- 506 **Sick, H. and Gersonde, K.** (1972). Theory and application of the diffusion technique for
507 measurement and analysis of O₂-binding properties of very autoxidizable
508 hemoproteins. *Anal. Biochem.* **47**, 46-56.

- 509 **Team, R. C.** (2013). R: A Language and Environment for Statistical Computing. Vienna,
510 Austria: R Foundation for Statistical Computing.
- 511 **Truchot, J. P.** (1976). Carbon dioxide combining properties of the blood of the shore crab,
512 *Carcinus maenas* (L.): CO₂-dissociation curves and Haldane effect. *J. Comp. Physiol.*
513 **112**, 283-293.
- 514 **van Holde, K. E., Miller, K. I. and Decker, H.** (2001). Hemocyanins and Invertebrate
515 Evolution. *J. Biol. Chem.* **276**, 15563-15566.
- 516 **Weber, R. E., Behrens, J. W., Malte, H. and Fago, A.** (2008). Thermodynamics of
517 oxygenation-linked proton and lactate binding govern the temperature sensitivity of O-
518 2 binding in crustacean (*Carcinus maenas*) hemocyanin. *J. Exp. Biol.* **211**, 1057-1062.
- 519 **Weber, R. E., Campbell, K. L., Fago, A., Malte, H. and Jensen, F. B.** (2010). ATP-
520 induced temperature independence of hemoglobin-O₂ affinity in heterothermic
521 billfish. *J. Exp. Biol.* **213**, 1579-1585.
- 522 **Wells, M. and Smith, P.** (1987). The performance of the octopus circulatory system: a
523 triumph of engineering over design. *Cell. Mol. Life Sci.* **43**, 487-499.
- 524 **Wells, R. and Weber, R.** (1989). The measurement of oxygen affinity in blood and
525 haemoglobin solutions. *Techniques in comparative respiratory physiology: an*
526 *experimental approach.* Cambridge University Press, Cambridge, 279-303.
- 527 **Wirkner, C. S. and Richter, S.** (2013). Circulatory system and respiration. *The natural*
528 *history of the Crustacea* **1**, 376-412.
- 529 **Zielinski, S., Sartoris, F. J. and Pörtner, H. O.** (2001). Temperature effects on hemocyanin
530 oxygen binding in an Antarctic cephalopod. *Biol. Bull. (Woods Hole)* **200**, 67-76.

