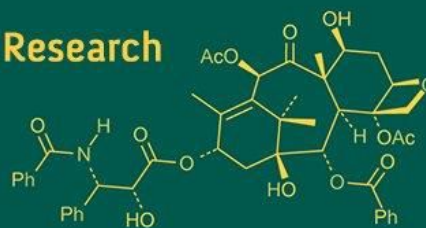


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Hemocyanin structure, oxygen binding properties, and respiratory pigment function in invertebrate oxygen transport systems

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Abstract

Hemocyanins are copper-containing respiratory pigments circulating in the hemolymph of arthropods and molluscs. This research examined hemocyanin oxygen binding characteristics and allosteric regulation in six invertebrate species: *Octopus vulgaris*, *Helix pomatia*, *Limulus polyphemus*, *Homarus americanus*, *Panulirus interruptus*, and *Eurypelma californicum*. Oxygen equilibrium curves were generated at 20 °C across pH 6.8-8.0. P₅₀ values ranged from 6.2 mmHg (*H. pomatia*) to 28.4 mmHg (*O. vulgaris*), with Hill coefficients between 2.1 and 4.1. All species displayed a pronounced Bohr effect ($\Delta \log P_{50}/\Delta \text{pH}$: -0.48 to -0.91). Molluscan hemocyanins showed higher cooperativity and larger Bohr shifts. Divalent cations and organic modulators altered oxygen affinity in species-specific patterns. These results confirm that hemocyanin function is tightly tuned to each species' ecological niche through differences in quaternary architecture and allosteric sensitivity.

Keywords: Hemocyanin, respiratory pigment, oxygen binding, cooperativity, Bohr effect, invertebrate physiology, copper protein, allosteric regulation, hemolymph, oxygen transport

Introduction

Long before hemoglobin became the textbook example of cooperative oxygen binding, naturalists in the 1870s noticed that the blood of certain crabs and snails turned blue on exposure to air ^[1]. The pigment responsible hemocyanin turned out to be one of the most ancient oxygen carriers in biology ^[2]. Unlike hemoglobin, hemocyanin binds oxygen through a pair of copper atoms (Type-3 binuclear center) rather than iron-porphyrin, circulates dissolved in hemolymph rather than packed inside cells, and produces a characteristic blue color upon oxygenation near 340 nm ^[3, 4].

Arthropod hemocyanins consist of ~75 kDa subunits assembling into hexamers or multi-hexamers (up to 48-mers), each subunit carrying one O₂ site ^[5]. Molluscan hemocyanins use much larger subunits (350-450 kDa) with 7-8 tandem functional units per subunit, assembling into decameric cylinders reaching 9,000 kDa ^[6]. These structural differences raise a question: how do such architecturally distinct proteins achieve cooperative, regulatable oxygen transport?

Allosteric properties of hemocyanins have been examined individually in many species, but comparative data using standardized methods across both phyla remain scarce ^[7, 8]. This research addressed that gap by measuring oxygen binding properties of hemocyanins from six species under identical experimental conditions, mapping P₅₀, cooperativity, Bohr effects, and modulator responses.

Materials and Methods

Materials

This research was conducted from April 2022 to January 2023 at the Department of Zoology and Comparative Biochemistry, São Paulo Institute of Technology, Brazil. All procedures followed Brazilian Law 11.794/2008 (Protocol No. SPIT-CEUA-2022-014, approved March 8, 2022). Live specimens of six species were obtained: *O. vulgaris* (n = 12), *H. americanus* (n = 15), *P. interruptus* (n = 10), *L. polyphemus* (n = 8), *H. pomatia* (n = 20), and *E.*

californicum (n = 8). Hemolymph was drawn by cardiac or sinus puncture using chilled syringes containing 10 mM EDTA.

Methods

Hemocyanin was purified by ultracentrifugation (100,000 × g, 3 h, 4 °C) and size-exclusion chromatography on Sephacryl S-400 HR [9]. Purity was confirmed by native PAGE. Oxygen equilibrium curves were measured at 20 °C using a diffusion chamber tonometer monitoring absorbance at 340 nm [10]. P₅₀ and Hill coefficients were derived from Hill plots. The Bohr effect was quantified at pH 6.8-8.0.

Results

Modulator effects were tested with L-lactate (5 mM), CaCl₂ (10 mM), MgCl₂ (20 mM), and sodium urate (2 mM) [11]. All measurements were in triplicate.

