

CHAPTER IV  
OXYGEN BINDING PROPERTIES OF WHOLE HEMOLYMPH  
AND PURIFIED HEMOCYANIN

Introduction

An ontogenic change in crustacean hemocyanin structure was first reported in 1982 by Terwilliger and Terwilliger. In that study on Cancer magister, the proportion of 16S molecules compared to 25S molecules is higher in the megalopa and early juvenile hemolymph than in the adult hemolymph. Also, in C. magister, the pattern of subunits seen by gel electrophoresis is different in the megalopa and juvenile hemocyanin compared to the adult hemocyanin (Terwilliger and Terwilliger, 1982). The stoichiometry of five subunits is different among the megalopa, juvenile and adult hemocyanins, and there is a sixth subunit which is unique to the adult hemocyanin. In earlier developmental stages, the subunits of the hemocyanin in Cancer productus, C. magister and C. gracilis oocytes are indistinguishable from the maternal hemocyanin. However, the embryonic hemocyanin subunit composition is different from the oocyte/maternal hemocyanin (Wache et al., 1988; Terwilliger, in press). In Homarus americanus, there are ontogenic

differences in subunit composition as seen by gel electrophoresis, and there is a greater proportion of 16S hemocyanin in larval than in adult hemolymph (Olson et al., 1988). In Carcinus maenas and Hyas araneus there is also a greater proportion of 16S hemocyanin in the larval hemolymph than in the adult hemolymph, as well as a change in the subunit composition between larval and adult hemocyanin (Markl et al. 1986).

There are to date only two published studies of larval hemocyanin function. The hemocyanin oxygen binding properties of C. magister are markedly different between the megalopa and the adult hemocyanin under the same experimental conditions. The oxygen affinity of the adult 25S hemocyanin is twice as great as the oxygen affinity of the megalopa /juvenile 25S hemocyanin. The two types of hemocyanin, megalopa/juvenile and adult, have the same relative sensitivity to L-lactate and protons (Terwilliger et al., 1986). Ontogenic differences in oxygen affinity in Homarus americanus hemocyanin have also been reported (Olson and McDowell Capuzzo, 1989; Olson et al., 1990). The sensitivity to protons changes in the hemolymph of H. americanus at the stage III larva (Olson et al., 1990).

The physiological significance of the ontogenic structural and functional changes in crustacean hemocyanin is unknown. How does the ontogenic change in hemocyanin structure and function affect oxygen transport in C.

magister during its life cycle?

The goal of this study was to examine the oxygen binding properties of the whole hemolymph from several stages in the life cycle of C. magister. The effect of specific ion concentrations on hemocyanin oxygen equilibria was also examined based on the ontogenic changes in ion regulation described in the preceding chapter.

### Materials and Methods

#### Animals

The different life stages of C. magister were caught and maintained as previously described in Chapter II.

#### Whole hemolymph

#### Sample Preparation

Hemolymph samples were obtained from the infrabranchial sinus of 5th juvenile and adult crabs with a needle and syringe. Hemolymph samples were taken from the same place in 1st juveniles but the sampling was done with a microcapillary pipette. Megalopa hemolymph was obtained by puncturing the heart with a microcapillary pipette. In order to avoid puncturing the yellowish-brown digestive tract, sampling of megalopae and 1st juveniles was done with the use of a dissecting microscope. The total number of animals sampled was 30 adults, 80 5th juveniles, about 900 1st

juveniles and about 2250 megalopae. Hemolymph from 5 adults, 10 5th juveniles, about 150 1st juveniles or about 250 megalopae were pooled for each whole hemolymph sample. The fresh, whole hemolymph samples were allowed to clot on ice for 30 minutes and then centrifuged at 12,000Xg for 10 minutes at 4°C to remove the aggregate. Hemolymph was stored at 4°C and used for oxygen equilibria within 24 hours.

#### Lactate and Urate Assays

L-lactate concentration in the whole hemolymph samples was measured enzymatically (Boehringer-Mannheim, no. 139 084). Urate concentration in the samples was measured enzymatically (Sigma Chemical Co., procedure no. 685).

#### Oxygen Carrying Capacity

The fresh whole hemolymph samples used in oxygen binding contained an unidentified substance with an absorbance peak at about 323 nm which was apparent in deoxyhemocyanin spectra. Due to the presence of this substance the absorbance used to calculate the carrying capacity was based on the difference between the oxyhemocyanin and the deoxyhemocyanin absorbances adjusted for the altered baseline. Examination of 47 paired sets of oxyhemocyanin and deoxyhemocyanin absorbances at 340 nm of C. magister 25S hemocyanin revealed that  $OD_{\text{deoxy}} = 0.09 (OD_{\text{oxy}} - OD_{\text{deoxy}})$  ( $R^2 = 0.86$ ). Given this relationship, the

difference in absorbance of oxygenated and deoxygenated whole hemolymph was adjusted and is referred to in the carrying capacity calculations as the absorbance of fully oxygenated hemocyanin in the sample.

The extinction coefficient at 280 nm of a 1 percent solution of C. magister hemocyanin in a 1 cm pathlength cuvette,  $E_{1\text{cm}}^{1\%}$ , is 15 (Nickerson and Van Holde, 1971). Based on a ratio of the absorbance of purified C. magister oxyhemocyanin at 340 nm and 280 nm of 0.2, the value of the extinction coefficient at 340 nm would be 3 (Graham, 1983). Since each hemocyanin subunit combines reversibly with one molecule of oxygen, the hemocyanin oxygen carrying capacities of whole hemolymph samples were calculated based on the absorbance at 340 nm of a fully oxygenated sample, a value of  $E_{1\text{cm}}^{1\%} = 3$  at 340 nm, and an average subunit molecular weight of 75 kD. The total oxygen carrying capacity of the hemolymph includes the amount of oxygen physically dissolved in solution as well.

### Oxygen Equilibria

Fresh whole hemolymph samples for oxygen binding were buffered in stage specific saline solutions. The ion concentrations for each stage specific saline were made up to correspond to the concentrations measured in the hemolymph of megalopae, 1st juveniles and 5th juveniles in 100% seawater as reported in Chapter III. The saline for the

adult hemolymph was made to match the formula used by Graham et al. (1983). Saline formulae are given in Table 1. Fresh whole hemolymph was diluted 1:2 to 1:3 in order to obtain the desired pH and an absorbance near 0.3 in a 1 mm cuvette at 340 nm.

Table 1. Formulae for stage specific salines for 100% seawater/10°C conditions

SALT	Megalopa	1st Juvenile	5th Juvenile	Adult
HCl	50	50	50	50
NaCl	322	322	353	454
KCl	5.4	5.4	4.7	11.5
CaCl <sub>2</sub>	7.4	7.4	8.5	13.5
MgCl <sub>2</sub>	32	32	23.2	18
Na <sub>2</sub> SO <sub>4</sub>	23.5	23.5	23.5	23.5

Note. Concentrations given in mmol/L. Saline solutions were titrated to the desired pH at 10°C with Trizma Base (Sigma Chemical Co.).

Oxygen equilibria were determined tonometrically (Benesch et al., 1965) using 0.4 to 0.5 ml of sample in tonometers with a volume of approximately 13.7 ml fitted with 1 mm pathlength cuvettes. All oxygen binding experiments were done at 10°C.

#### Purified 25S Hemocyanin

##### Sample Preparation

Purified 25S hemocyanin from adult crabs was obtained by chromatographing an aliquot of centrifuged hemolymph (as

described above) on a Bio-Gel A-5m column (1.8 X 135 cm) equilibrated with 0.05 ionic strength Tris-HCl (pH 7.5), 0.1 mol/L in NaCl, 10 mmol/L in MgCl<sub>2</sub> and 10 mmol/L in CaCl<sub>2</sub> at 10°C. The eluted 25S hemocyanin peak was concentrated using Centricon 30 tubes (Amicon Div. of W. R. Grace and Co.).

In order to obtain purified 25S hemocyanin from 1st juveniles, the crabs were cut in two across the lateral edge of one gill chamber and packed 50 per 15 ml centrifuge tube. A-5m column buffer (0.2 ml) containing 1 mmol phenylmethylsulfonyl fluoride was added to inhibit protease activity. The 1st juveniles were centrifuged at 3,000Xg for 10 minutes at 4°C. The supernatant was collected and respun at 12,000Xg for another 10 minutes. Three hundred 1st juveniles yielded 4 to 5 ml of supernatant. The supernatant was chromatographed on a Bio-Gel A-5M column and the 25S hemocyanin fractions concentrated as described above for the adult hemocyanin.

#### Oxygen equilibria

The effects of magnesium ion and calcium ion concentration on the oxygen equilibria of adult and 1st juvenile purified 25S hemocyanin were measured. Fresh adult 25S hemocyanin was dialyzed against saline solutions containing 0, 16, 32 or 100 mmol/L magnesium, and a constant concentration of other ions (Table 2). The 25S hemocyanin samples were dialyzed against 1 L of saline for a total of

24 hours; there were four changes of saline, each lasting no less than four hours.

Frozen purified 25S adult hemocyanin samples were thawed and dialyzed against either 16 or 32 mmol/L magnesium saline to assay the effect of freezing on C. magister hemocyanin. They will be referred to as frozen samples.

Table 2. Formulae for saline containing 0, 16, 32 and 100 mmol/L magnesium

<u>SALT</u>	<u>0</u>	<u>16</u>	<u>32</u>	<u>100</u>
HCl	50	50	50	50
NaCl	454	454	454	454
KCl	11.5	11.5	11.5	11.5
CaCl <sub>2</sub>	13.5	13.5	13.5	13.5
MgCl <sub>2</sub>	0	16	32	100
Na <sub>2</sub> SO <sub>4</sub>	23.5	23.5	23.5	23.5

Note. Concentrations given in mmol/L. Saline solutions were titrated to the desired pH at 10°C with Trizma Base (Sigma Chemical Co.).

The effect of calcium on adult 25S hemocyanin was determined on frozen adult 25S hemocyanin samples dialyzed against salines with varying calcium ion concentrations (Table 3). The samples had previously been used to assay the effect of magnesium on fresh 25S hemocyanin and frozen at -73°C. These samples were reused in this fashion in order to parallel the treatment of 1st juvenile 25S hemocyanin of which there was a limited quantity.

The first juvenile 25S hemocyanin samples were used in three sets of oxygen binding experiments. The fresh 25S

hemocyanin samples were first used to determine the effect of stage specific salines (see below) and then stored at  $-73^{\circ}\text{C}$ . The samples were thawed and dialyzed against either 16 or 32 mmol/L magnesium saline (Table 2) and oxygen binding performed. After the magnesium experiments the samples were refrozen at  $-73^{\circ}\text{C}$ . They were subsequently thawed, pooled and aliquots dialyzed against either 16 or 32 mmol/L calcium saline (Table 3) to determine the effect of calcium on 1st juvenile 25S hemocyanin oxygen affinity.

Table 3. Formulae for saline containing 0, 16, 32 and 100 mmol/L calcium

SALT	0	16	32	100
HCl	50	50	50	50
NaCl	454	454	454	454
KCl	11.5	11.5	11.5	11.5
CaCl <sub>2</sub>	0	16	32	100
MgCl <sub>2</sub>	18	18	18	18
Na <sub>2</sub> SO <sub>4</sub>	23.5	23.5	23.5	23.5

Note. Concentrations given in mmol/L. Saline solutions were titrated to the desired pH at  $10^{\circ}\text{C}$  with Trizma Base (Sigma Chemical Co.).

The effects of stage specific ion concentrations (100% seawater) on oxygen binding of 25S hemocyanin were determined by dialyzing the 25S hemocyanin from adult and 1st juveniles against stage specific salines (Table 1). The effects of stage specific salines on the oxygen equilibria of 25S hemocyanin samples were all done using fresh samples.

## Data analysis

The Bohr coefficients (the slope of  $\log P_{50}$  vs. pH) and the oxygen affinities were compared by analysis of covariance (ANCOVA). Mean values of cooperativity ( $n_{50}$ ) were compared by Student's T-test.  $P < 0.05$  was considered significant. Statistical analyses were done using SYSTAT version 4.1 (SYSTAT, Inc.).

## Results

### Whole hemolymph

#### Lactate and Urate

No urate was detected in any of the whole hemolymph samples from any of the four stages examined. L-lactate levels were variable between individual samples and between stages. The lowest detectable level of L-lactate was 0.05 mmol/L. L-lactate concentrations in the megalopa whole hemolymph samples ranged from the limit of detection to 0.57 mmol/L. Only one 1st juvenile whole hemolymph sample had any detectable L-lactate, 0.47 mmol/L. There was no detectable L-lactate in any of the 5th juvenile hemolymph samples. L-lactate in adult whole hemolymph samples ranged from 0.18 to 3.44 mmol/L.

#### Oxygen Carrying Capacity

Hemocyanin concentration, [Hc], maximum hemocyanin

oxygen carrying capacity,  $C_{Hc}O_2$ , dissolved oxygen,  $C_{diss}O_2$  (10°C, in seawater at atmospheric pressure) and total oxygen carrying capacities,  $C_{tot}O_2$  (volumes % = ml  $O_2$ /100 ml) of the hemolymph of the four stages are given in Table 4. The concentration of hemocyanin in the hemolymph changes during development.

Table 4. The hemocyanin concentrations and oxygen carrying capacities of four stage of C. magister

STAGE	[Hc]	$C_{Hc}O_2$	$C_{diss}O_2$	$C_{tot}O_2$
MEGALOPA	20.6 ±0.8	0.62 ±0.03	0.64	1.26
1st JUVENILE	6.2 ±0.4	0.19 ±0.01	0.64	0.83
5th JUVENILE	11.1 ±2.2	0.33 ±0.07	0.64	0.97
ADULT	38.0 ±2.1	1.14 ±0.06	0.64	1.75

Note. Hemocyanin concentration in mg/ml; carrying capacities in ml  $O_2$ /100 ml. Mean ± S. E..

#### Oxygen Equilibria

The megalopa, 1st juvenile and 5th juvenile oxygen affinities of whole hemolymph with endogenous L-lactate and ion concentrations are indistinguishable. The oxygen affinity of adult whole hemolymph, with endogenous L-lactate and ion concentrations, is higher (log  $P_{50}$  is lower) than

the megalopa and juvenile stages (Fig. 1). There are no significant differences in the Bohr coefficients ( $\log P_{50}/\text{pH} = -1.2 \pm 0.1$ ) of the whole hemolymph from the four stages. The cooperativities of the whole hemolymph samples from the four stages are not significantly different (Fig. 2).

The measured  $P_{50}$  of each sample in Figure 1 was adjusted to what it would be if the samples contained a uniform concentration of L-lactate (equal to the lowest detectable limit, 0.05 mmol/L) rather than its endogenous lactate level. The value used to adjust the  $P_{50}$  values of all four stages was -0.29. This value is based on the coefficients for the relationship of  $\log P_{50}$  vs.  $\log$  L-lactate for adult C. magister hemocyanin (-0.287) and for the juvenile type hemocyanin is (-0.291) (Terwilliger et al., 1986). When the  $P_{50}$  values for each of the whole hemolymph samples are thus adjusted for the effect of L-lactate, there is no significant difference in oxygen affinity between any of the four stages (Fig. 3).

#### Purified 25S Hemocyanin

##### Magnesium Ion Effect

The effect of varying magnesium ion concentration on the oxygen affinity of fresh adult 25S hemocyanin is shown in Figure 4. An increase in magnesium ion concentration increases the oxygen affinity (decreases  $P_{50}$ ); this effect

Figure 1. The oxygen affinity C. magister whole hemolymph with endogenous L-lactate and inorganic ions.  
■ adult; + 5th juvenile; \* 1st juvenile;  
□ megalopa; --- regression for adult  $P_{50}$  vs. pH; — regression for megalopa and juvenile log  $P_{50}$  vs. pH.

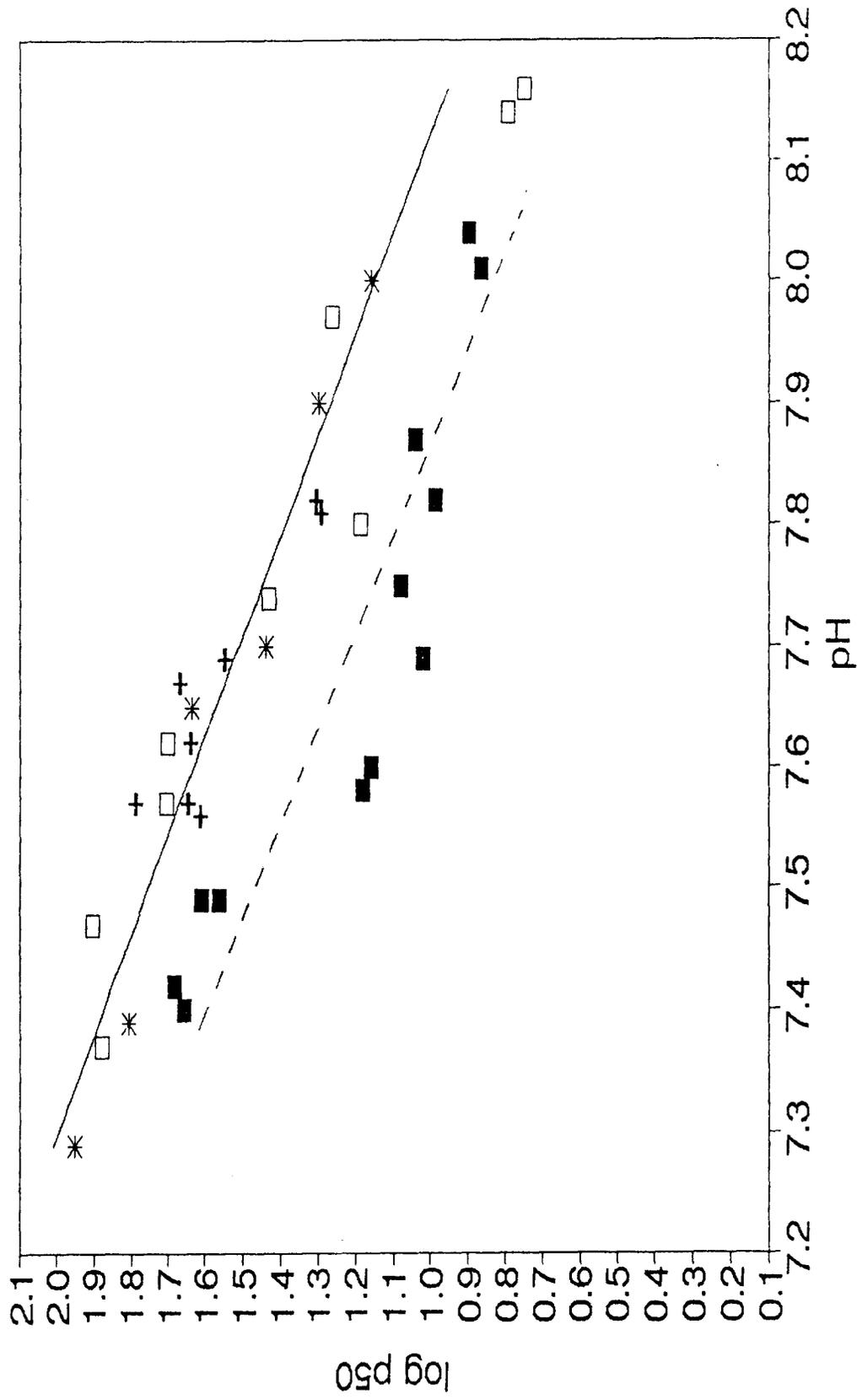


Figure 2. The cooperativity of C. magister whole hemolymph with endogenous L-lactate and inorganic ions.  
■ adult; + 5th juvenile; \* 1st juvenile; □ megalopa.

