Supplementary materials 5.

Putative respiratory pigment in the haemolymph of *P. brevicornis*.

The absorbance peak detected at λ = 332 nm in oxygenated, but not deoxygenated, hemolymph from *Prostebbingia brevicornis in vitro* we interpret as indicating the presence of hemocyanin (Hc), a respiratory pigment that reversibly and cooperatively binds oxygen, and is used in oxygen transport in this species, for the following reasons.

(1) In all crustaceans examined to date oxygenated Hc is detected by an intense absorption peak at, or around, λ =340 nm [1,2,14]. This is close to the absorbance of the peak of λ = 332 nm detected for hemolymph from *P. brevicornis*.

(2) In all crustaceans examined to date, this oxygenated Hc peak disappears when haemolymph is deoxygenated [1,2] and the same happens in hemolymph from *P. brevicornis*.

(3) Haemocyanin that binds oxygen reversibly and cooperatively has been found in the haemolymph of most gammaridean amphipods in which it has been sought [3-10] including giant amphipods in Lake Baikal [11], (exceptions being *Paraceradocus miersi* and *Shraderia gracilis* in this present study – see below for implications of this finding). Non gammaridean groups such as the cyamid whale lice possess haemoglobin and not Hc [12] and the planktonic hyperiids possess neither [13]. This makes our interpretations in points 1 and 2 more likely for *P. brevicornis*.

(4) If we use the absorbance measures obtained at λ = 332 nm, and assume that they are associated with the presence of a haemocyanin subunit of 74 kD in mass [3] we can use the extinction coefficients for Hc produced by Nickerson and Van Holde [14] ($E^{1\%}_{cm}$ = 2.83, equating to $E^{mmol.l^{-1}}_{cm}$ = 17.26 at λ =340 nm) to calculate putative Hc concentration. For *P. brevicornis* the calculated putative Hc concentration ranges between 0.33 and 0.39 $mmol.l^{-1}$ at the upper end of the range reported for other amphipod species, 0.14 – 0.41 $mmol.l^{-1}$ [3]. This congruence strengthens the suggestion that there is Hc in the hemolymph of *P. brevicornis*.

Taken together we have presented a number of different lines of evidence that there is a Hc in the hemolymph of *P. brevicornis*. However, a number of puzzles, incongruities and alternative explanations also exist which need to be aired. They are as follows.

(1) We have not directly measured oxygen-binding by the pigment, or isolated, identified, and verified the hemocyanin molecule itself. Even if there is oxygen binding present we have no evidence that it is cooperative.
(2) While the congruence between the Hc values we might expect and what we estimate for *P. brevicornis* is good when using the extinction coefficient for the copper peak at 340 nm, the congruence is not so good when we measure the protein peak at 280 nm and assume that it is likely to be 60-95% Hc in line with other amphipods investigated [3]. In that case the protein concentration is always lower than the value calculated based on how much Hc there seems to be. In *P. brevicornis* brought back to the UK from the Antarctic the haemolymph protein levels can be very low even though the animals are well fed and have sufficient copper in their food (JIS, pers obs). Therefore we have been unable to substantiate the claim of Hc being present in the haemolymph by (i) measuring the oxygen content of the hemolymph or (ii) replicating the disappearance of any peak at λ= 332 nm when the haemolymph is equilibrated with nitrogen gas.

Aside from oxygen transport, Hcs are also known to participate in homeostatic and physiological processes: moulting, hormone transport, osmoregulation and protein storage and as precursors of antimicrobial and antiviral peptides.

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