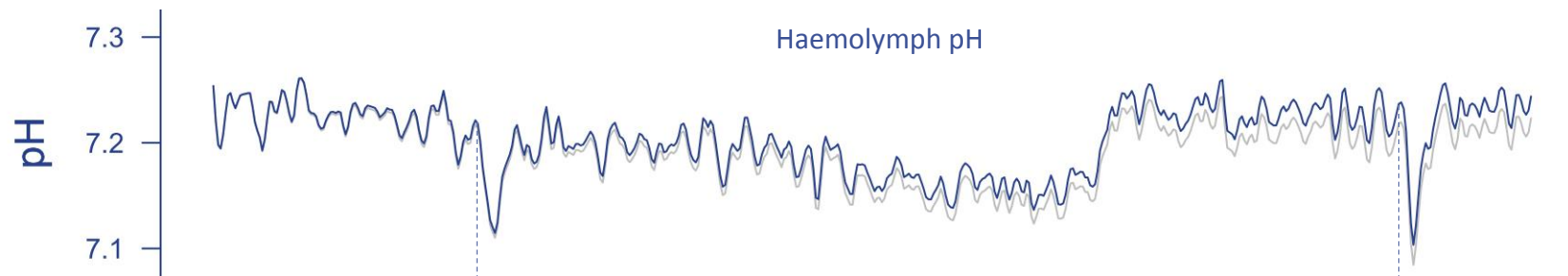




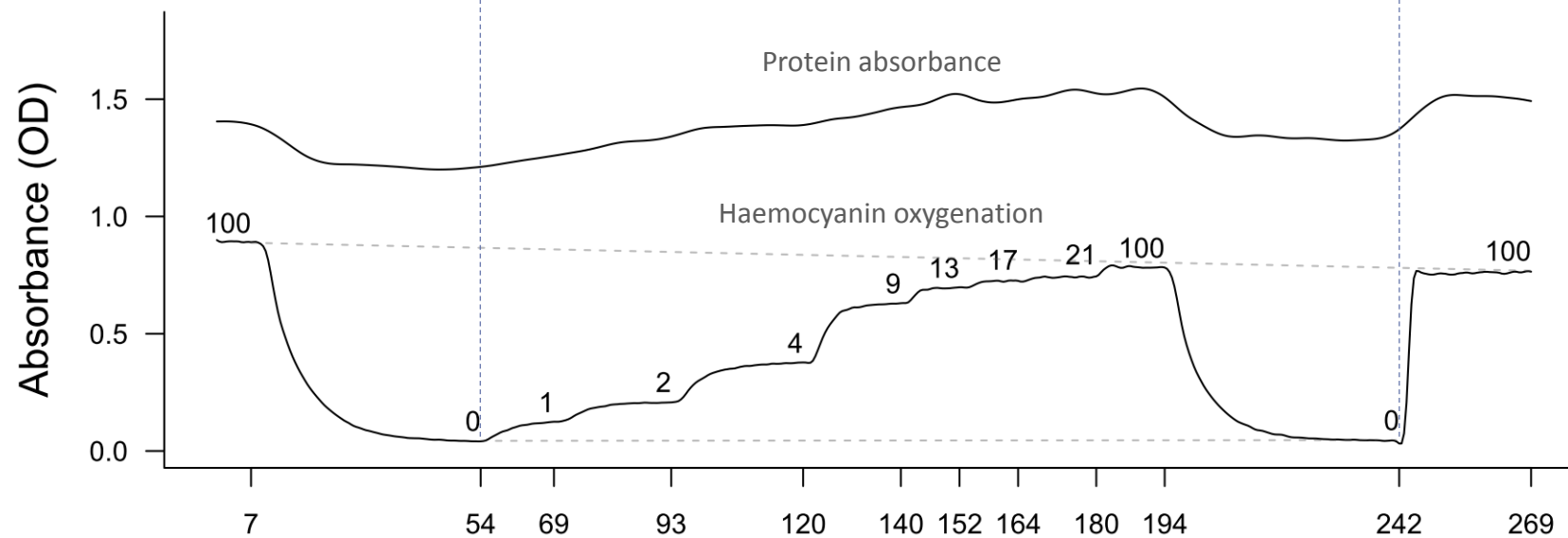


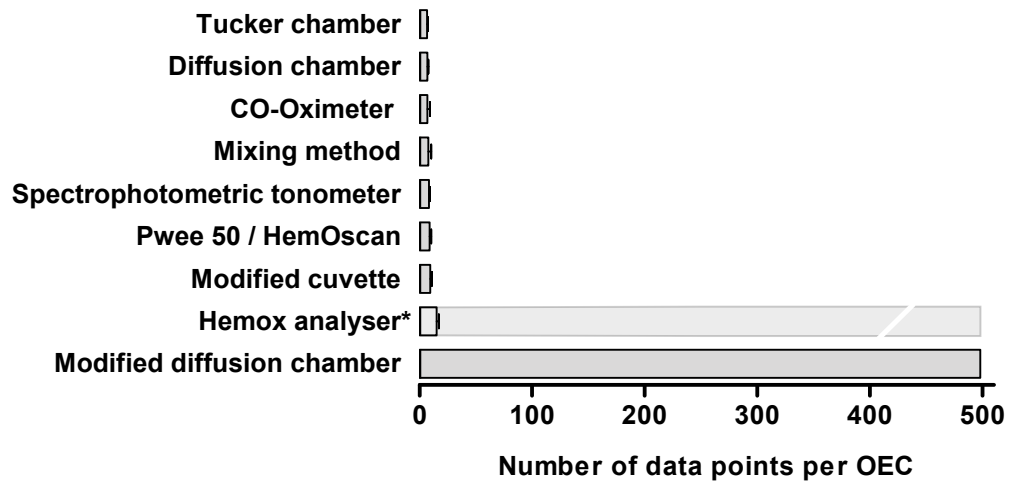


A



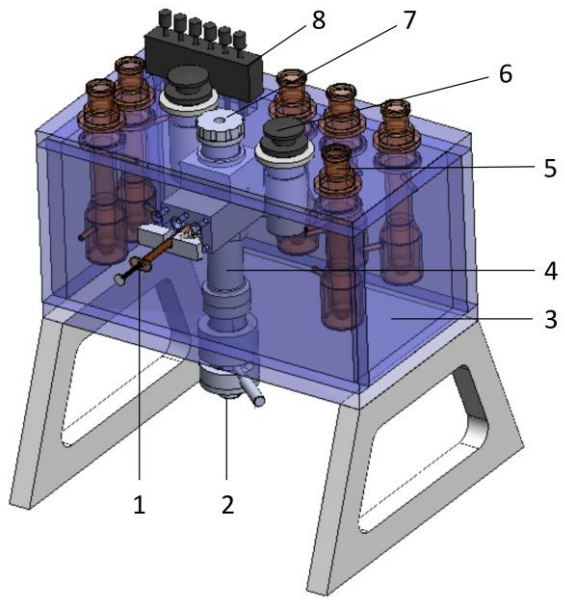