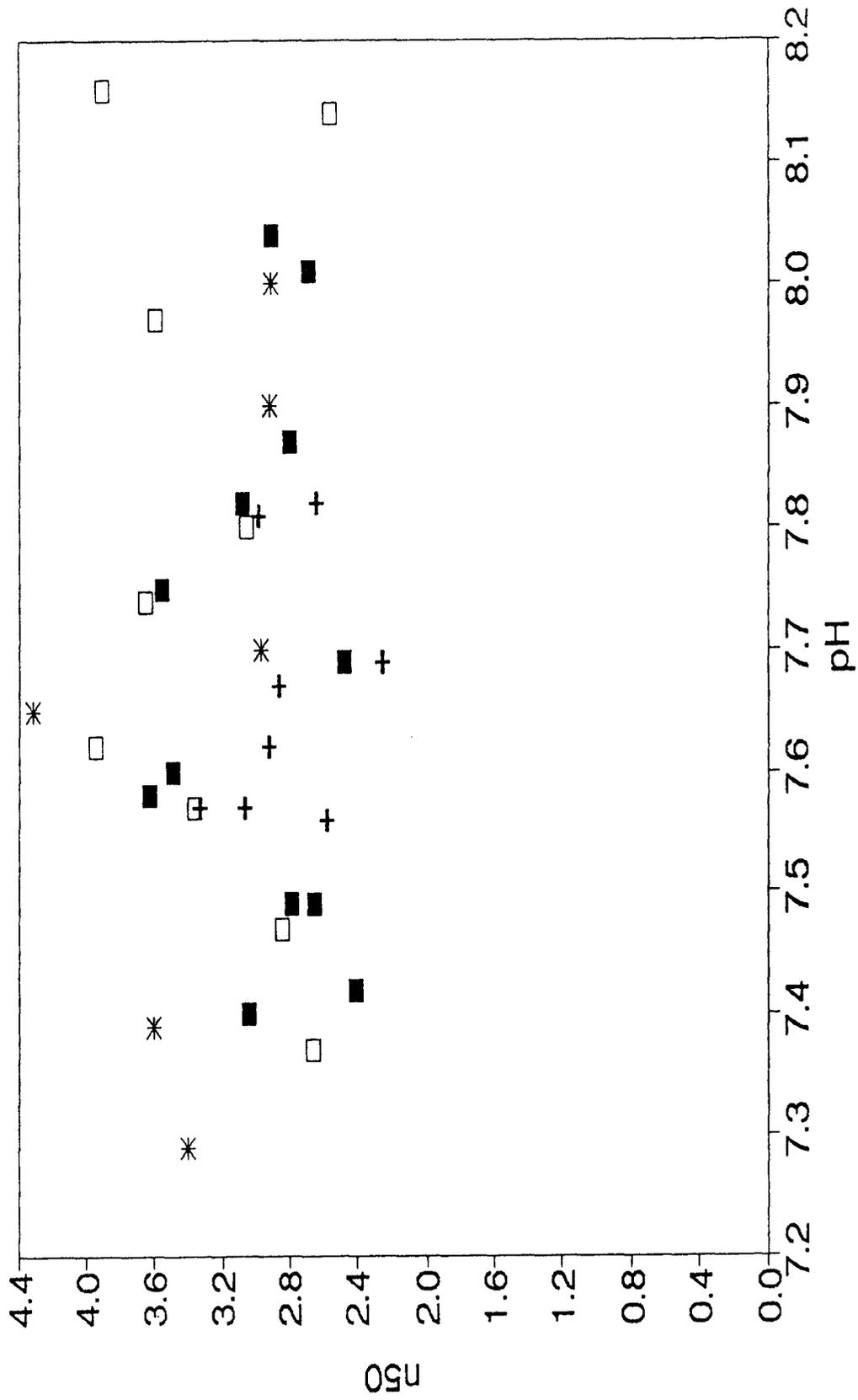


Figure 3. The oxygen affinity of *C. magister* whole hemolymph with log  $P_{50}$  values adjusted to 0.05 mmol/L L-lactate. ■ adult; + 5th juvenile; \* 1st juvenile; □ megalopa; — regression for all four stages.

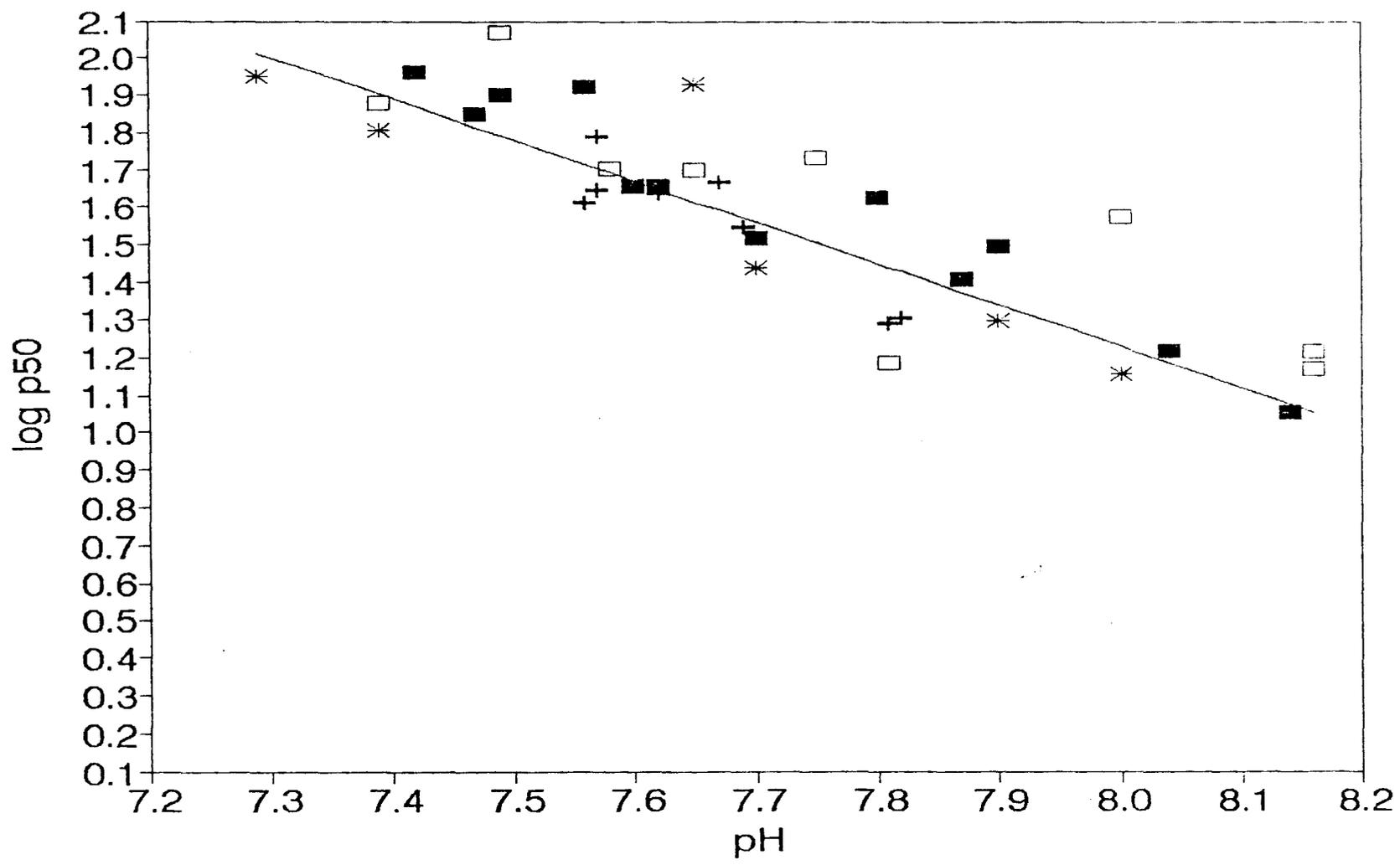
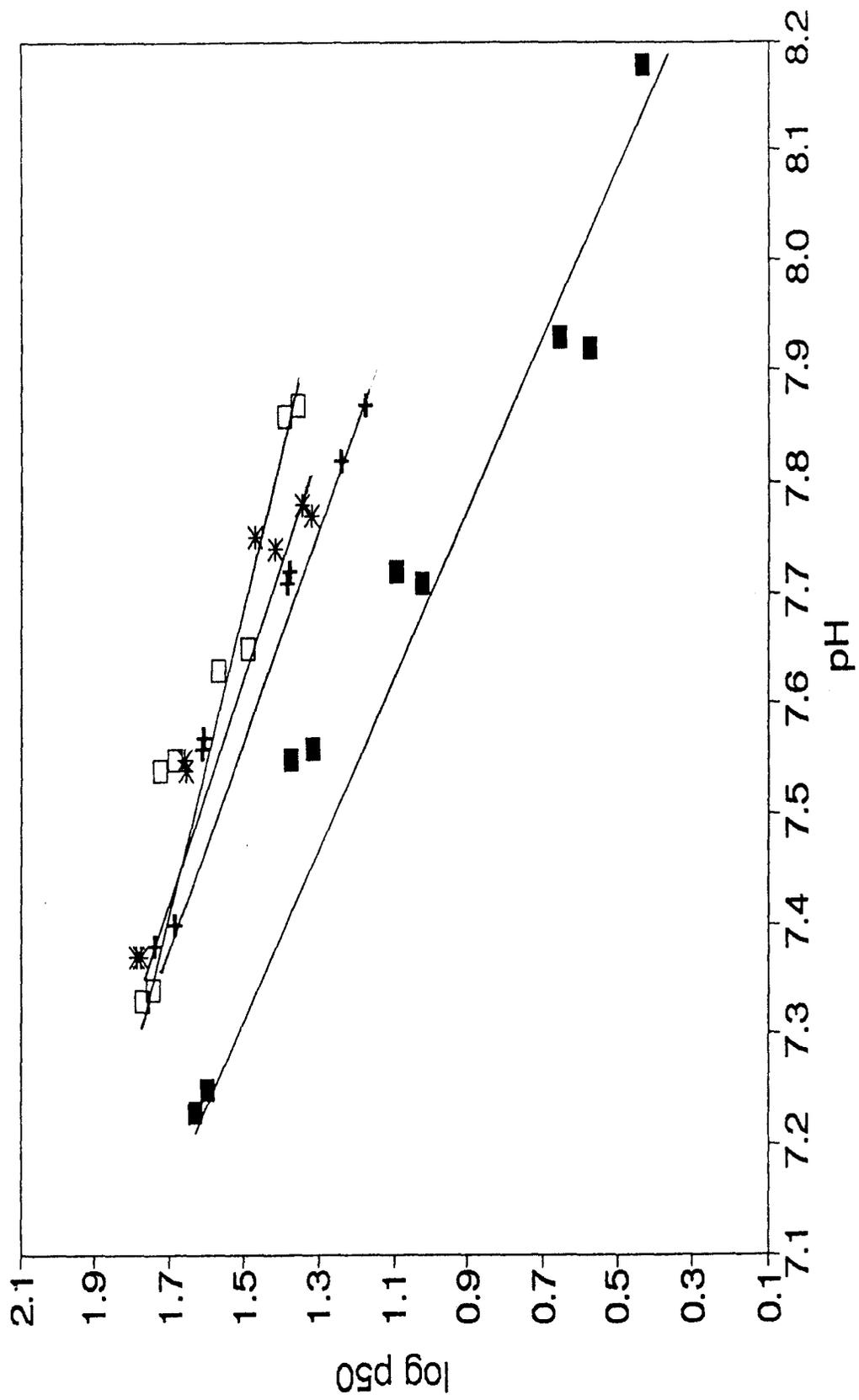


Figure 4. The effect of magnesium on oxygen affinity of C. magister fresh adult 25S hemocyanin.

□ 0 mmol/L; \* 16 mmol/L; + 32 mmol/L;  
■ 100 mmol/L.



is enhanced at higher pH. An increase in magnesium ion concentration from 0 to 100 mmol/L Mg<sup>++</sup> significantly increases the Bohr coefficient as well, although the Bohr coefficients are not significantly different between 0 and 32 mmol/L magnesium (Table 5).

Table 5. Regression equations for log P<sub>50</sub> vs. pH for adult C. magister fresh 25S hemocyanin in saline with varying magnesium concentration

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log P <sub>50</sub> = -0.76 (pH) + 7.37	(R <sup>2</sup> : 0.90),	0 mmol/L Mg <sup>++</sup>
log P <sub>50</sub> = -1.05 (pH) + 9.53	(R <sup>2</sup> : 0.95),	16 mmol/L Mg <sup>++</sup>
log P <sub>50</sub> = -1.14 (pH) + 10.16	(R <sup>2</sup> : 0.96),	32 mmol/L Mg <sup>++</sup>
log P <sub>50</sub> = -1.38 (pH) + 11.66	(R <sup>2</sup> : 0.95),	100 mmol/L Mg <sup>++</sup>

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There is a linear relationship between increasing magnesium and log P<sub>50</sub> at a given pH:

$$\log P_{50} = -0.005 [\text{Mg}^{++}] + 1.44 \quad (R^2 = 0.99) \quad \text{at pH } 7.8 \quad (\text{Fig. } 5).$$

The cooperativity is significantly less at pH lower than 7.4 ( $n_{50} = 2.47 \pm 0.07$ , SE) than at pH greater than 7.4 ( $n_{50} = 2.97 \pm 0.05$ , SE) (Fig. 6). There is no significant difference in cooperativity related to magnesium ion concentration.

All of the 1st juvenile 25S hemocyanin samples used in the magnesium experiments had been stored at -73°C. Therefore, in order to be able to make valid comparisons on the effect of magnesium between adult and 1st juvenile 25S hemocyanin, it was necessary to determine the effect of freezing on adult samples. Adult 25S hemocyanin was stored

Figure 5. The relationship between  $\log P_{50}$  and magnesium concentration for C. magister fresh adult 25S hemocyanin at pH 7.8.

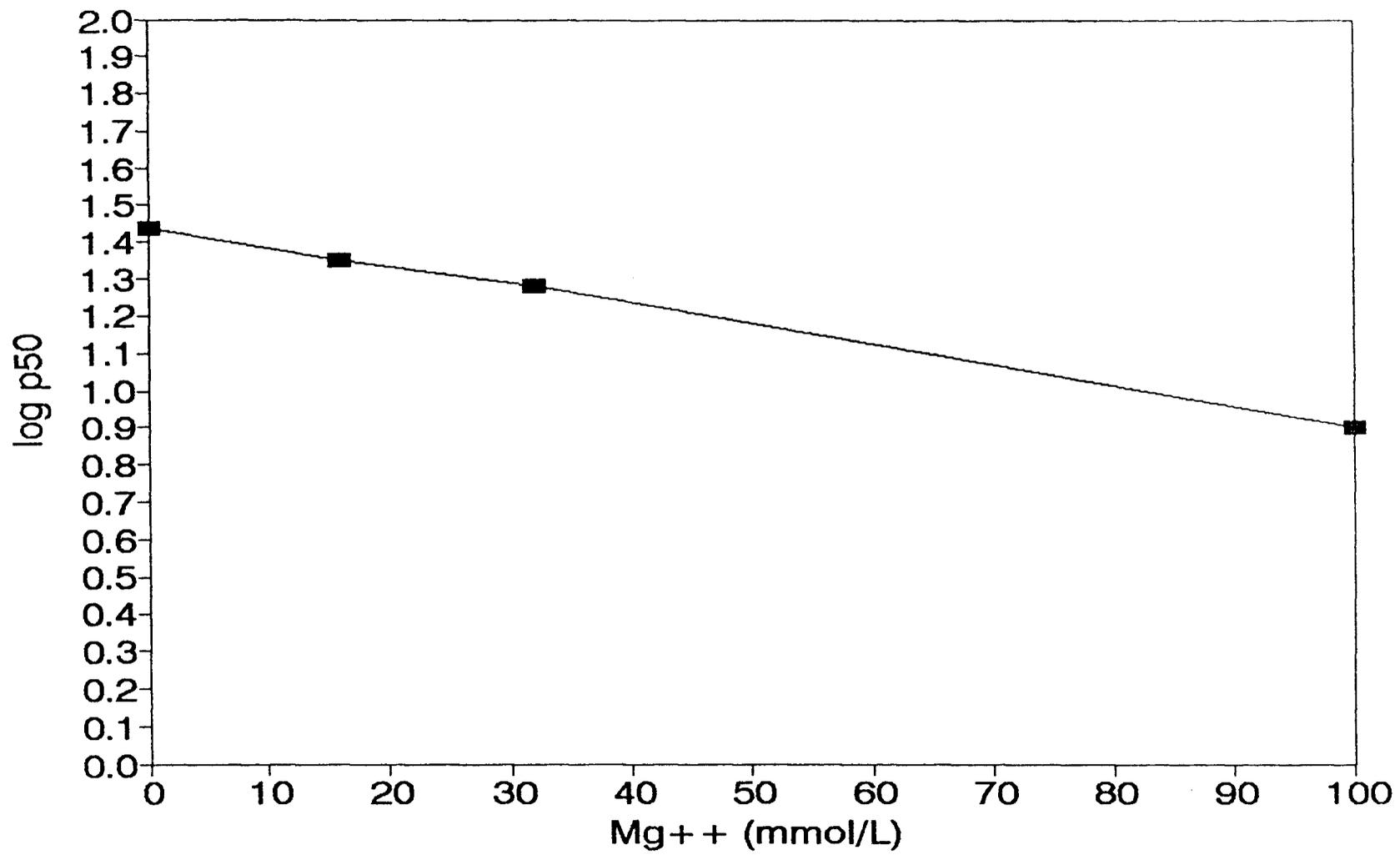
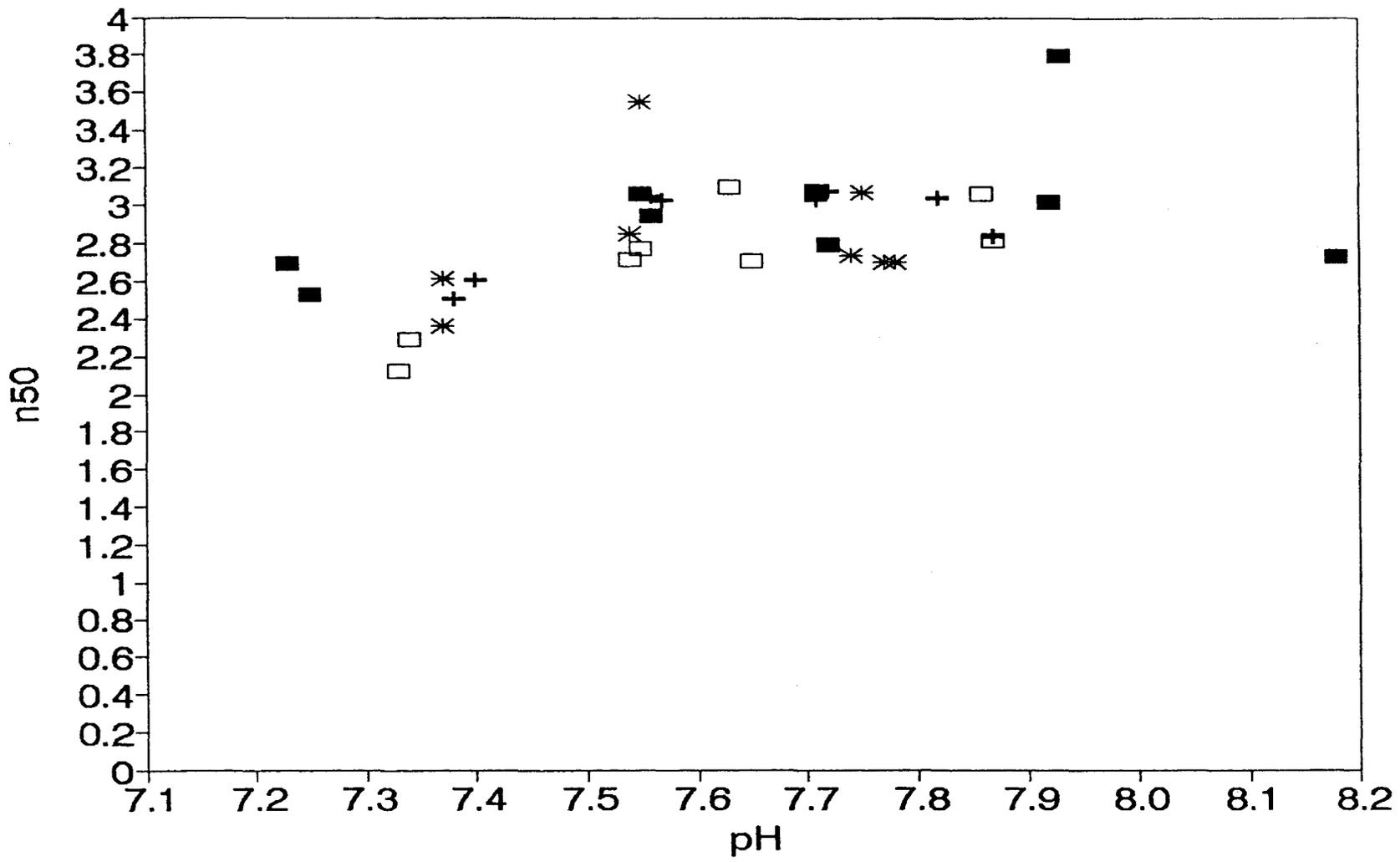