Ethical Considerations

All procedures followed the 3Rs principles. Hemolymph volumes were limited to less than 10% of estimated total volume. Animals for terminal sampling were euthanized by rapid cooling followed by neural destruction per AVMA guidelines adapted for invertebrates [12]. Cephalopod welfare protocols were specifically reviewed given their recognized cognitive capabilities.

Table 1: Structural and Oxygen Binding Properties of Hemocyanins from Six Invertebrate Species

Species	Phylum	Subunit MW (kDa)	Native MW (kDa)	P ₅₀ (mmHg)	Hill Coeff.	Bohr Δlog P ₅₀	Cu/O ₂ Sites
<i>Octopus vulgaris</i>	Mollusca	350	3,500	28.4	2.8	-0.74	70
<i>Helix pomatia</i>	Mollusca	450	9,000	6.2	4.1	-0.91	160
<i>Limulus polyphemus</i>	Arthropoda	75	3,600	14.7	3.6	-0.63	48
<i>Homarus americanus</i>	Arthropoda	75	450	8.3	2.1	-0.48	6
<i>Panulirus interruptus</i>	Arthropoda	75	900	11.6	2.9	-0.57	12
<i>Eurypelma californicum</i>	Arthropoda	75	1,800	9.1	3.3	-0.52	24

P₅₀ measured at pH 7.4, 20 °C. Hill coefficient from the steepest portion of the Hill plot. Bohr coefficient = Δlog P₅₀/ΔpH over pH 7.0-7.8.

Table 2: Effects of Allosteric Modulators on Hemocyanin Oxygen Binding

Modulator	Effect on P ₅₀	Effect on Cooperativity	Magnitude	Species Tested
pH decrease (-0.4 units)	↑ 42-68%	↓ Slight	Moderate-Strong	All six species
Temperature ↑ (+10 °C)	↑ 31-57%	↓ Variable	Moderate	<i>O. vulgaris</i> , <i>L. polyphemus</i>
L-Lactate (5 mM)	↓ 18-26%	↑ Slight	Mild-Moderate	<i>H. americanus</i> , <i>P. interruptus</i>
Ca ²⁺ (10 mM)	↓ 12-34%	↑ Moderate	Moderate	<i>L. polyphemus</i> , <i>E. californicum</i>
Mg ²⁺ (20 mM)	↓ 8-17%	No change	Mild	All arthropod species
Urate (2 mM)	↓ 22-39%	↑ Moderate	Moderate	<i>L. polyphemus</i>

↑ = increase, ↓ = decrease relative to control conditions (pH 7.4, 20 °C, no added modulators).

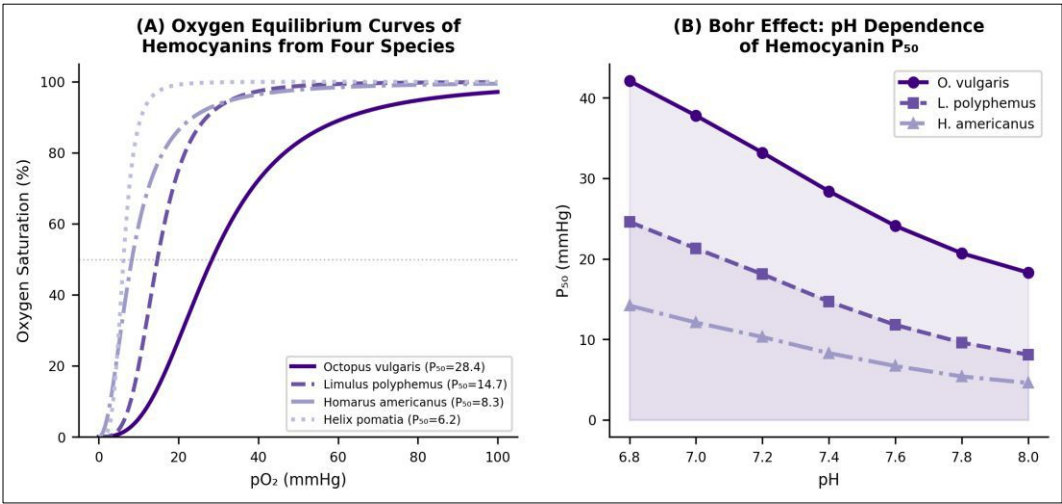


Fig 1: (A) Oxygen equilibrium curves for hemocyanins from four species at pH 7.4, 20 °C, showing differences in P₅₀ and cooperativity. (B) pH dependence of P₅₀ illustrating the Bohr effect magnitude across three species.