B



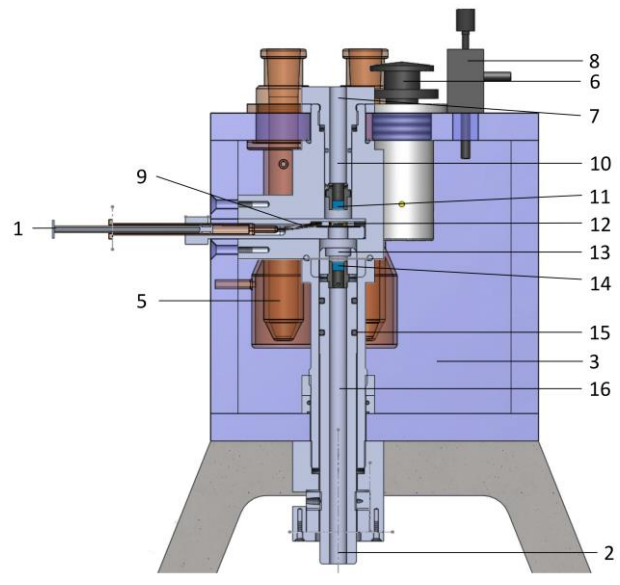


*While some studies record fewer data points, recording intervals may be maximised to 1/sec