Figure 6. The effect of magnesium on the cooperativity of C. magister fresh adult 25S hemocyanin.  0  
mmol/L; \* 16 mmol/L; + 32 mmol/L;  100  
mmol/L.



at  $-73^{\circ}\text{C}$  for 7 weeks, and oxygen binding was done in 16 and 32 mmol/L magnesium saline. A comparison of fresh and frozen adult 25S hemocyanin oxygen affinity is shown in Figure 7. Freezing the hemocyanin decreases the  $P_{50}$  by 50% and decreases cooperativity (Fig. 8). Freezing does not significantly affect the Bohr coefficient.

Comparison of the effect of magnesium ion concentration on the oxygen affinity of frozen adult and 1st juvenile 25S hemocyanin is shown in Figure 9. Once again, when the 25S hemocyanins from the two stages are dialyzed against identical saline solutions, the  $P_{50}$  of the 1st juvenile is about 50% higher than the  $P_{50}$  of the adult. The increase in magnesium from 16 to 32 mmol/L has a significant effect on the affinity and the Bohr coefficient of the 1st juvenile 25S hemocyanin. This magnitude of change in magnesium concentration does not have a statistically significant effect on the  $P_{50}$  or Bohr coefficient of adult 25S hemocyanin. Increasing magnesium from 16 to 32 mmol/L has no effect on the cooperativity of frozen 25S 1st juvenile hemocyanin (Fig. 10). The cooperativity of the 1st juvenile 25S hemocyanin ( $n_{50} = 1.44 \pm 0.07$ , SE) is less than that of the adult 25S hemocyanin ( $n_{50} = 2.02 \pm 0.09$ , SE) under these conditions.

#### Calcium Ion Effect

Increasing calcium ion concentration increases the

Figure 7. The effect of magnesium on the oxygen affinity of C. magister fresh and frozen adult 25S hemocyanin.

⊗	fresh, 16 mmol/L;	■	frozen, 16 mmol/L;
*	fresh, 32 mmol/L;	□	frozen, 32 mmol/L.

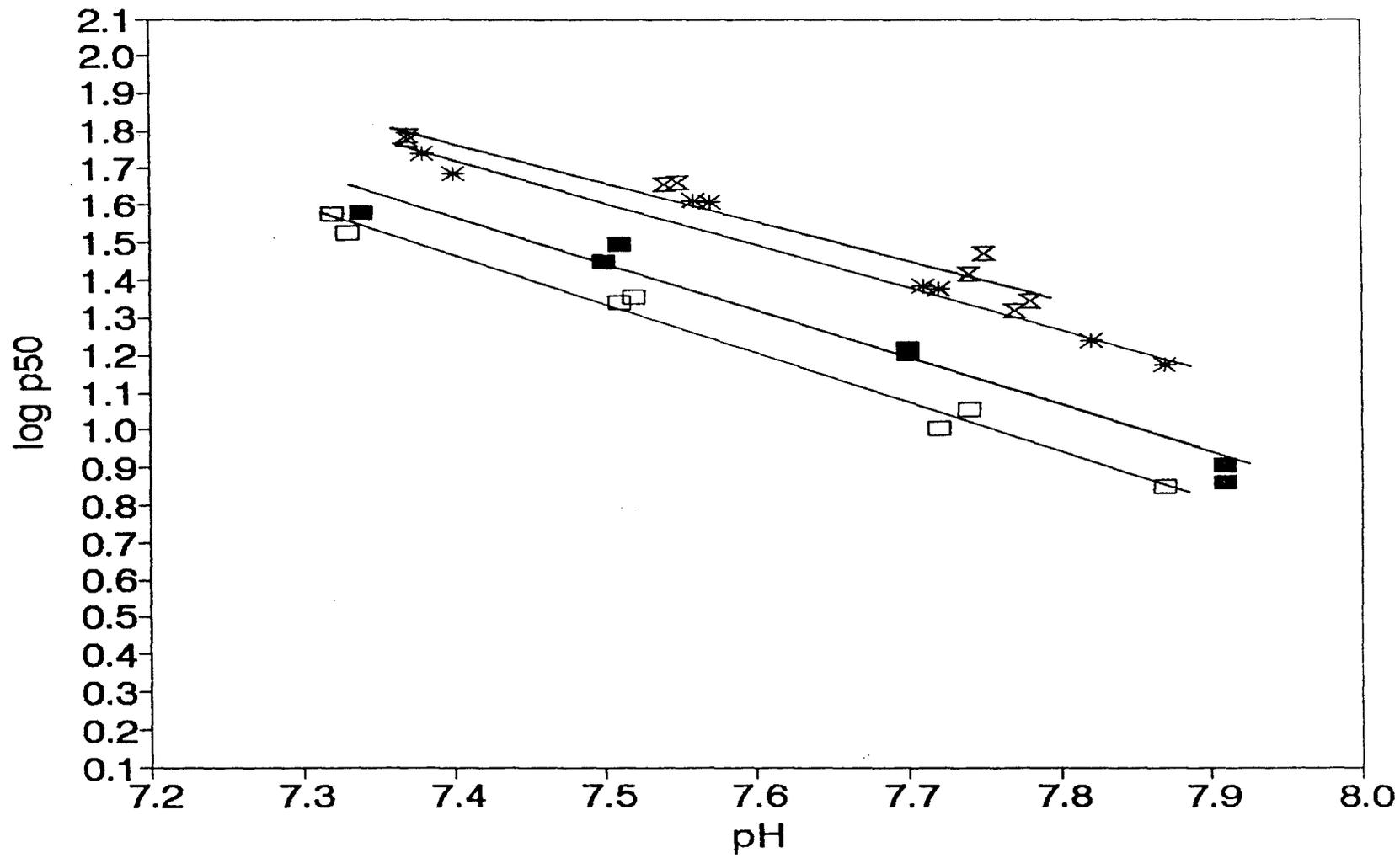


Figure 8. The effect of magnesium on the cooperativity of C. magister fresh and frozen adult 25S hemocyanin.

⊗	fresh, 16 mmol/L;	■	frozen, 16 mmol/L;
*	fresh, 32 mmol/L;	□	frozen, 32 mmol/L.

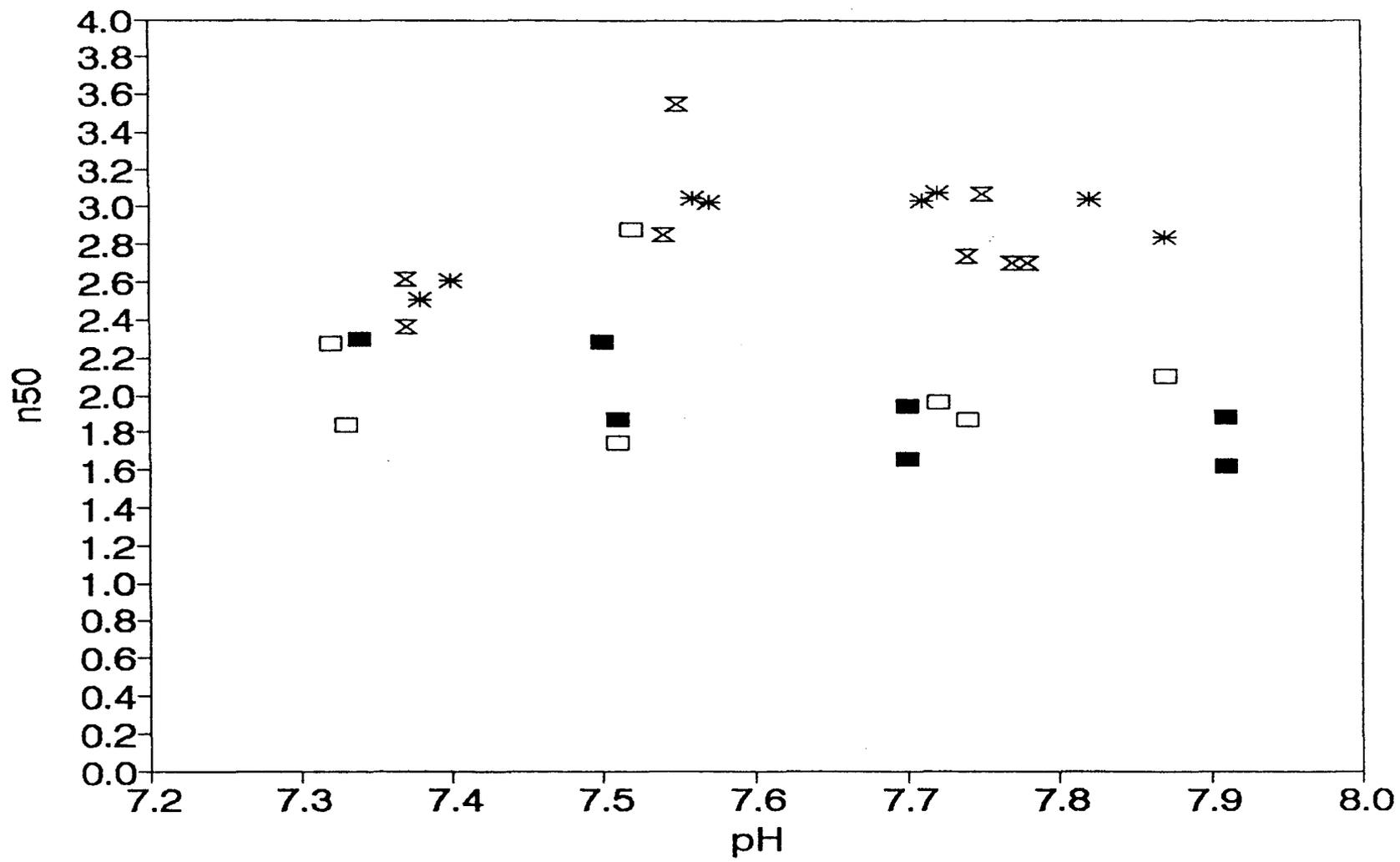


Figure 9. The effect of magnesium on the oxygen affinity of C. magister frozen adult 25S hemocyanin and frozen 1st juvenile 25S hemocyanin. □ adult, 16 mmol/L; ⋈ 1st juvenile, 16 mmol/L; ■ adult, 32 mmol/L; \* 1st juvenile, 32 mmol/L.

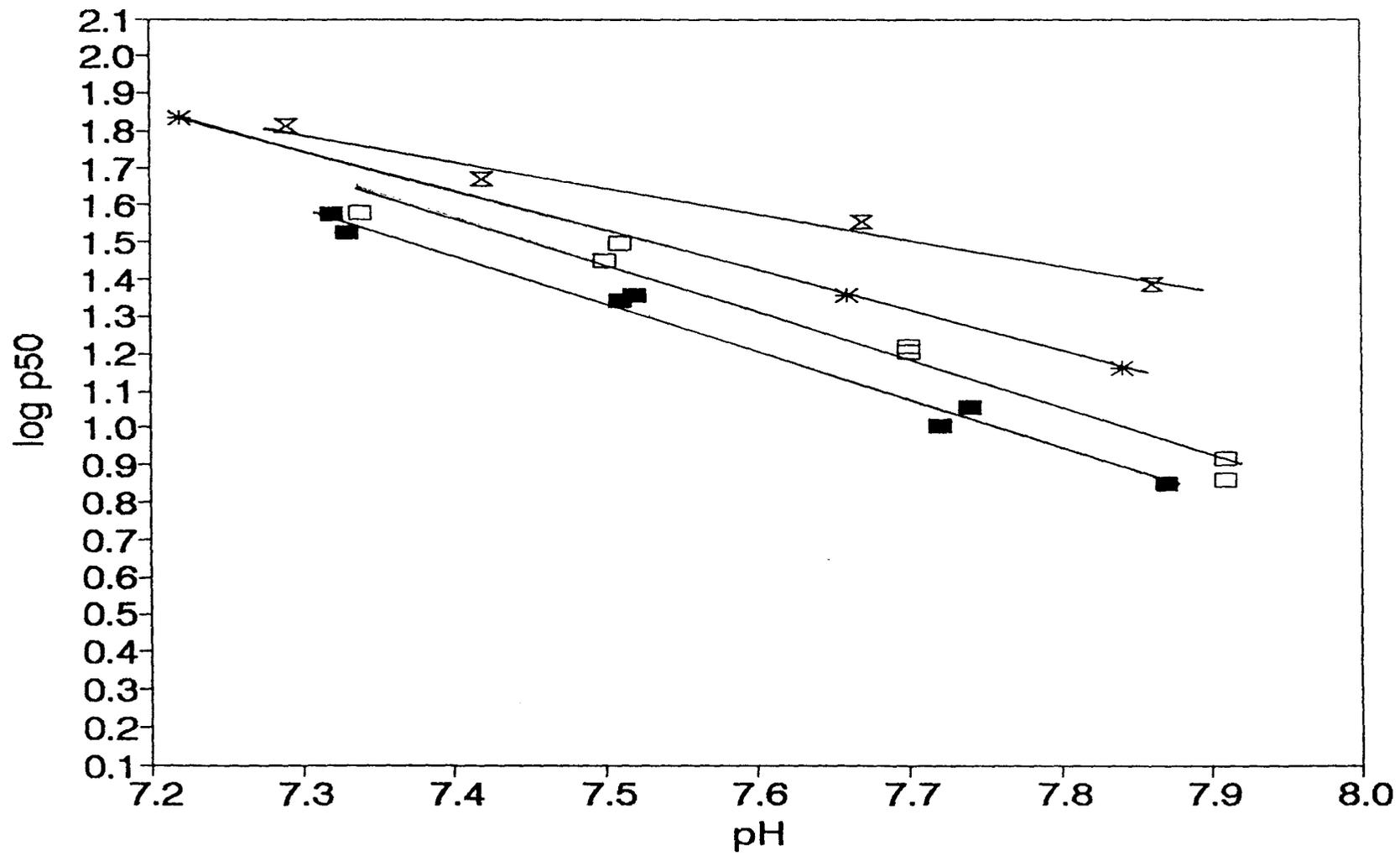
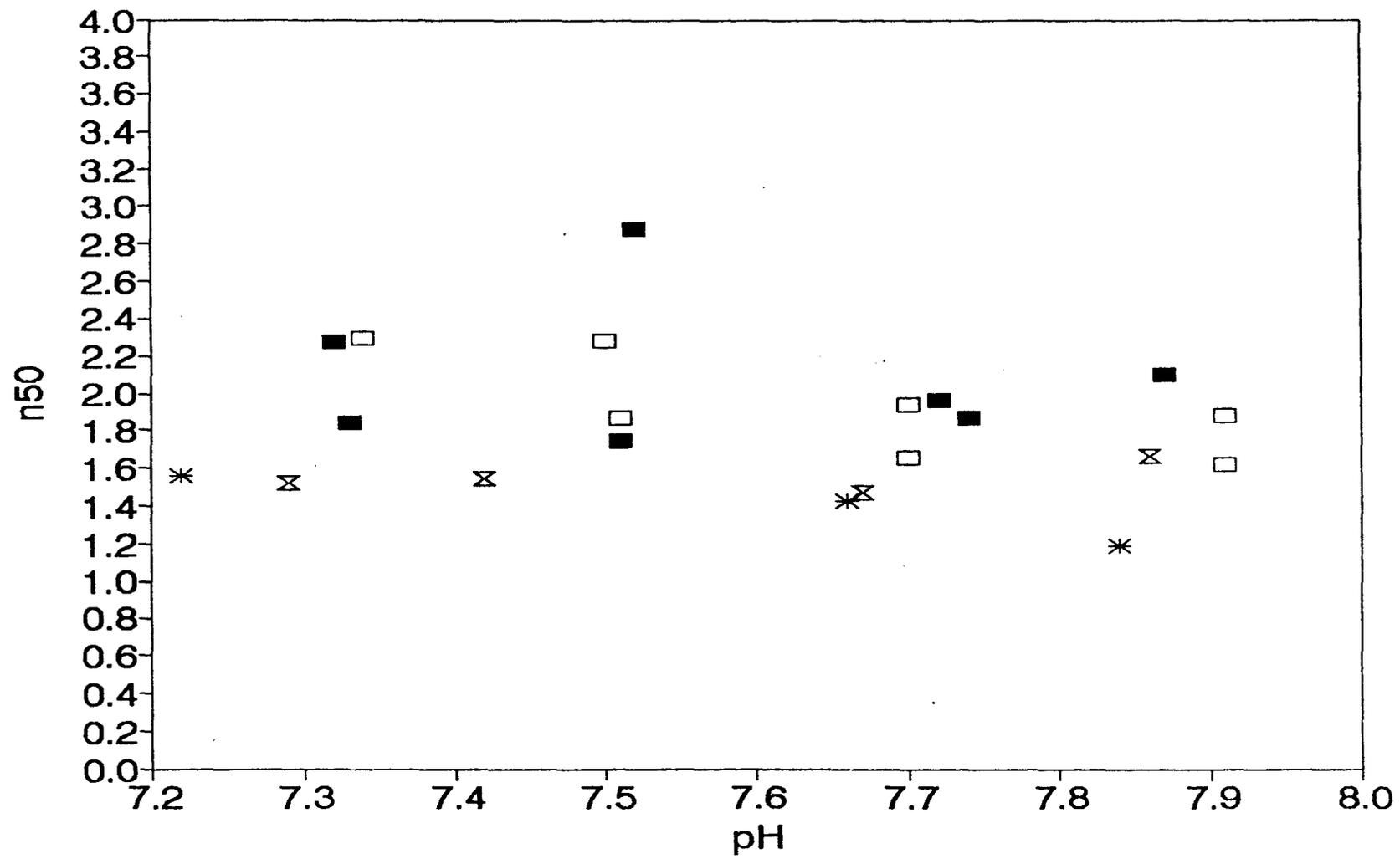


Figure 10. The effect of magnesium on the cooperativity of C. magister frozen adult 25S hemocyanin and frozen 1st juvenile 25S hemocyanin. □ adult, 16 mmol/L; ⊗ 1st juvenile, 16 mmol/L; ■ adult, 32 mmol/L; \* 1st juvenile, 32 mmol/L.



oxygen affinity of frozen adult 25S hemocyanin (Fig. 11). The effect of calcium on the Bohr coefficient is irregular. The Bohr coefficient changes with changing calcium concentration (Table 6) but not in as consistent a fashion as with the effect of magnesium.