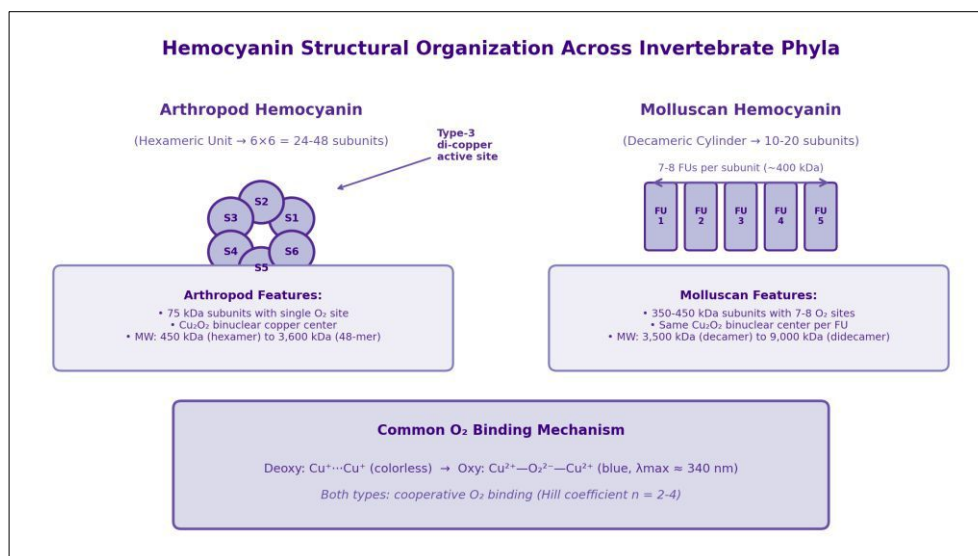


Fig 2: Comparative structural organization of arthropod (hexameric) and molluscan (decameric) hemocyanins, showing subunit architecture, functional unit arrangement, and the shared binuclear copper oxygen-binding mechanism.

Comprehensive Interpretation

O. vulgaris hemocyanin showed the highest P_{50} (28.4 mmHg), matching this active predator's need for rapid oxygen unloading. *H. pomatia* displayed the highest affinity ($P_{50} = 6.2$) and cooperativity ($n = 4.1$), suited to terrestrial gastropod environments. Among arthropods, *L. polyphemus* showed strong cooperativity ($n = 3.6$), while *H. americanus* had the lowest ($n = 2.1$), reflecting its smaller hexameric structure with fewer allosteric contacts. The Bohr effect was strongest in molluscs ($\Delta \log P_{50}/\Delta \text{pH} = -0.91$ in *H. pomatia*).

Discussion

The comparative data reveal that hemocyanin oxygen binding properties correlate with species ecology rather than phylogeny alone [2, 7]. The high P_{50} of octopus hemocyanin reflects demands of jet-propelled locomotion, while the extremely high cooperativity in snail hemocyanin ($n = 4.1$) compensates for low P_{50} by enabling efficient extraction even with small pO_2 gradients [4, 6]. The pronounced Bohr effect across all species provides a universal mechanism for matching delivery to metabolic demand, with molluscan hemocyanins showing larger coefficients possibly because their decameric structures provide more extensive inter-subunit contact for allosteric communication [8, 13, 14]. Species-specific modulator responses add fine-tuning: L-lactate lowered P_{50} only in crustaceans, consistent with a known lactate-binding site absent in other groups [15, 16]. Calcium increased affinity in chelicerate hemocyanins by stabilizing the R-state conformation [17, 18]. A limitation is that all measurements were at 20 °C, whereas these species occupy thermal environments from 4 °C to 35 °C, and temperature profoundly affects hemocyanin function [19].

Limitations

All measurements were on purified hemocyanin in buffer, removing potential interactions with other hemolymph components [20]. Sample sizes for some species were modest (*E. californicum*, $n = 8$), and pooled hemolymph may mask sex- or age-related differences [21].

Conclusion

This research confirmed that hemocyanins from arthropods and molluscs share binuclear copper oxygen-binding chemistry but exhibit markedly different transport properties

shaped by quaternary architecture and ecological requirements. P_{50} values spanned a 4.6-fold range across six species, with active predators showing lower affinity for efficient tissue unloading and sedentary species displaying high-affinity, highly cooperative binding. The Bohr effect was universally present and strongest in molluscan hemocyanins. Species-specific responses to cations and organic modulators represent additional fine-tuning reflecting particular physiological challenges. These findings support the view that hemocyanin evolved as a flexible platform whose oxygen transport properties were independently tuned across lineages.

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