A

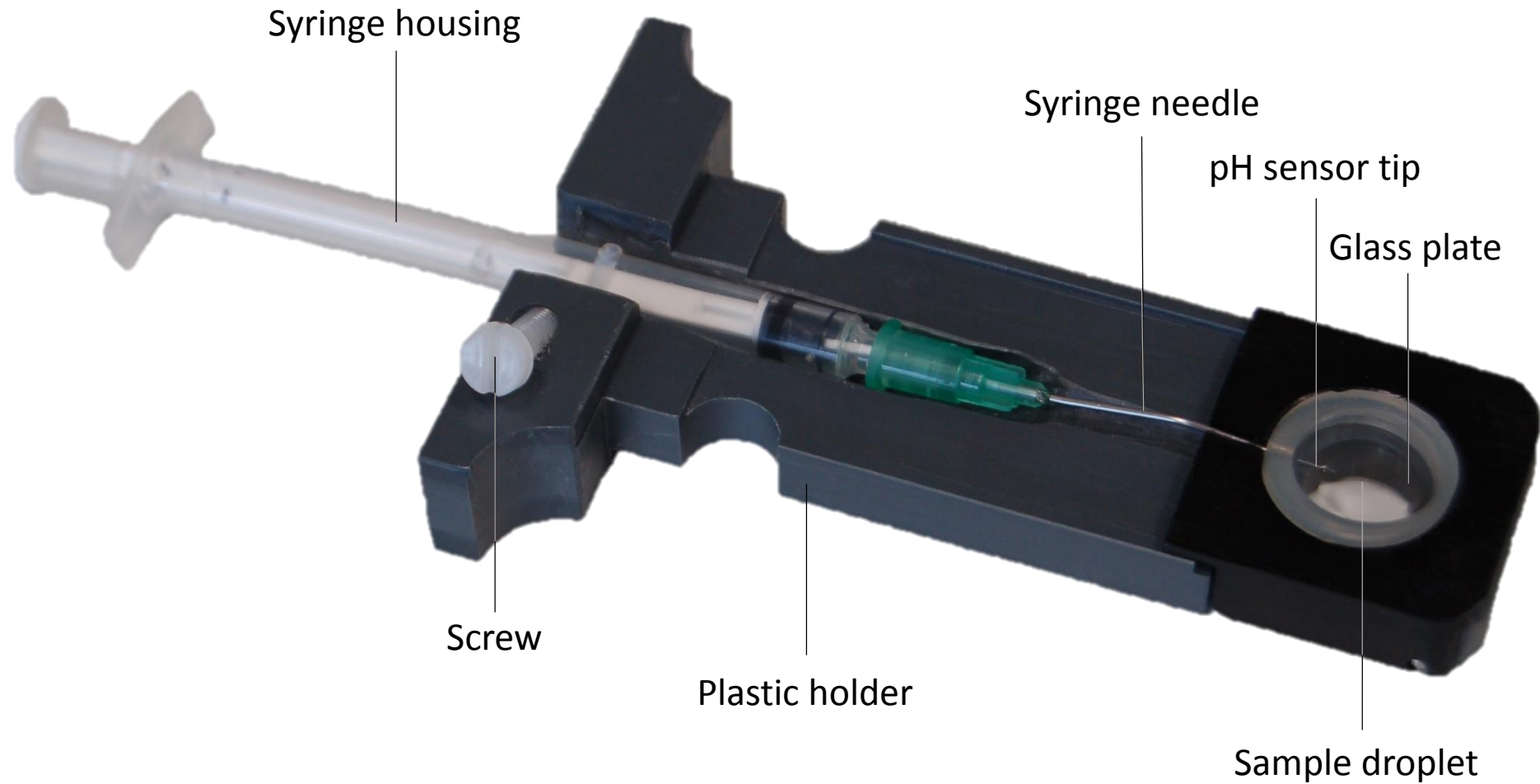


B



1. Syringe housing for the pH micro optode
2. Fibre optic cable exit to the spectrophotometer
3. Temperature controlled water reservoir
4. Central cylinder containing optical devices
5. Gas-washing flask
6. Control wheel for gas distribution
7. Fibre optic cable inlet from the light source
8. Control panel for gas inflow
9. Needle housing for the fibre optic sensor tip
10. Upper custom made tube, housing a collimating lens and the fibre optic cable from the light source

11. Upper collimating lens
12. Blood sample spread on a glass plate
13. Spacer to keep 10 mm minimal distance between collimating lens and sample
14. Lower collimating lens
15. Rubber to seal lower tubing
16. Lower custom made tube, housing a collimating lens and the fibre optic cable leading to the spectrophotometer



Syringe housing

Syringe needle

pH sensor tip

Glass plate

Screw

Plastic holder

Sample droplet