Table 6. Regression equations of  $\log P_{50}$  vs. pH for adult frozen 25S hemocyanin from C. magister in salines with varying calcium concentrations

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$\log P_{50} = -0.92$	(pH) + 8.77	( $R^2 = 0.97$ ),	0 mmol/L $\text{Ca}^{++}$
$\log P_{50} = -1.38$	(pH) + 11.92	( $R^2 = 0.93$ ),	16 mmol/L $\text{Ca}^{++}$
$\log P_{50} = -0.97$	(pH) + 8.49	( $R^2 = 0.97$ ),	32 mmol/L $\text{Ca}^{++}$
$\log P_{50} = -1.35$	(pH) + 10.91	( $R^2 = 0.98$ ),	100 mmol/L $\text{Ca}^{++}$

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The Bohr coefficients at 0 and 32 mmol/L calcium are not significantly different from each other, but they are different from the Bohr coefficients at 16 and 100 mmol/L calcium. The Bohr coefficients at 16 and 100 mmol/L calcium are not significantly different from one another. The relationship between  $\log P_{50}$  and  $\log$  calcium concentration is linear at a given pH (Fig. 12):

$$\log P_{50} = -0.99 \log [\text{Ca}^{++}] + 2.4 \quad (R^2 = 0.99) \quad \text{at pH } 7.8.$$

Cooperativity of adult hemocyanin is not dependent on pH (Fig. 13). However, cooperativity decreases significantly with each increase in calcium concentration from 16 to 100 mmol/L (Table 7).

The relative effect of 16 and 32 mmol/L calcium on oxygen affinity is the same on 1st juvenile frozen 25S

Figure 11. The effect of calcium on the oxygen affinity of C. magister frozen adult 25S hemocyanin.

□ 0 mmol/L; \* 16 mmol/L; + 32 mmol/L;  
■ 100 mmol/L.

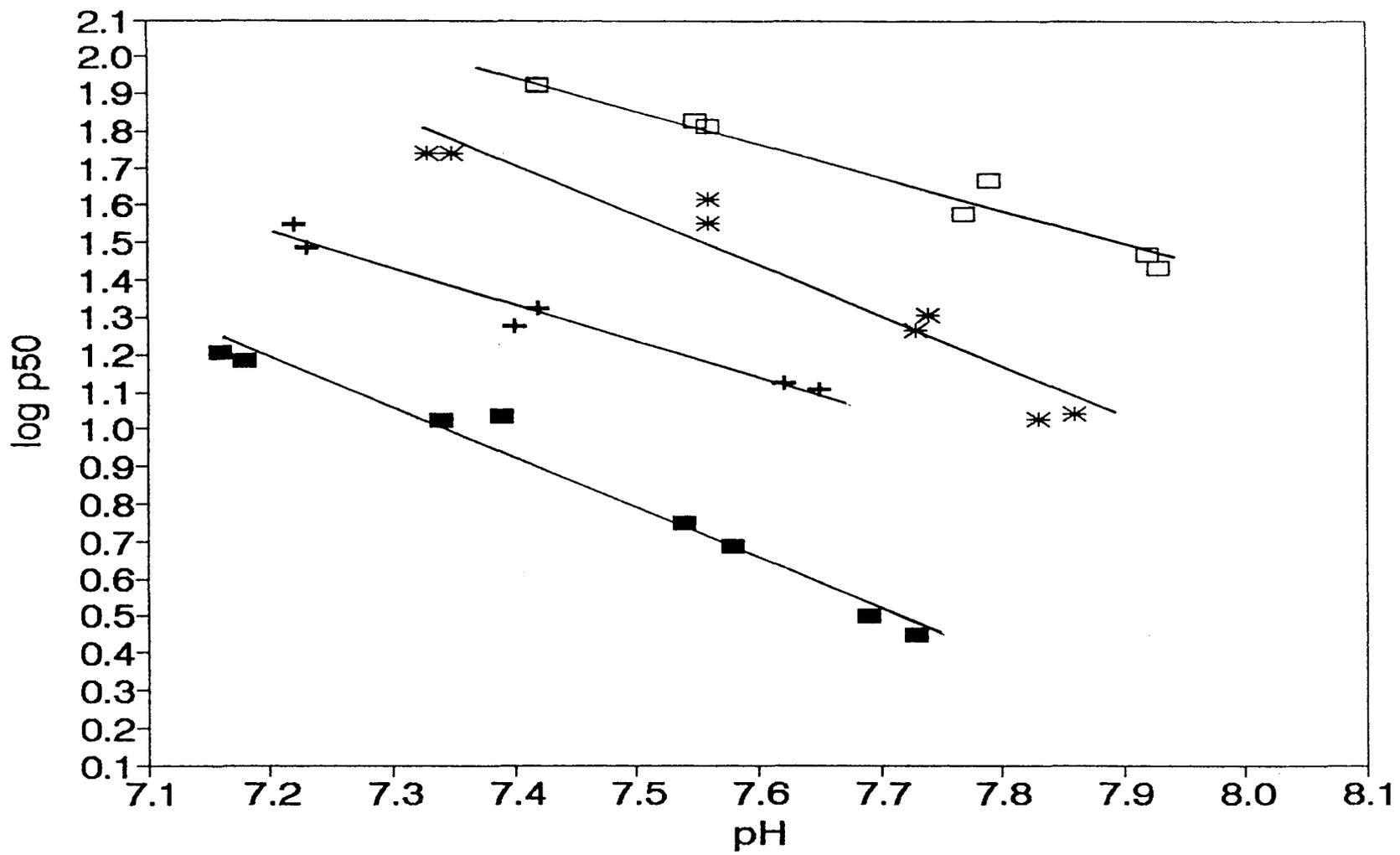


Figure 12. The relationship between  $\log P_{50}$  and  $\log$  calcium concentration for C. magister frozen adult 25S hemocyanin at pH 7.8.

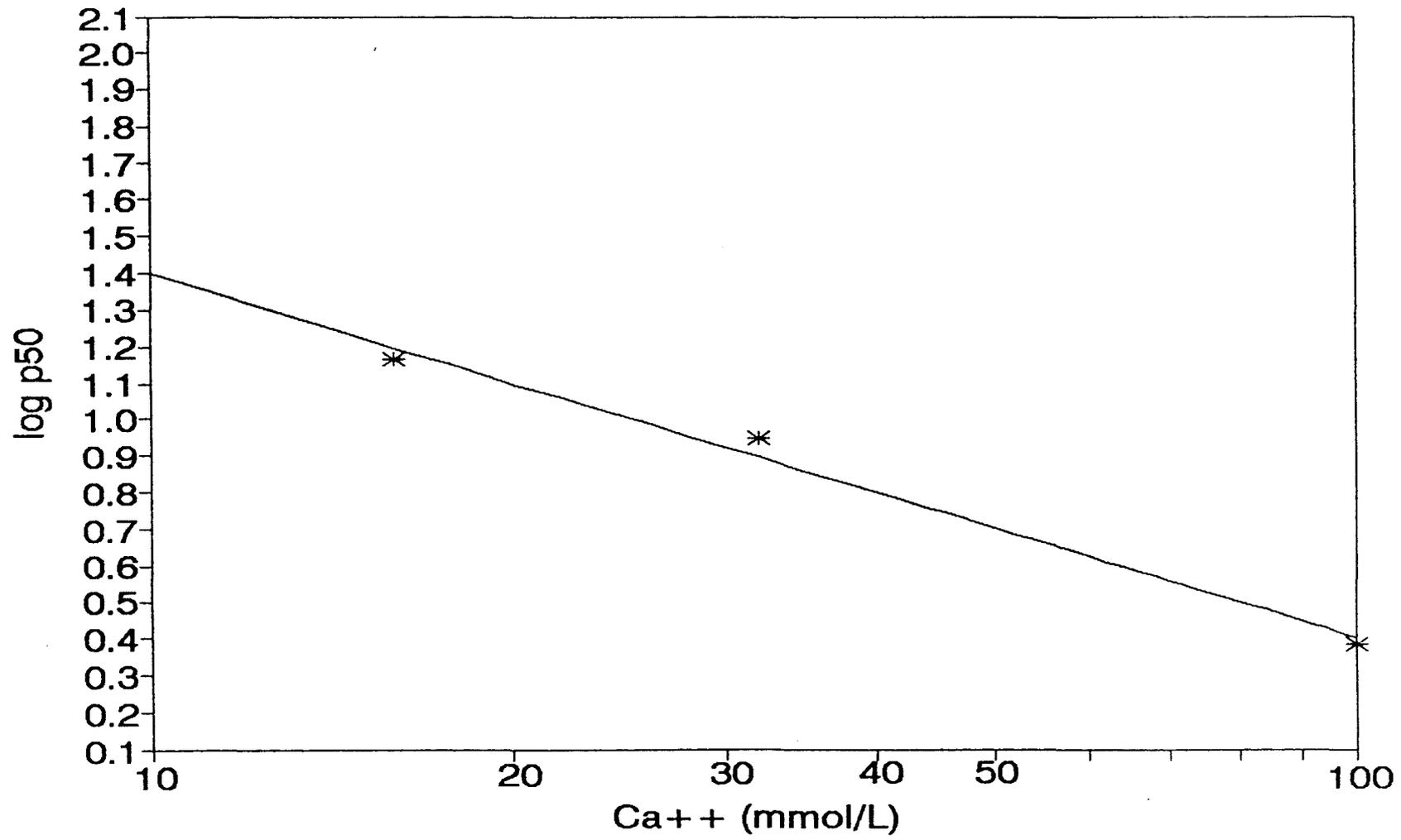


Figure 13. The effect of calcium on the cooperativity of C. magister frozen adult 25S hemocyanin.

□ 0 mmol/L; \* 16 mmol/L; + 32 mmol/L;  
■ 100 mmol/L.

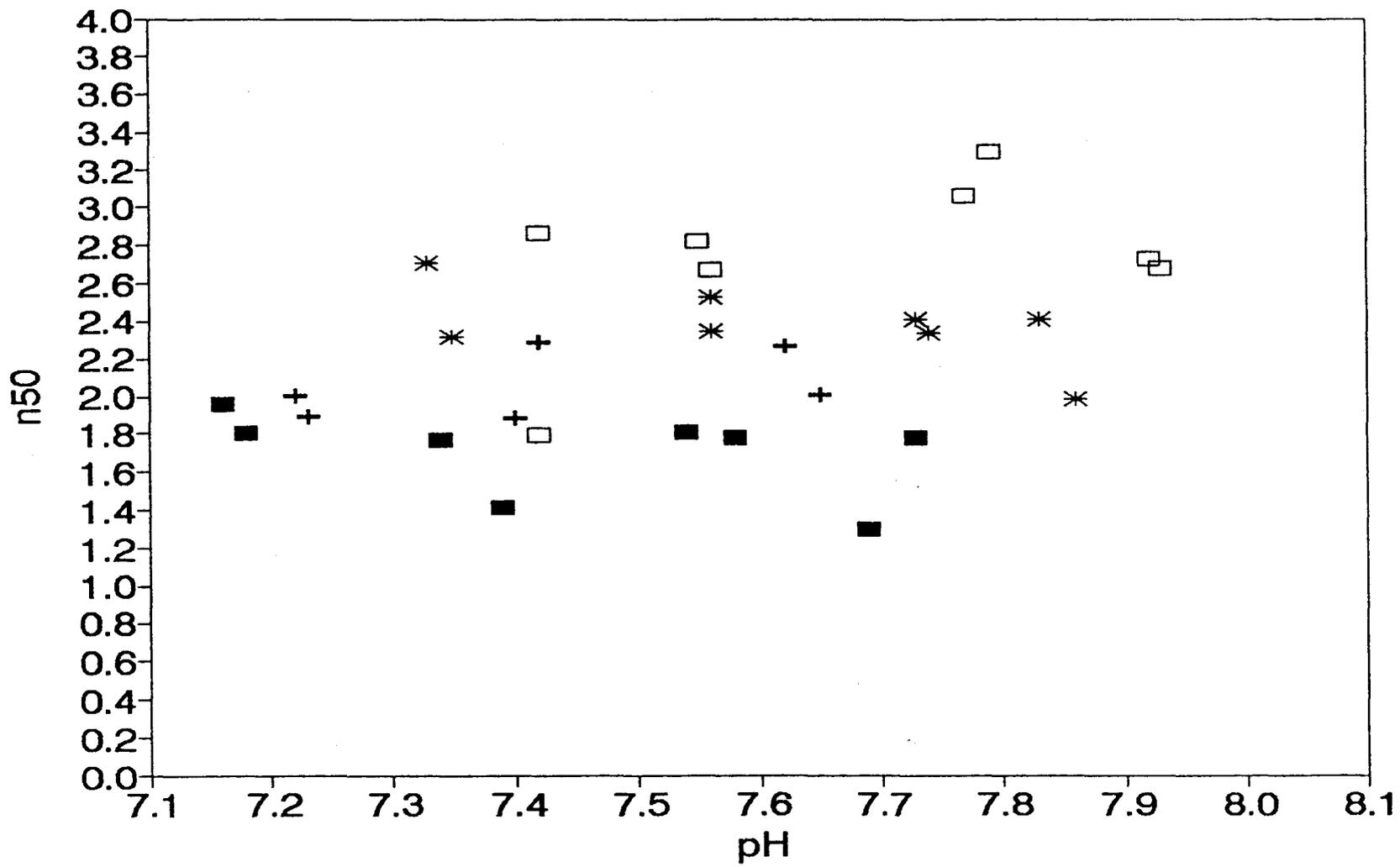


Table 7. Cooperativity of frozen adult 25S hemocyanin at different calcium ion concentrations

<u>Calcium conc.</u>	<u>n<sub>50</sub></u>
0 mmol/L	2.74 ( $\pm$ 0.16) n=8
16 mmol/L	2.35 ( $\pm$ 0.09) n=8
32 mmol/L	2.11 ( $\pm$ 0.09) n=6
100 mmol/L	1.70 ( $\pm$ 0.08) n=8

Note. Mean  $\pm$  S. E..

hemocyanin as on adult frozen 25S hemocyanin (Fig. 14). At pH 7.8 the P<sub>50</sub> of 1st juvenile 25S hemocyanin in 16 mmol/L calcium is 35% higher than the P<sub>50</sub> of the 1st juvenile 25S hemocyanin in 32 mmol/L calcium; the P<sub>50</sub> of adult 25S hemocyanin is 39% higher in 16 mmol/L than in 32 mmol/L. The Bohr coefficients are different between the adult and 1st juvenile hemocyanin in both 16 and 32 mmol/L calcium. However the relative change in the Bohr coefficient with a change in calcium from 16 to 32 mmol/L is the same for the 25S hemocyanin from both stages.

Cooperativities of the 1st juvenile frozen hemocyanin in 16 (n<sub>50</sub> = 1.44  $\pm$  0.07, S.E.) and 32 mmol/L calcium (n<sub>50</sub> = 1.29  $\pm$  0.10, S.E.) are significantly less than the cooperativities of the adult frozen hemocyanin in 16 and 32 mmol/L calcium (Fig. 15).

#### Stage Specific Salines

When both fresh adult 25S and fresh 1st juvenile 25S

Figure 14. The effect of calcium on the oxygen affinity of C. magister frozen adult 25S hemocyanin and frozen 1st juvenile 25S hemocyanin.  
—□— adult, 16 mmol/L; —X— 1st juvenile, 16 mmol/L; —■— adult, 32 mmol/L; —\*— 1st juvenile, 32 mmol/L.

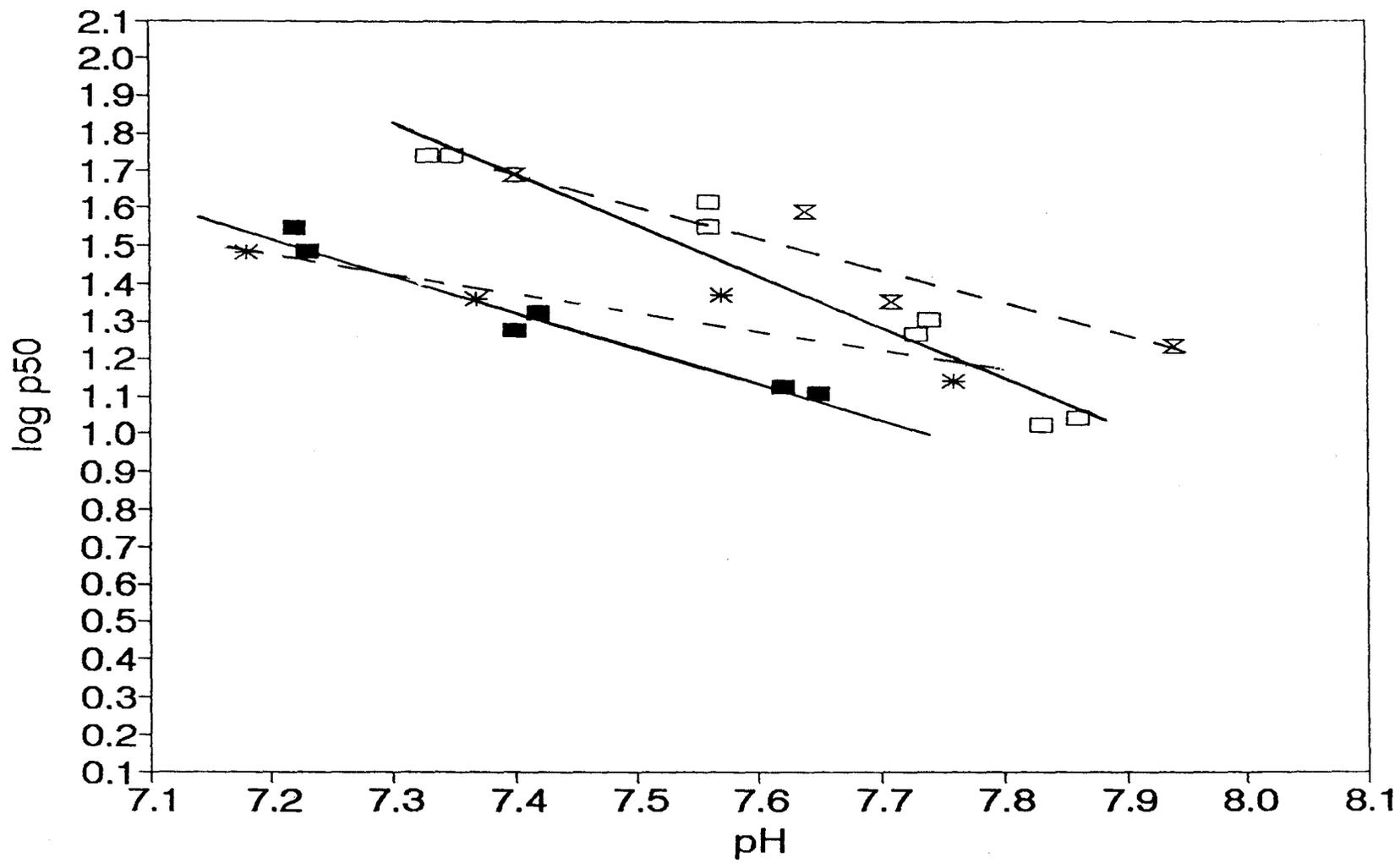
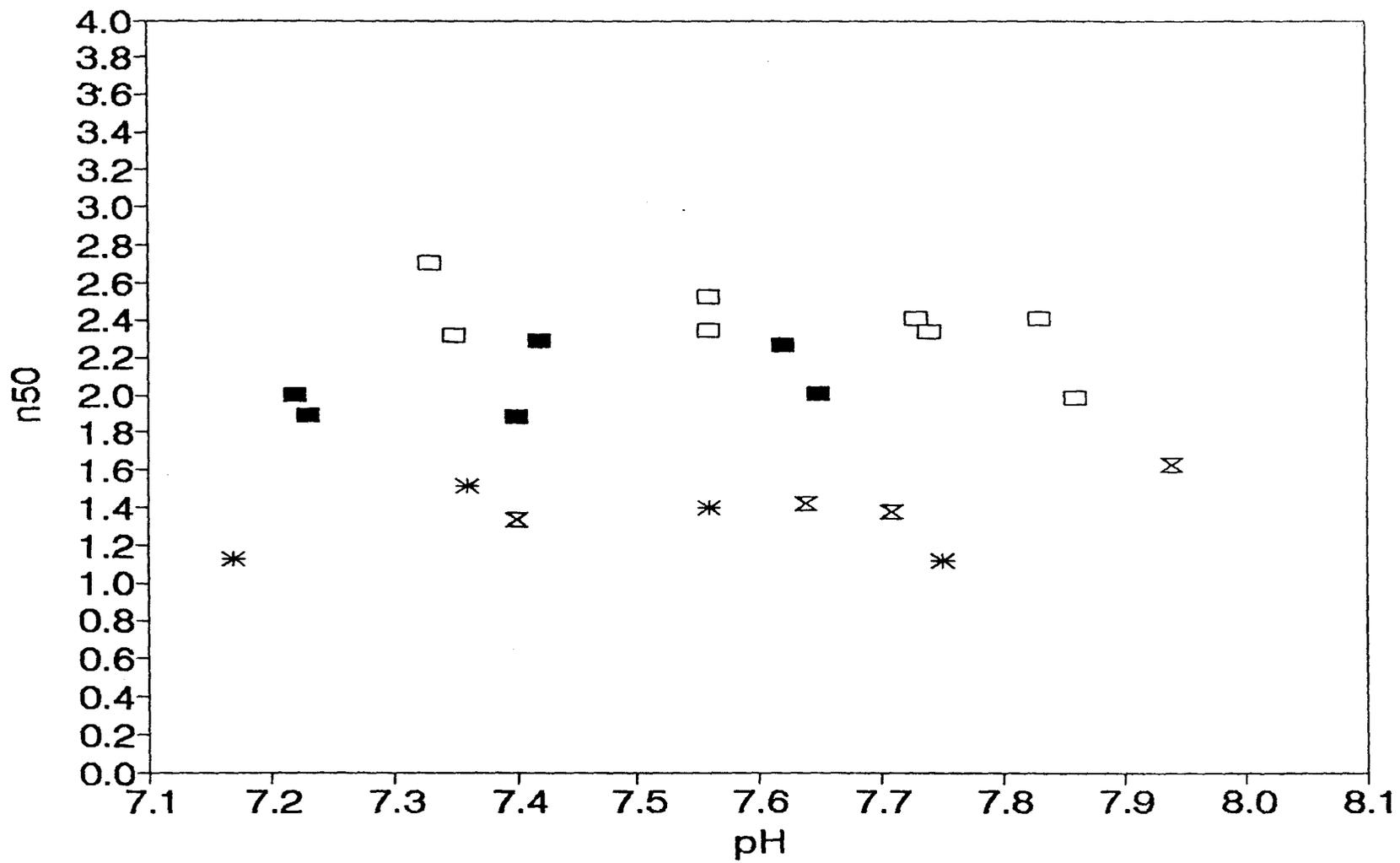


Figure 15. The effect of calcium on the cooperativity of C. magister frozen adult 25S hemocyanin and frozen 1st juvenile 25S hemocyanin. □ adult, 16 mmol/L; ⊗ 1st juvenile, 16 mmol/L; ■ adult, 32 mmol/L; \* 1st juvenile, 32 mmol/L.



hemocyanin are dialyzed against identical 1st juvenile saline, the  $P_{50}$  of the 1st juvenile 25S hemocyanin (at pH 7.8,  $P_{50} = 35.1$  torr  $O_2$ ) is 54% higher than that of the adult 25S hemocyanin (at pH 7.8,  $P_{50} = 16.2$  torr  $O_2$ ), a result similar to that reported by Terwilliger et al. (1986). The Bohr coefficients are not significantly different between stages in identical saline solutions (Table 8 and Fig. 16).

Table 8. Regression equations for  $\log P_{50}$  vs. pH for C. magister fresh 1st juvenile and fresh adult 25S hemocyanins in stage specific salines

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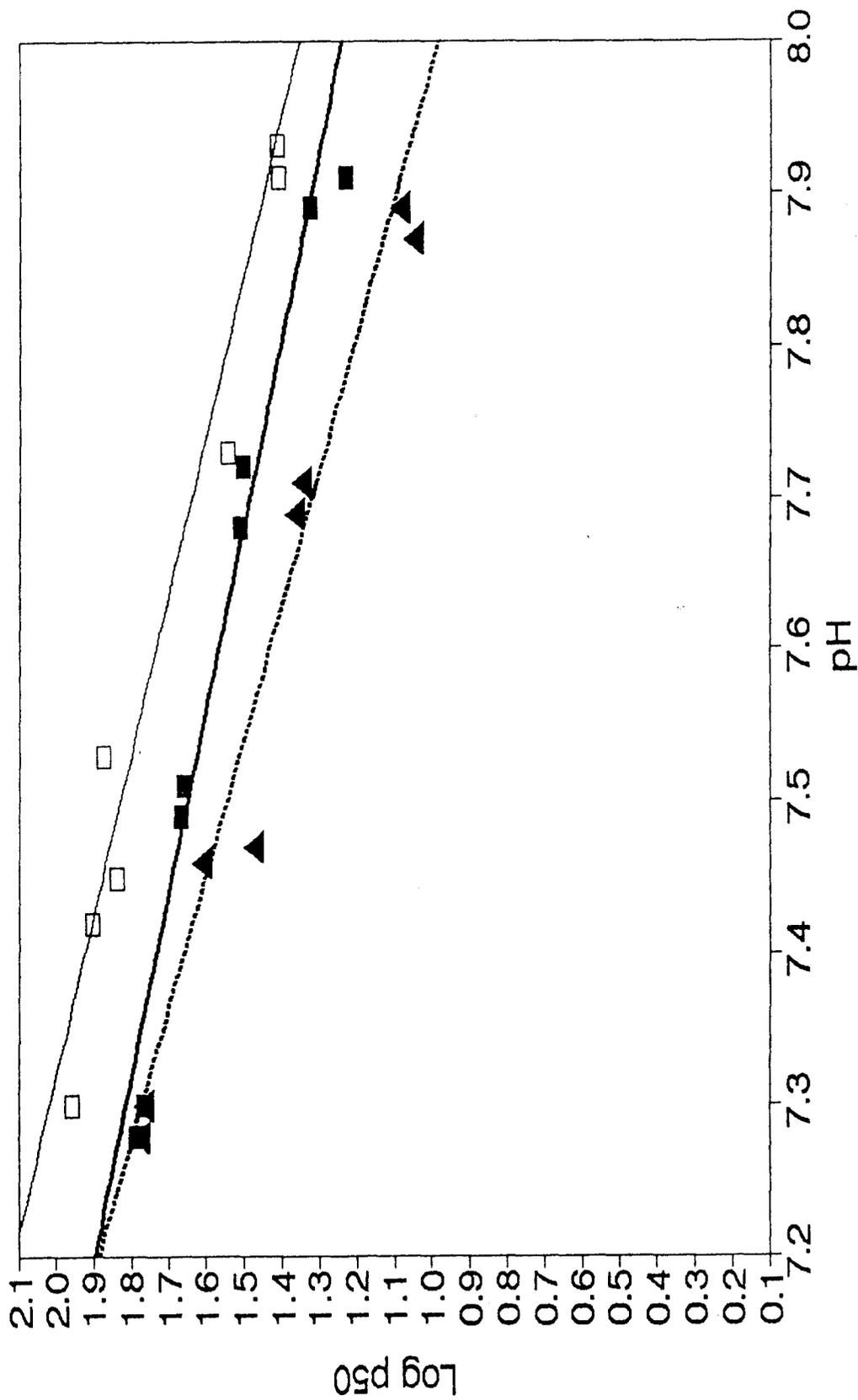
$\log P_{50} = -0.95$ (pH) + 8.93 ( $R^2: 0.96$ ), 1J in 1J
$\log P_{50} = -0.82$ (pH) + 7.81 ( $R^2: 0.95$ ), AD in AD
$\log P_{50} = -1.13$ (pH) + 10.03 ( $R^2: 0.97$ ), AD in 1J

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The Bohr coefficients of fresh 1st juvenile 25S hemocyanin in its stage specific saline (1J in 1J) and of fresh adult 25S hemocyanin in its stage specific saline (AD in AD) are not significantly different. The Bohr coefficient of AD in AD, however, is significantly lower than for AD in 1J. When the 1st juvenile 25S and the adult 25S hemocyanins are in their stage specific salines the  $P_{50}$  of the 1st juvenile (at pH 7.8,  $P_{50} = 25.4$  torr  $O_2$ ) is only about 28% greater than the adult (at pH 7.8,  $P_{50} = 35.1$  torr  $O_2$ ). About half of the difference in oxygen affinity observed when the hemocyanins are in identical salines is eliminated when the hemocyanins are in stage specific salines containing the endogenous

Figure 16. The effect of stage specific salines on the oxygen affinity of C. magister fresh adult 25S hemocyanin and fresh 1st juvenile 25S hemocyanin.

■ adult 25S hemocyanin in adult stage specific saline; ▲ adult 25S hemocyanin in 1st juvenile stage specific saline; □ 1st juvenile 25S hemocyanin in 1st juvenile stage specific saline.



hemolymph concentrations of ions.

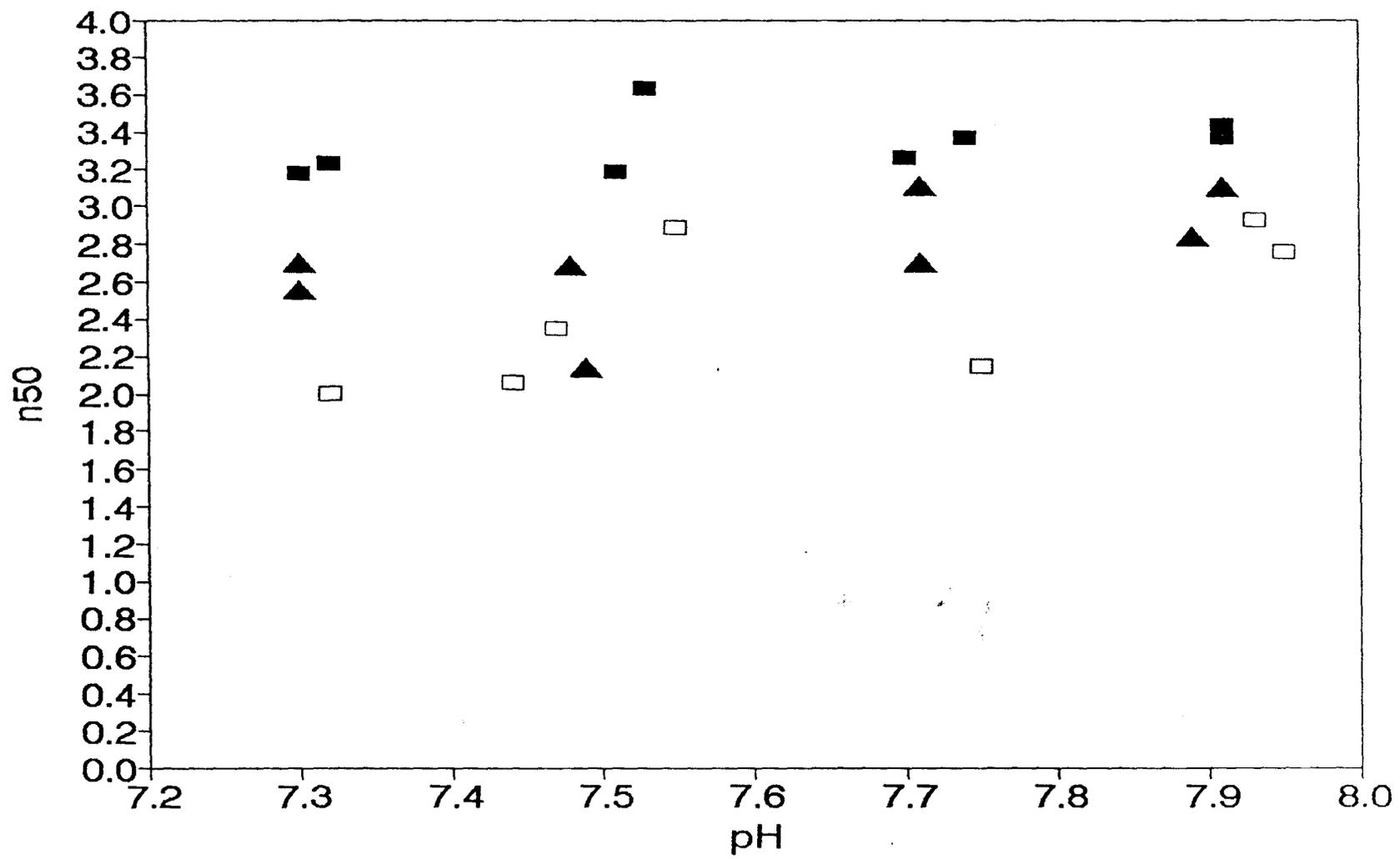
There is no significant relationship between pH and cooperativity for 1J in 1J, for AD in AD or for AD in 1J (Fig. 17). There are significant differences in cooperativity (averaged across pH) among the three above mentioned combinations. The average cooperativity for 1J in 1J ( $n_{50} = 2.45 \pm 0.15$ , S.E.) is significantly less than the cooperativity of AD in AD ( $n_{50} = 3.37 \pm 0.07$ , S.E.). The cooperativity of AD in AD is different from AD in 1J ( $n_{50} = 2.73 \pm 0.11$ , S.E.). The cooperativity of AD in 1J is slightly higher but is not statistically significantly different from 1J in 1J.

#### Discussion

The oxygen carrying capacity of C. magister hemocyanin changes with developmental stage. Compared to the other stages, the 1st juveniles have a very low hemocyanin concentration and therefore a very low oxygen carrying capacity. The implications of this will be discussed further in Chapter V. The carrying capacity calculated for the adult (1.75 vol %) is comparable to the values reported by other researchers for C. magister, 1.01 vol % (McMahon et al., 1978) and for Callinectes sapidus, 1.3 vol % (Booth et al., 1982).

The oxygen affinities of the whole hemolymph from all four stages are indistinguishable when the effect of

Figure 17. The effect of stage specific saline on the cooperativity of C. magister fresh adult 25S hemocyanin and fresh 1st juvenile 25S hemocyanin. ■ adult 25S hemocyanin in adult stage specific saline; ▲ adult 25S hemocyanin in 1st juvenile stage specific saline; □ 1st juvenile 25S hemocyanin in 1st juvenile stage specific saline.



L-lactate is taken into account. This is extremely interesting, because previous measurements of the affinity of the purified 25S hemocyanin from C. magister 1st juveniles and adults in identical salines showed that the oxygen affinity of the 1st juvenile 25S hemocyanin is about 50% lower than the oxygen affinity of the adult 25S hemocyanin. This difference in affinity of the two 25S hemocyanins has been corroborated in the present study. At the same time, there are clearly factors present in the hemolymph of the different stages of C. magister which alter the affinity of the hemocyanin molecule. Either the relatively high intrinsic oxygen affinity of the adult 25S hemocyanin is reduced in the hemolymph or the lower intrinsic oxygen affinity of the larval/juvenile type 25S hemocyanin in the megalopae and juveniles is increased.

Effectors of hemocyanin oxygen binding properties include both organic molecules and inorganic ions. The organic molecules which may alter the affinity and/or cooperativity of hemocyanin include L-lactate, urate and dopamine. L-lactate is the main end product of anaerobiosis in decapod crustaceans. L-lactate was first identified as a modulator of hemocyanin oxygen affinity in Carcinus maenas and Cancer pagurus (Truchot, 1980). Subsequently it has been shown that L-lactate increases the oxygen affinity in other species, including Callinectes sapidus (Booth et al., 1982) and C. magister (Graham et al., 1983). The effect of L-

lactate on a variety of crustacean hemocyanins has been reviewed by Mangum (1983) and by Bridges and Morris (1986). The magnitude of the effect varies between species; the hemocyanin of some species is very sensitive to L-lactate, other hemocyanins are less sensitive. Urate is another recently identified hemolymph factor which increases the oxygen affinity of hemocyanin from decapod crustaceans, including Austropotamobius pallipes (Morris et al., 1985; Morris et al., 1986), Carcinus maenas (Lallier and Truchot, 1989a) and Penaeus japonicus (Lallier and Truchot, 1989b) and Callinectes sapidus (deFur et al., 1990). Dopamine has also been identified as strongly increasing the oxygen affinity of hemocyanin from C. magister (Morris and McMahon, 1989a,b).

Changes in hemolymph inorganic ion concentrations, in particular divalent cations, can affect the aggregation state, oxygen affinity and the cooperativity of decapod crustacean hemocyanins. The importance of divalent cations in the aggregation of hemocyanin subunits has been demonstrated in C. magister (Ellerton et al., 1970) and in Callinassa californiensis (Roxby et al., 1974; Arisaka and Van Holde, 1979), for example. Ten mmol/L magnesium is sufficient to maintain the 25S hemocyanin of C. magister (Ellerton et al., 1970). The presence of calcium or magnesium ions also maintains the aggregation of C. californiensis hemocyanin in the physiological pH range

(Arisaka and Van Holde, 1979).

Divalent cations have variable effects on hemocyanin functional properties. The oxygen affinity of the hemocyanin from Procambarus simulans is increased by raising both calcium and magnesium concentrations; calcium and magnesium also increase the cooperativity (Larimer and Riggs, 1964). In Panulirus interruptus, however, calcium and magnesium decrease the hemocyanin oxygen affinity (Johnson et al., 1983). The oxygen affinity and cooperativity of C. californiensis hemocyanin is increased with increasing magnesium from 0 to 100 mmol/L; calcium also increases oxygen affinity (Miller and Van Holde, 1974). In Carcinus maenas magnesium and calcium increase the oxygen affinity of the hemocyanin (Truchot, 1975). The effect of magnesium on the oxygen affinity of hemocyanin from C. maenas is stronger at higher pH (Truchot, 1975). This interaction in the binding of magnesium and protons has also been described for C. californiensis hemocyanin (Arisaka and Van Holde, 1979). The hemocyanins from Penaeus setiferus (Brouwer et al., 1978), Callinectes sapidus (Mason et al., 1983), Austropotamobius pallipes (Morris et al., 1986) and Birgus latro (Morris et al., 1988) all increase in oxygen affinity with increasing calcium concentration, but the cooperativities do not change. Furthermore, in B. latro magnesium ion does not affect either oxygen affinity or cooperativity. When the results from the present study are

compared with those on other decapod hemocyanins it is clear that C. magister adult 25S hemocyanin shows similarities to other hemocyanins as well as some unique properties.

The results of the present study show that the affinities of the whole hemolymph from different stages in the life cycle of C. magister were indistinguishable from each other when the affinities were adjusted for the effect of L-lactate. No urate was detected in any of the whole hemolymph samples. Therefore L-lactate and urate do not appear to be the factors involved in equalizing the intrinsically different stage specific oxygen affinities of the hemocyanins. Clearly, there must be other factors present in the hemolymph which affect the stage specific hemocyanins in such a way as to make the whole hemolymphs functionally similar. Because of the specific ionic and osmotic regulatory responses of different life stages described in Chapter III, it was decided to examine the effects of magnesium, calcium and stage specific salines on the oxygen equilibria of 25S hemocyanin from 1st juvenile and adult crabs.

The effect of magnesium on C. magister hemocyanin is stronger at higher pH (Fig. ) as mentioned above for both C. maenas (Truchot, 1975) and C. californiensis (Arisaka and Van Holde, 1979) hemocyanins. However, magnesium does not affect the cooperativity of C. magister hemocyanin (Fig. ) as it does C. californiensis hemocyanin (Miller and Van

Holde, 1974).

The effect of calcium on C. magister hemocyanin is clearly different from the effect of calcium on the hemocyanin of other species. The variable effect of calcium on the Bohr coefficient ( $\log P_{50}/\text{pH}$ ) is unique. The linear relationship between  $\log P_{50}$  and  $\log$  calcium concentration, however, is also true for Carcinus maenas (Truchot, 1975), Callinectes sapidus (Mason et al., 1983), Austropotamobius pallipes (Morris et al., 1986) and Birgus latro (Morris et al., 1988) hemocyanins. The effect of calcium on C. magister hemocyanin ( $\log P_{50}/\log [\text{Ca}^{++}] = -0.99$ ) is stronger than for C. maenas ( $\log P_{50}/\log [\text{Ca}^{++}] = -0.28$ ), C. sapidus ( $\log P_{50}/\log [\text{Ca}^{++}] = -0.82$ ), A. pallipes ( $\log P_{50}/\log [\text{Ca}^{++}] = -0.74$ ) and B. latro ( $\log P_{50}/\log [\text{Ca}^{++}] = -0.39$ ). The effect of an increase in calcium causing a decrease in cooperativity of adult C. magister hemocyanin is also unprecedented. Because of the role of divalent cations in maintaining the aggregation of the 25S, two hexamer, hemocyanin molecule, the general understanding has been that an increase in the concentration of divalent cations would increase the interaction between subunits, thereby increasing cooperativity. In the present study, the oxygen binding under conditions of varying calcium was done in the presence of 18 mmol/L magnesium which is sufficient to maintain the aggregation of the 25S molecule (Ellerton et al. 1970). Therefore the decrease in

cooperativity seen with increasing calcium is probably not due to a change in aggregation state. L-lactate, in addition to affecting affinity, also causes a decrease in cooperativity of the hemocyanin in some species, including C. magister (Graham et al., 1983), but not in all species (reviewed by Mangum, 1983; Bridges and Morris, 1986). It has been proposed that L-lactate decreases cooperativity by binding between the subunits (Johnson et al., 1987). The binding sites of other allosteric ligands are not fully characterized and seem to be variable between species. As with L-lactate, the effects of calcium on oxygen affinity and cooperativity appear to be species specific.

Physiologically, a decrease in cooperativity coincident with an increase in affinity, as observed here for C. magister hemocyanin, may cause a decrease in the oxygenation at the gills and an increase in the venous oxygen reserve. The implications of this will be discussed in the next chapter.

Before comparing the effects of magnesium and calcium on adult 25S and 1st juvenile 25S hemocyanin, a brief comment about the effect of freezing on hemocyanin is in order. According to the literature the effect of freezing hemocyanin varies from species to species. In the few species examined, freezing of crustacean hemocyanin does not significantly affect affinity or the Bohr coefficient ( $\log P_{50}/pH$ ) but causes a significant decrease in cooperativity (Morris, 1988). In the present study it was found that

freezing adult 25S hemocyanin not only decreased cooperativity but also significantly increased oxygen affinity. The same effect appears to occur after freezing 1st juvenile 25S hemocyanin; there is a decrease in cooperativity.

Comparison of oxygen binding of frozen adult and 1st juvenile 25S hemocyanin in 16 and 32 mmol/L calcium reveals that the magnitude of the calcium effect is relatively the same between stages. The effect of magnesium on 1st juvenile 25S hemocyanin, however, is greater than on adult 25S hemocyanin; not only does the  $P_{50}$  change to a greater extent for the 1st juvenile but the Bohr coefficient is increased. The greater sensitivity of 1st juvenile hemocyanin to magnesium is particularly intriguing in light of the high magnesium concentrations in the hemolymph of megalopae, 1st and 5th juveniles in 100% seawater compared to adult hemocyanin (Chapter III).

The importance of the difference in hemolymph magnesium concentrations and the different magnesium sensitivities of the two types of hemocyanin is emphasized when the results of the functional studies of adult 25S and 1st juvenile 25S hemocyanin in stage specific salines are compared. As noted earlier, when the two types of 25S hemocyanin are in identical salines the affinity of the 1st juvenile hemocyanin is about 50% less than that of the adult. However, in stage specific salines the 1st juvenile

oxygen affinity is only about 28% lower than that of the adult. There are still unknown factors in the hemolymph of the megalopae, juvenile and adult crabs which shift the affinities and cooperativities so that they are indistinguishable in the whole hemolymph of these stages. The specific ion differences in the whole hemolymph, however, particularly magnesium, appear to play a major role in vivo in compensating for the intrinsic differences in oxygen affinity.

CHAPTER V  
INTEGRATION OF OXYGEN CONSUMPTION, ION REGULATION  
AND HEMOCYANIN FUNCTION

Introduction

There are many interdependent physiological processes which occur simultaneously in living animals. In this thesis the effects of a narrow set of environmental conditions on several processes related to the respiratory physiology of C. magister have been examined. Calculations based on the results of this thesis, including oxygen consumption rates, oxygenation properties of whole hemolymph and ion effects on hemocyanin, and based on values reported in the literature, including hemolymph post-branchial and pre-branchial pH and  $P_{O_2}$  and the effect of temperature on hemocyanin oxygen affinity, lead to predictions about changes which may occur in other facets of the oxygen transport system, such as cardiac output.

Cardiac output can be viewed as an estimate of how hard an animal has to work to maintain sufficient oxygen supply to the tissues and thereby sustain aerobic metabolism. The hemolymph carries the oxygen necessary to fuel oxidative metabolism and is circulated in the hemolymph through the body by the heart. Higher rates of metabolism cause an

increase in cardiac output. Cardiac output,  $Q_H$ , is the volume of hemolymph pumped by the heart per unit body mass per unit time (ml/gm/hr).

#### Calculations

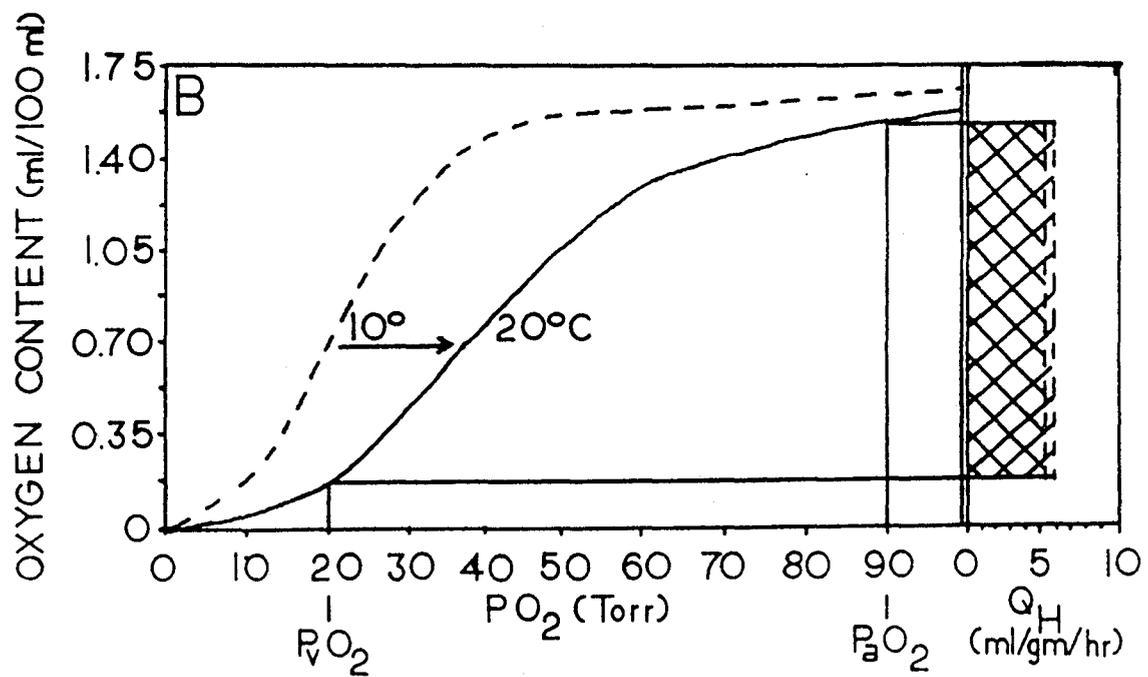
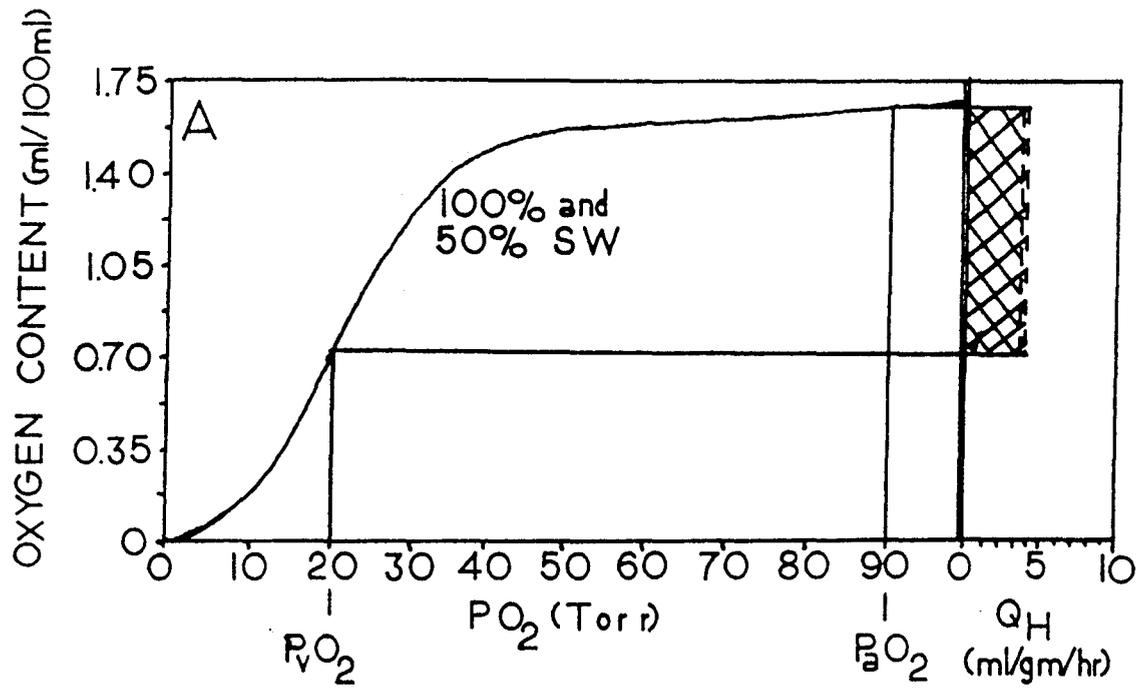
Using the oxygen equilibria curves of the whole hemolymph adjusted to constant L-lactate (Chapter IV), the oxygen carrying capacity of the hemolymph,  $C_{\text{tot}}\text{O}_2$  (ml  $\text{O}_2$ /100 ml) (Chapter IV) and the weight specific rate of oxygen consumption,  $Q_{\text{O}_2}$  (ml  $\text{O}_2$ /gm/hr) (Chapter II), it is possible to estimate the weight specific cardiac output,  $Q_H$  (ml/gm/hr) according to the Fick principle:  $Q_{\text{O}_2} = Q_H (C_{\text{post}}\text{O}_2 - C_{\text{pre}}\text{O}_2)$ .  $C_{\text{post}}\text{O}_2$  is the amount of oxygen in the hemolymph at post-branchial  $p\text{O}_2$  and  $C_{\text{pre}}\text{O}_2$  is the amount of oxygen in the hemolymph at pre-branchial  $p\text{O}_2$ .

The average post-branchial and pre-branchial pHs for adult C. magister at rest generally do not differ by more than 0.01 to 0.05 pH units and range from 7.8 to 7.9 (Johansen et al., 1970; McMahon et al., 1978; McDonald et al., 1979; Wheatly, 1985). When the crabs are active, the pre-branchial pH may drop as low as pH 7.3 and post-branchial pH to pH 7.5 (McDonald et al., 1979). The post-branchial partial pressure of oxygen ( $p_a\text{O}_2$ ) is 90 torr, and the pre-branchial partial pressure of oxygen ( $p_v\text{O}_2$ ) is 20 torr in immersed C. magister crabs at rest and may be lower than 10 torr when the animals are active (Johansen et al.,

1970). Cancer magister hemolymph oxygen affinity is decreased by about 50% with a 10°C increase in temperature (Johansen et al., 1970; Burnett et al., 1988). By using the ion concentrations measured in the hemolymph of the different stages in reduced salinity and the in vitro effects of these ions on the hemocyanin oxygen affinity it is possible to estimate the extent to which oxygen delivery by the hemolymph may be affected by these ion changes. The relative effect of calcium ions is assumed to be similar in both adult and juvenile hemocyanin, and the effect of magnesium to be slightly stronger in juvenile hemocyanin based on results in Chapter IV. In the following discussion the values for post-branchial and pre-branchial pH, partial pressure of oxygen and the effect of temperature on oxygen affinity are assumed to be the same for the juvenile and larval stages as for the adults.

Figure 1 shows an experimentally derived oxygen equilibrium curve for C. magister adult whole hemolymph at pH 7.9, 10°C, adjusted to 0.05 mmol/L L-lactate ( $\log P_{50} = 1.35$ ,  $n_{50} = 3$ ). The ordinate indicates the oxygen content or oxygen carrying capacity (ml O<sub>2</sub>/100 ml) of the hemolymph. The oxygen contents at post-branchial and pre-branchial partial pressures of oxygen are indicated. The total amount of oxygen given up or delivered by the hemolymph as it circulates can then be estimated by subtracting the oxygen content at  $p_aO_2$  from that at  $p_vO_2$ . The amount of oxygen

Figure 1. Estimated cardiac output for adult C. magister.  
(A) 10°C,  100% seawater,  50% seawater.  
(B) 20°C,  100% seawater,  50% seawater



delivered by the hemolymph ( $C_{\text{post}}\text{O}_2 - C_{\text{pre}}\text{O}_2$ ) is represented by the dashed vertical lines on the right side of the graph. According to the Fick principle the oxygen consumption (Chapter II, Table 1), shown as the diagonally striped area in the right side of the figure, divided by the amount of oxygen delivered is equal to the cardiac output, plotted on the abscissa on the right side of the figure:  $Q_H = Q_{\text{O}_2} / (C_{\text{post}}\text{O}_2 - C_{\text{pre}}\text{O}_2)$ . Note that the scales of the oxygen content axis and of the cardiac output axis are different for the different stages (Figs. 1, 2, 3 and 4).

In Figures 1B, 2, 3 and 4 the  $\log P_{50}$  of the equilibrium curves are derived from the effects of divalent cations on oxygen affinity (Chapter IV) and the effects of temperature on oxygen affinity. The slope of the curves between 25% and 75% oxygen content equals 3, the average cooperativity of whole hemolymph at 10°C in 100% seawater. The curves below 25% and above 75% are approximations based on actual curves with  $n = 3$  and  $\log P_{50}$ 's close to the derived value.

### Results

Table 1 shows the cardiac output calculated for the four stages of C. magister in 100% and 50% seawater at 10°C and 20°C. Using the average weight of the different life stages used for oxygen consumption measurements the log of weight specific cardiac output ( $\log Q_H$ ) is linearly related

Table 1. Cardiac output of four life stages of C. magister

STAGE	SEAWATER	CARDIAC OUTPUT	
		10°C	20°C
Megalopa	100%	168	240
	50%	158	311
1st Juvenile	100%	105	423
	50%	128	436
5th Juvenile	100%	23	32
	50%	18	34
Adult	100%	4	5
	50%	4	5

Note. Cardiac output in ml O<sub>2</sub>/gm/hr.

to the log of wet weight (log W) (Table 2 and Figure 5).

Table 2. Regression equations for log  $Q_H$  vs. log W

log $Q_H$	=	-0.41 (log W) + 1.64	( $R^2 = 0.99$ ),	10°C,	100% SW
log $Q_H$	=	-0.43 (log W) + 1.62	( $R^2 = 0.99$ ),	10°C,	50% SW
log $Q_H$	=	-0.47 (log W) + 1.92	( $R^2 = 0.96$ ),	20°C,	100% SW
log $Q_H$	=	-0.50 (log W) + 1.95	( $R^2 = 0.97$ ),	20°C,	50% SW

Figures 1A and 1B illustrate how the amount of oxygen delivered by the hemolymph of the adult can be affected by temperature. First, since there is no significant change in hemolymph magnesium and calcium when the adult crab is in 50% seawater, there is no change in effect of ions on the whole hemolymph oxygen affinity. At 10°C (Fig. 1A) the cardiac output is not substantially different between 100% and 50% seawater because the measured rate of oxygen consumption of the adult is not affected by salinity. At 20°C (Fig. 1B) the shift of the oxygen equilibrium curve to the right means that at  $p_vO_2$  a greater amount of oxygen is released, but a high level of saturation is still maintained at  $p_aO_2$ . The decrease in oxygen affinity due to increased temperature thus enables an overall larger delivery of oxygen to the tissues. So, despite the fact that  $Q_{O_2}$  more than doubles from 10°C to 20°C, the cardiac output is only slightly increased from what it was at 10°C. The decrease in adult hemocyanin oxygen affinity at 20°C results in a lower venous reserve of oxygen at 20°C than at 10°C. Further shift

of the curve to the right would only marginally increase oxygen unloading and might decrease loading at the arterial  $pO_2$ . The adult crab, therefore, can cope with increased temperature without having to work much harder, but it has little oxygen reserve left.

Figures 2, 3 and 4 show the estimated effects of temperature and hemolymph magnesium and calcium changes on the oxygen delivery and on the cardiac output of megalopa and 1st juvenile and 5th juvenile. In 50% seawater at both 10°C and 20°C, the megalopae have half the concentration of magnesium in their hemolymph as they have in 100% seawater; hemolymph calcium does not change significantly. As a result of the decrease in hemolymph magnesium the oxygen affinity of the hemolymph will be somewhat reduced as shown in Figures 2A and 2B. At 10°C (Fig. 2A) in 50% seawater, this small reduction in affinity at means a larger delivery of oxygen, and since the  $Q_{O_2}$  of megalopae is not significantly altered by salinity at 10°C the cardiac output would actually be reduced. However, the venous reserve is also reduced. The cardiac output calculated for megalopae at 20°C in 100% seawater is higher than at 10°C in 100% seawater. Furthermore, the venous reserve is reduced even more under these conditions. The rate of oxygen consumption at 20°C in 50% seawater for the megalopae is significantly higher than at 20°C in 100% seawater and cardiac output is also higher. The cardiac output at 20°C in 50% seawater is almost twice

Figure 2. Estimated cardiac output for C. magister megalopa.  
(A) 10°C,  100% seawater,  50% seawater.  
(B) 20°C,  100% seawater,  50% seawater

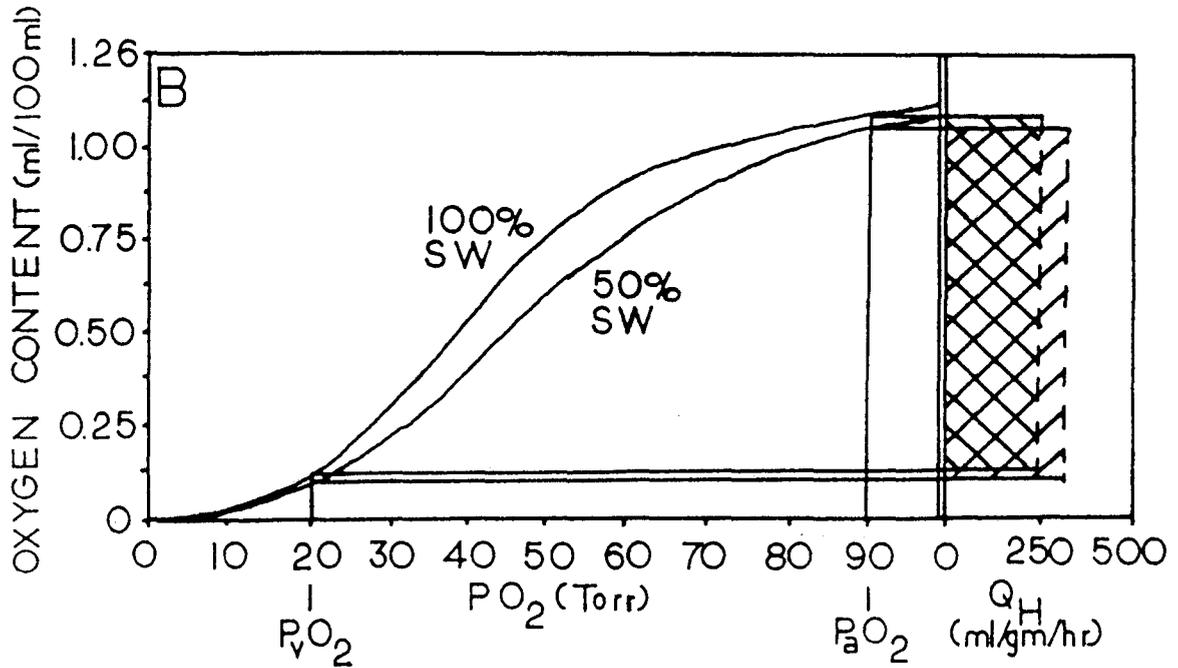
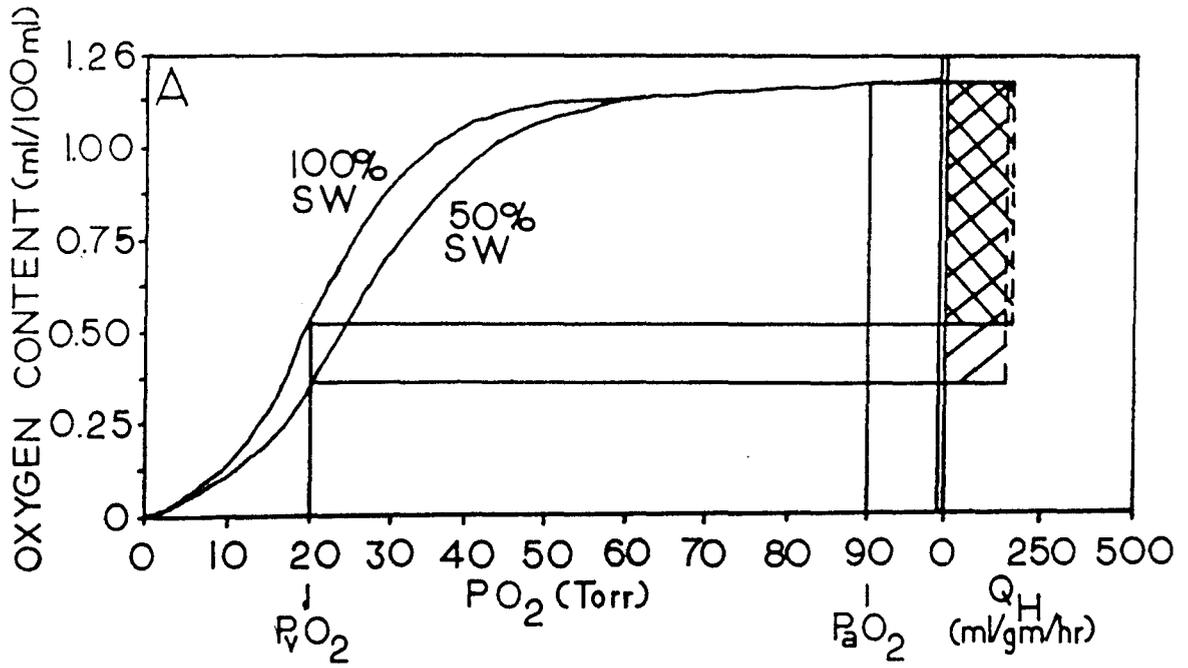


Figure 3. Estimated cardiac output for C. magister 1st juvenile.

(A) 10°C,  100% seawater,  50% seawater.

(B) 20°C,  100% seawater,  50% seawater

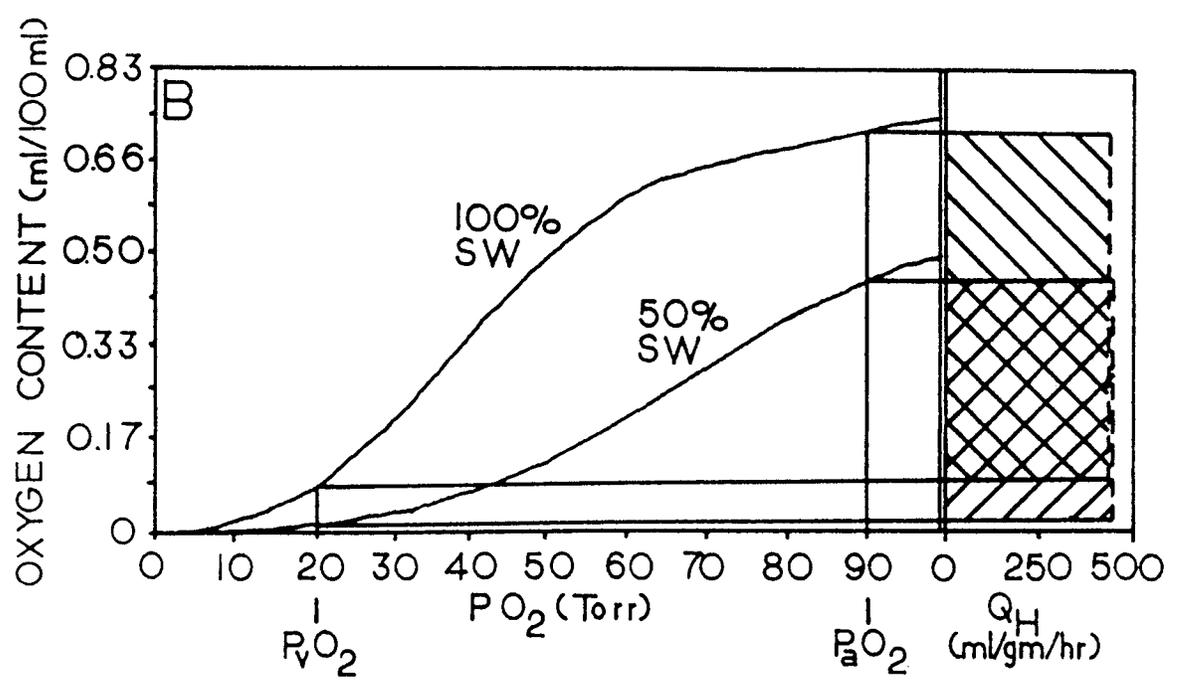
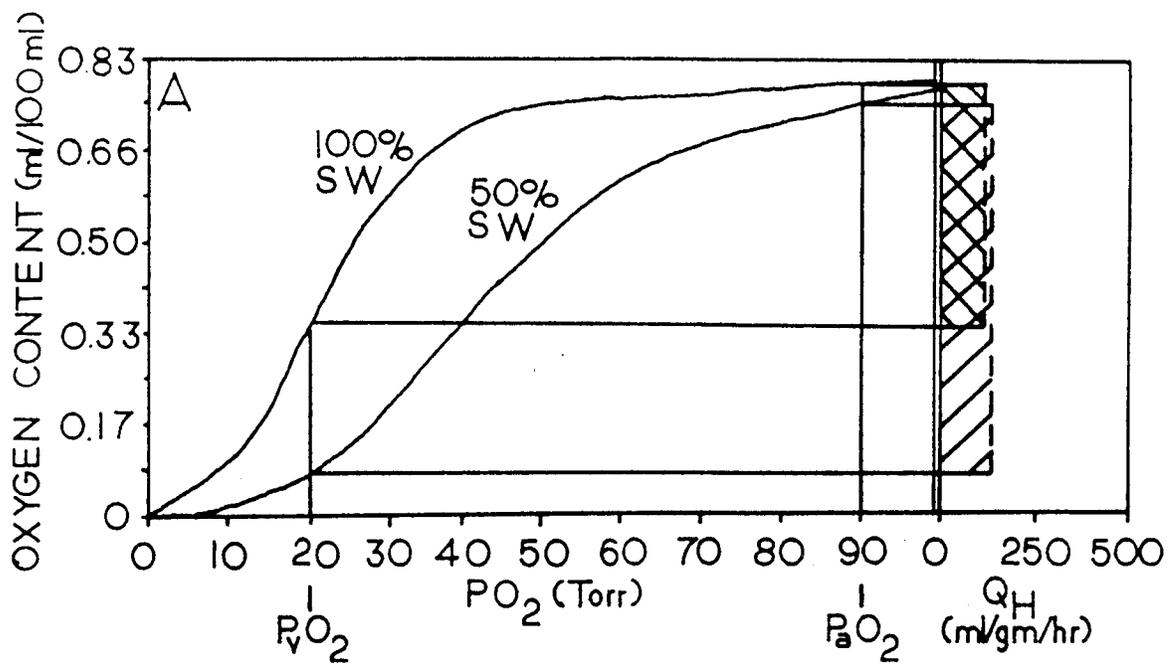


Figure 4. Estimated cardiac output for C. magister 5th juvenile.

(A) 10°C,  100% seawater,  50% seawater.

(B) 20°C,  100% seawater,  50% seawater

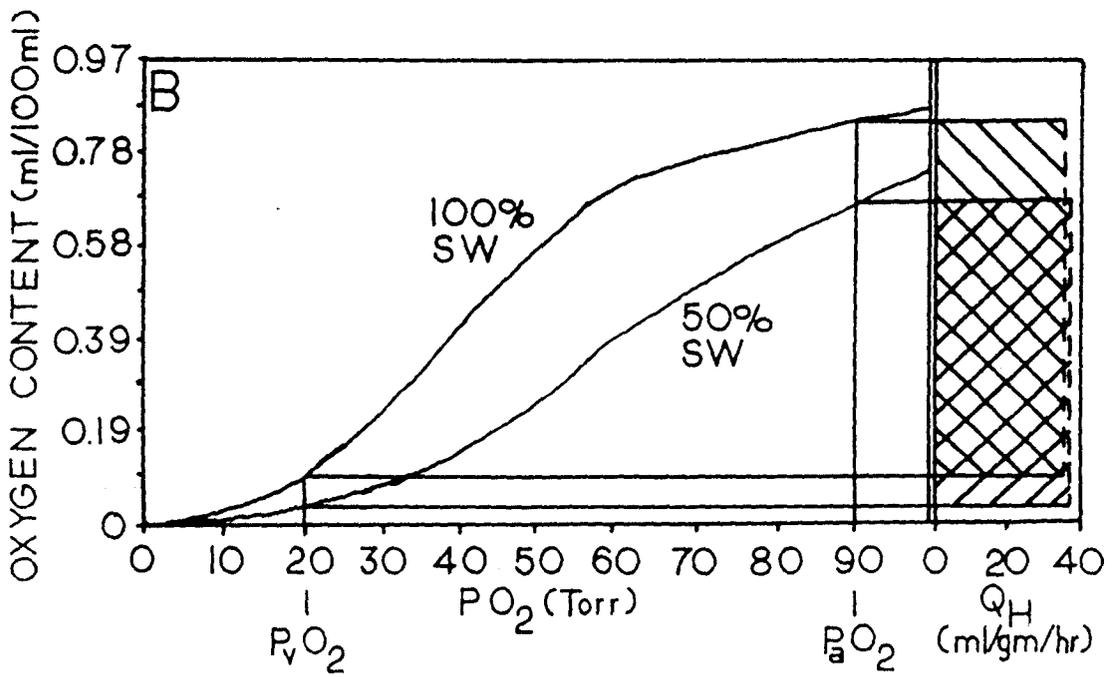
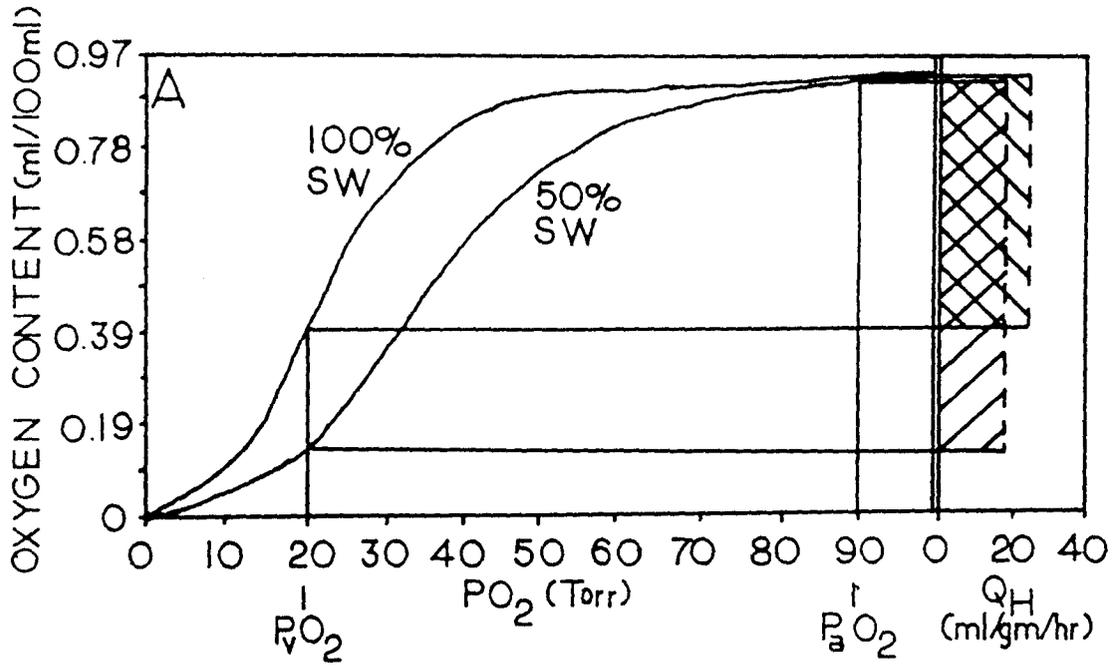
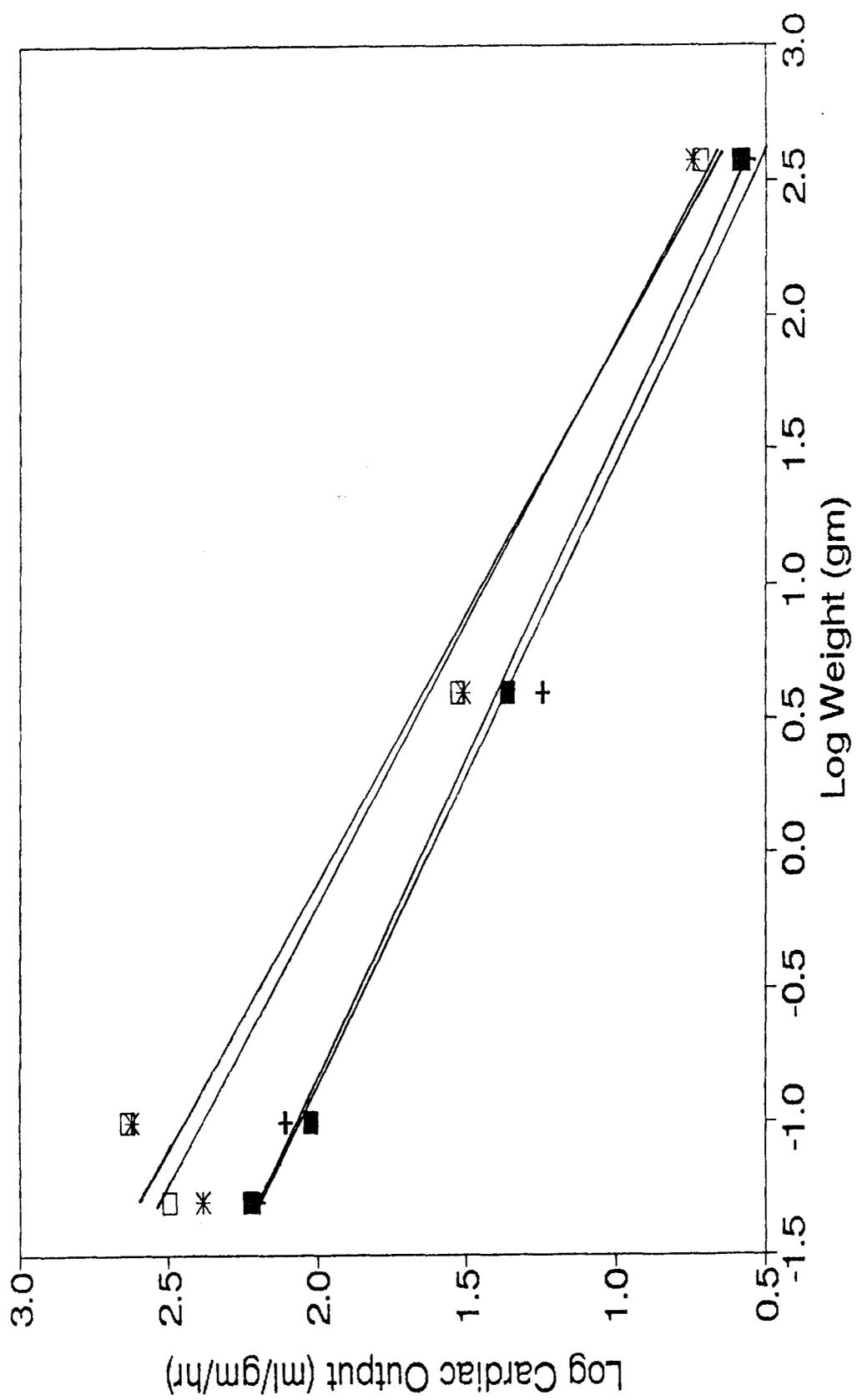


Figure 5. The relationship of  $\log Q_H$  and  $\log W$  for four life stages of C. magister.  
■ 10°C, 100% seawater;  
+ 10°C, 50% seawater; \* 20°C, 100% seawater;  
□ 20°C, 50% seawater.



as high as at 10°C in 100% seawater. The combination of increased cardiac output and reduced venous reserve leads one to suppose that the potential for increased aerobic activity (higher rate of oxygen consumption) is limited at 20°C and 50% seawater for the megalopae.

The 1st juvenile hemolymph has significantly lower concentrations of magnesium and calcium in 50% seawater than in 100% seawater at both 10°C and 20°C. The magnitude of the effect due to calcium is very large because of the steepness of the relationship between  $\log P_{50}$  and  $\log$  calcium concentration (Chapter IV, Fig. 12). Although magnesium concentration changes to a greater degree than calcium, the experimentally determined change in affinity due to magnesium is less than that from calcium. The potential increase in cooperativity due to the decrease in calcium is very small. The decrease in oxygen affinity based on ion differences would mean a much lower affinity in 50% seawater than in 100% seawater (Figs. 3A and 3B). At 10°C (Fig. 3A), despite the greater unloading of oxygen caused by the lower affinity in 50% seawater, the cardiac output is slightly higher in the low salinity condition. At 20°C (Fig. 3B) in 50% seawater, the decrease in hemocyanin oxygen affinity due to temperature in addition to the effect of ions would severely decrease the amount of oxygen delivered since the hemolymph is not very highly saturated with oxygen at arterial  $pO_2$ . The extremely high rate of oxygen consumption

combined with the small amount of oxygen delivered at 20°C in 50% seawater mean that cardiac output would be increased 4-fold from the 100% seawater 10°C conditions.

The reductions in hemolymph magnesium and calcium concentrations in the hemolymph of the 5th juvenile in 50% seawater are not as large as those which occur in the hemolymph of the 1st juvenile. Also, the rate of oxygen consumption of the 5th juvenile is not as sensitive to temperature as that of the 1st juvenile. The effects of changes in hemolymph divalent cations and temperature on the oxygen delivery and cardiac output of 5th juveniles is shown in Figure 4A and 4B. In comparison with the estimated oxygen equilibria curves of the 1st juvenile, it is clear that the smaller change in hemolymph ions in the 5th juvenile may be very important, particularly at 20°C. At 20°C (Fig. 4B) in 50% seawater, the hemolymph of the 5th juvenile is much more saturated with oxygen at  $p_aO_2$  than the 1st juvenile. The difference in oxygen loading can have a large effect on the amount of oxygen delivered and therefore on the cardiac output. As in all of the stages, the potential for increased activity is limited by the low venous reserve in the 5th juveniles at 20°C in 50% seawater.

#### Discussion

The  $Q_H$  calculated for the adult in 100% seawater at 10°C, 4 ml/gm/hr, is comparable to those previously

determined for C. magister, 4.3 ml/gm/hr (McMahon et al., 1979) and another related crab of similar size, Cancer productus, 3.6 ml/gm/hr (deFur and McMahon, 1984).

From the calculations presented in this chapter it is apparent that at high temperature and low salinity the oxygen transport system of all stages, particularly the megalopae and juveniles, is probably near the limit of its function. The rate of oxygen consumption used to calculate the cardiac output is a routine rate; the crabs were not totally inactive nor were they hyperactive. Thus, a certain low level of activity is incorporated in the values given for cardiac output. If it were necessary for a crab to increase its activity, i.e. escape from a predator, its rate of oxygen consumption would increase, possibly beyond the capacity of the oxygen transport system.

It is known that increasing activity in adult C. magister causes a rapid decrease in hemolymph pH, both venous and arterial, and there is an increase in hemolymph L-lactate, a product of anaerobiosis (McDonald et al., 1979). The presence of lactate in the hemolymph only partially counteracts the decrease in hemocyanin oxygen affinity caused by the decrease in pH (reviewed by McMahon, 1986). The net reduction in oxygen affinity of the hemolymph during activity may decrease the amount of oxygen loaded at the gills, a serious problem compounded by high temperature and low salinity. A possible compensating factor, urate, has

been shown to increase oxygen affinity of hemocyanin (Morris et al., 1965; Morris et al., 1986). However, urate does not increase in the hemolymph as a result of increased activity in C. magister (McDonough, 1990) or in Callinectes sapidus (Lallier and Walsh, 1990). It has also been proposed that dopamine has a role as a modulator of hemocyanin oxygen affinity in vivo, and it has been shown to increase the oxygen affinity of C. magister hemocyanin in vitro (Morris and McMahon, 1989a,b). Dopamine also increases the rate of beating of the scaphognathite (the appendage which draws water into the gill chamber) (Wilkins et al., 1985), which would in turn increase the rate and volume of water passing over the gills. Assuming the crabs are in an area with ample oxygen, an increase in ventilation would maintain a high oxygen gradient across the gills and could increase  $p_{O_2}$ . Because of this dual action of dopamine it may be a significant factor in the ability of C. magister to cope with salinity and temperature stress. This thesis proposes that dopamine may be particularly important in the early juvenile crabs, where the rate of oxygen consumption is greatly increased with temperature.

Dopamine is a catecholamine which is produced in the pericardial organs in crustaceans. The pericardial organs are neurohemal organs situated in the pericardium and release catecholamines, phenolamines and peptides into the circulatory system of crustaceans (Belamarich and

Terwilliger, 1966; Terwilliger et al., 1970; Cooke and Sullivan, 1982). Injection of dopamine into the hemolymph of Carcinus maenas causes a respiratory and cardiac response similar to that which occurs when the crabs are stressed by handling (Wilkins et al., 1985). Given this information it seems likely that dopamine is released from the pericardial organs in response to stress or disturbance imposed on the animal. As mentioned above, when adult C. magister are active the L-lactate level in the hemolymph increases, but reaches maximal levels 1-2 hours after the crabs have stopped being active (McDonald et al., 1979). L-lactate only partially compensates for the effect of pH on the affinity of the hemocyanin. However, it may be that dopamine is released from the pericardial organs when the crabs need to suddenly increase their level of activity, i.e. escape from predators. The rapid release of dopamine from the pericardial organs would have a much more immediate effect than the gradual increase of L-lactate. The possibility that dopamine increases both  $p_aO_2$  and oxygen affinity in vivo would lead to an increase in the amount of oxygen delivered to the tissues by increasing the oxygen content of arterial hemolymph. Thus, although the oxygen transport system of C. magister may be near the limits of its ability to provide oxygen for increased activity when the animals are at 20°C in 50% seawater, the effect of dopamine may be to provide the potential for activity by increasing the amount of

oxygen delivered to the tissues.

The increase in  $Q_{10}$  and  $Q^H$  at 20°C in 50% seawater in the megalopa is substantial. However, the megalopae are in the estuary for a very brief time. Shortly after they enter the coastal and estuarine waters during the late spring and early summer, they metamorphose into 1st juveniles. During the time the megalopae are in the estuary they are in the water column and not on the tideflats. Hence, they are not exposed to the extreme changes in both temperature and salinity to which the juveniles are exposed on the tideflats.

In addition to the high stress condition of combined high temperature and low salinity which the juveniles encounter when they are on the tideflats at low tide, they also spend a large amount of time foraging at lower temperatures in more saline water when the tide is high. It may be under these conditions, 100% seawater at 10°C, that the high magnesium concentration in the hemolymph of megalopae and juveniles is most important. The hemocyanin of the megalopae and juveniles has an intrinsically lower affinity for oxygen compared to the adult. From Chapter IV it is clear that differences in endogenous ion concentrations are important in partially compensating for the difference in oxygen affinity between adult and juvenile type hemocyanin. This slight increase in hemocyanin affinity may make a large difference with regard to overall oxygen

transport. A slightly higher affinity would increase the venous reserve. This in turn may play an important role in increasing oxygen delivery to the tissues when the crabs become active and the resulting decrease in pH lowers affinity. The magnesium related potential for higher activity levels when the tide is high may be very important in allowing the rapidly growing young crabs to move about and take advantage of the rich food source on the submerged tideflats.

The adult crabs are rarely found on the tideflats, yet they appear to be the least stressed based on the changes one sees in  $Q_{10}$  (Chapter II) and  $Q_H$  in response to reduced salinity and increased temperature. These results indicate that they have the capacity to survive the environmental conditions examined here. However, their size may be a significant reason they are not on the tideflats. The tiny juvenile stages are in pools of water in the algae on the tideflats and small depressions in the mud; when they are on the tideflats at low tide they are in effect submerged. The adults on the other hand would be partially or totally emersed, or buried in the mud. In either situation, access to water with which to ventilate the gill chamber is likely to be inadequate to maintain water flow over the gills of the adult crab.

Examining cardiac output provides a relative measure of how hard an animal has to work to sustain aerobic

metabolism. The combination of low salinity and high temperature cause a tremendous increase in the amount of work the different stages of C. magister must do to provide sufficient oxygen to the respiring tissues. Yet the juvenile crabs, which are the most sensitive to temperature and salinity, are abundant on the tideflats where the changes in temperature and salinity can be extreme. The high cost of remaining on the tideflats in low salinity and high temperature when the tide is low must be outweighed by the benefit of being in the tideflat habitat when the tide is high.

## CHAPTER VI

## CONCLUSION

The goal of the research presented in this thesis was to elucidate the effects of short term, tidal changes in salinity and temperature on the respiratory physiology of different life stages of C. magister. The data available, prior to the commencement of this study, on the ontogenic change in hemocyanin structure (Terwilliger and Terwilliger, 1982) and function (Terwilliger et al., 1986) raised questions about the function of the stage specific hemocyanins in oxygen transport. The purified hemocyanin molecule from the megalopa and juvenile crabs has an affinity for oxygen 50% lower than the affinity of the adult hemocyanin when they are in identical saline solutions (Terwilliger et al., 1986). Important considerations in formulating the goals of the present study included possible changes in mode of existence, habitat utilization and the concomitant changes in environmental conditions that occur during development of C. magister from megalopa to adult.

Cancer magister, like many other decapod crabs, undergoes a dramatic change in mode of existence from planktonic to benthic at metamorphosis from megalopa to juvenile. Just preceding metamorphosis, the megalopae enter

the nearshore and estuarine waters from the offshore waters. This transition from oceanic planktonic larval stage to benthic coastal and estuarine juvenile means that the different stages are exposed to different magnitudes of change in environmental salinity and temperature. During development from the juvenile stages to the adult crab there is a transition from inhabiting the tideflats in large numbers as juveniles to inhabiting the coastal areas and deeper channels in the bay, and only rarely inhabiting the lowtide mudflats as adults. The seasonal timing of settlement of the juveniles in the tideflat environment means that they are exposed to extreme variations in salinity and temperature as the tides change. The adults are also exposed to changes in salinity in the subtidal areas, particularly during the winter; temperature variations are less extreme at the bottom of the channels than on the exposed tideflats because of the high thermal capacitance of the water. Given these changes in mode of existence, habitat utilization and changes in environmental conditions that occur during the development from megalopa to adult crab, the question of the physiological significance of the change in hemocyanin structure and function during development is important.

The results presented in this thesis bring to light many important differences at the molecular, physiological and whole animal levels, in the responses of specific stages

to environmental changes. As discussed in Chapter II, the metabolic response to fluctuations in salinity and temperature changes during development. The rate of oxygen consumption of the megalopa is more sensitive to low salinity at 20°C than at 10°C. None of the other stages examined, 1st juvenile, 5th juvenile and adult, showed this type of response to high temperature and low salinity. In fact, the rate of oxygen consumption of these three other stages is not significantly affected by salinity at either 10°C or 20°C. However, the 1st juvenile is far more sensitive to an increase in temperature from 10°C to 20°C than are the other stages examined. There are changes in the metabolic response to salinity and temperature throughout development from megalopa to adult, but the change in sensitivity to salinity at metamorphosis is remarkable.

There are also major changes in ionic and osmotic regulation (described in Chapter III) which occur during development. The juvenile crabs are less able to regulate hemolymph osmolality and ions than are the megalopae. None of the early stages can regulate hemolymph osmolality as well as the adults. An extremely interesting result presented in this thesis is the high concentration of magnesium in the hemolymph of the megalopa and juvenile crabs, compared to the adult crabs in 100% seawater. All four stages have the same calcium ion concentration in their hemolymph in 100% seawater, and only the megalopa and adult

regulate calcium in reduced salinity. The reduction in regulation of ions, in particular magnesium and calcium, has a potentially substantial effect on hemocyanin oxygen affinity.

The results presented in Chapter IV on the oxygen binding function of whole hemolymph from all four stages and of purified 25S hemocyanin from the 1st juvenile and the adult are very interesting. The previously reported difference in the oxygen affinity of the purified hemocyanins from the early juveniles and the adults in identical saline solutions is corroborated in this thesis. However, an important finding of the present study is that this difference in affinity of the stage specific hemocyanin molecules is not apparent in the whole hemolymph when the affinities are adjusted for the effect of L-lactate. Examination of the effects of divalent cations on the oxygen affinity of stage specific hemocyanins indicates that the relative effect of calcium appears to be the same on both the juvenile and adult type hemocyanins. However, the relative effect of magnesium on oxygen affinity appears to be greater on the juvenile hemocyanin than on the adult hemocyanin. This is a particularly interesting finding given the high concentration of magnesium found in the hemolymph of the megalopa and juveniles. Another major result is that when the oxygen binding of the purified hemocyanin is done in salines with the stage specific level of hemolymph ions

present, the difference in affinity is only half of what it was in identical saline solutions. Thus, the difference in magnesium concentrations in the hemolymph of different stages is important in the function of the hemocyanin in the whole animal.

The relationship, described in Chapter V, between the oxygen transport function of hemocyanin under different environmental conditions and changes in cardiac output as a result of altered hemocyanin oxygen affinity and rate of oxygen consumption give some insight into the limitations of the oxygen transport system. The oxygen necessary for oxidative metabolism is carried to the tissues in the hemolymph. Cardiac output, as a measure of the work needed to supply sufficient oxygen to the tissues, can be used as a way of assessing the relative stress imposed on an animal by changes in environmental conditions. The estimations of cardiac output in Chapter V indicate that the 1st juvenile crabs, when exposed to 50% seawater at 20°C, have a far greater increase in the amount of work necessary to maintain an adequate oxygen supply to the tissues than do the other stages examined. This is counterintuitive, given the fact that the juveniles are exposed to these extremes of salinity and temperature far more often than are the other stages. It is proposed in this thesis that dopamine may be a major factor, particularly in the young juvenile crabs, in increasing the delivery of oxygen to the tissues when the

crabs are stressed (see Chapter V). Under less stressful conditions, at 10°C in 100% seawater, the high hemolymph magnesium in the megalopa and juveniles may be a very significant factor in increasing the amount of oxygen carried in the hemolymph to the tissues. The increase in hemolymph oxygen content may in turn be reflected in the ability of the juvenile crabs to sustain higher levels of activity when the tide is high and they can move about and forage.

In this thesis some of the capacities and the limitations of the respiratory physiology of the different life stages of C. magister are brought to light. One of the conclusions is that the extremes of low salinity and high temperature encountered by C. magister during its life cycle bring these animals close to the limit of their physiological abilities.

Another major conclusion of this thesis is that the ontogeny of ion regulation, particularly changes in magnesium regulation, and the ontogeny of hemocyanin structure and function are complementary processes in the development of C. magister, from the megalopa to the adult crab. It is clear that the ontogenic change in hemolymph magnesium regulation, resulting in high hemolymph magnesium in megalopa and juvenile crabs compared to the adults, at 10°C in 100% seawater, partially compensates for the low intrinsic oxygen affinity of the juvenile type hemocyanin.

The relatively high hemolymph magnesium in megalopa and juvenile crabs increases the capacity of the megalopae and juveniles to maintain sufficient oxygen supply and therefore high levels of activity when they are at 10°C in 100% seawater. This is important since the opportunity for foraging by the juveniles on the tideflats is limited by the duration of tidal immersion.

Some further questions arise out of the consideration of the response to environmental stress in C. magister. These include the possible role of dopamine in modulation of hemocyanin oxygen affinity and ventilation, especially in the early juvenile crabs. Under what conditions do hemolymph dopamine levels increase? How long lasting is the effect of dopamine on ventilation and oxygen affinity?

The developmental change in ion regulation raises several questions. The role of the antennal gland in magnesium regulation on adult C. magister is well documented (Holliday, 1980). However, the data presented here show profound changes in magnesium regulation during development. What is the functional capacity of the antennal gland in larval and juvenile crabs and how is it controlled?

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