

1                   **Differential effects of cobalt ions *in vitro* on gill (Na<sup>+</sup>, K<sup>+</sup>)-ATPase kinetics**  
2                   **in the blue crab *Callinectes danae* (Decapoda, Brachyura)**

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21 **Running title:** Cobalt ions and gill (Na<sup>+</sup>, K<sup>+</sup>)-ATPase kinetics

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23 **Keywords:** Cobalt ions; K<sup>+</sup>-phosphatase activity; *Callinectes danae*; gill (Na<sup>+</sup>,K<sup>+</sup>)-ATPase; *p*-  
24 nitrophenylphosphate

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32

33 **Abstract**

34 To evaluate the crustacean gill (Na<sup>+</sup>, K<sup>+</sup>)-ATPase as a molecular marker for toxic  
35 contamination by heavy metals of estuarine and coastal environments, we provide a  
36 comprehensive analysis of the effects of Co<sup>2+</sup> *in vitro* on modulation of the K<sup>+</sup>-phosphatase  
37 activity of a gill (Na<sup>+</sup>, K<sup>+</sup>)-ATPase from the blue crab *Callinectes danae*. Using *p*-nitrophenyl  
38 phosphate as a substrate, Co<sup>2+</sup> can act as both stimulator and inhibitor of K<sup>+</sup>-phosphatase  
39 activity. Without Mg<sup>2+</sup>, Co<sup>2+</sup> stimulates K<sup>+</sup>-phosphatase activity similarly but with a ≈4.5-fold  
40 greater affinity than with Mg<sup>2+</sup>. With Mg<sup>2+</sup>, K<sup>+</sup>-phosphatase activity is almost completely  
41 inhibited by Co<sup>2+</sup>. Substitution of Mg<sup>2+</sup> by Co<sup>2+</sup> slightly increases enzyme affinity for K<sup>+</sup> and  
42 NH<sub>4</sub><sup>+</sup>. Independently of Mg<sup>2+</sup>, ouabain inhibition is unaffected by Co<sup>2+</sup>. Mg<sup>2+</sup> displaces bound  
43 Co<sup>2+</sup> from the Mg<sup>2+</sup>-binding site in a concentration dependent mechanism. However, at  
44 saturating Mg<sup>2+</sup> concentrations, Co<sup>2+</sup> does not displace Mg<sup>2+</sup> from its binding site even at  
45 elevated concentrations. Saturation by Co<sup>2+</sup> of the Mg<sup>2+</sup> binding site does not affect *p*NPP  
46 recognition by the enzyme. Given that the interactions between heavy metal ions and enzymes  
47 are particularly complex, their toxic effects at the molecular level are poorly understood. Our  
48 findings elucidate partly the mechanism of action of Co<sup>2+</sup> on a crustacean gill (Na<sup>+</sup>, K<sup>+</sup>)-  
49 ATPase.

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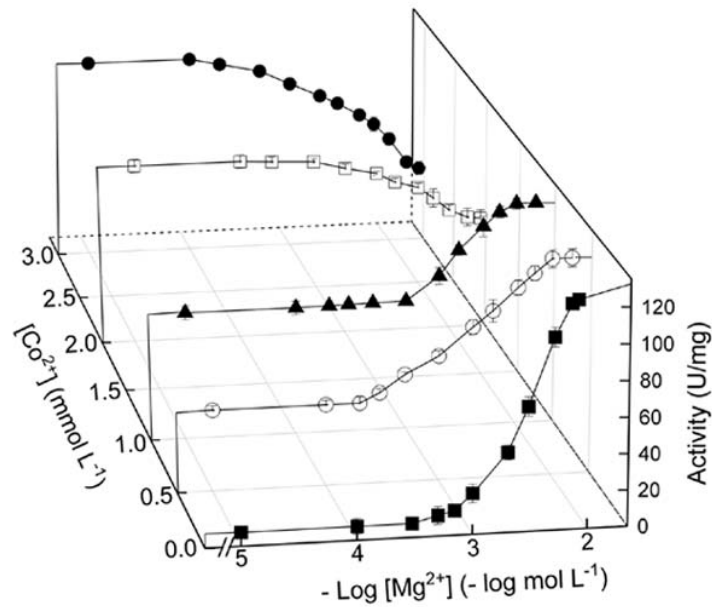
52 **Highlights**

- 53 1. Without Mg<sup>2+</sup>, cobalt ions stimulate the gill (Na<sup>+</sup>, K<sup>+</sup>)-ATPase
- 54 2. Co<sup>2+</sup> has a 4.5-fold greater affinity for the gill (Na<sup>+</sup>, K<sup>+</sup>)-ATPase than does Mg<sup>2+</sup>
- 55 3. Mg<sup>2+</sup> displaces Co<sup>2+</sup> from the Mg<sup>2+</sup>-binding site in a concentration dependent manner
- 56 4. Ouabain inhibition with Co<sup>2+</sup> or Mg<sup>2+</sup> is identical
- 57 5. Saturation by Co<sup>2+</sup> of Mg<sup>2+</sup>-binding sites does not affect substrate recognition

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59 **Graphical Abstract**

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63 **Graphical abstract (synopsis)**

64 Using a crab gill ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase, we demonstrate that  $\text{Co}^{2+}$  inhibits  $\text{K}^+$ -phosphatase

65 activity with  $\text{Mg}^{2+}$ , which is stimulated without  $\text{Mg}^{2+}$ .  $\text{Mg}^{2+}$  displaces  $\text{Co}^{2+}$  from the  $\text{Mg}^{2+}$ -

66 binding site but  $\text{Co}^{2+}$  cannot displace  $\text{Mg}^{2+}$ . Ouabain inhibition is unaffected by  $\text{Co}^{2+}$ ,

67 independently of  $\text{Mg}^{2+}$ . The molecular mechanism of  $\text{Co}^{2+}$  toxicity is partly elucidated.

68

## 69 1. INTRODUCTION

70 Estuarine and coastal environments accumulate toxic contaminants owing to natural  
71 phenomena and/or anthropogenic activities [1]. Such pollutants include a wide variety of  
72 heavy metal ions, organic compounds and various micro/nano-particles [2–4]. Transition  
73 metals stand out specifically as the most abundant contaminants found in aquatic  
74 environments and contribute largely to toxicity [5,6]. Heavy metal ions are particularly  
75 harmful to organisms as they are not degradable and accumulate acutely or chronically in  
76 cells and tissues, altering biochemical and physiological homeostatic mechanisms, which can  
77 lead to demise at the organismal level [7].

78 The uptake and toxicity of heavy metals in aquatic organisms is determined by various  
79 ambient parameters like pH, temperature and salinity [8]. The biotic ligand model is the model  
80 most used to predict metal toxicity in aquatic environments [9]. A profusion of ecotoxicological  
81 studies has highlighted the importance of heavy metal toxicity in aquatic environments [e.g.,  
82 10,11,12,13]. Most studies of heavy metal toxicity in aquatic organisms concern their  
83 bioaccumulation in different tissues [1,14,15] and effects on physiological and biochemical  
84 processes like osmoregulatory capability and aerobic and oxidative stress metabolism [1,11,16].  
85 The toxic effects of heavy metals at the molecular level are poorly known since the  
86 interactions between such metal ions and enzymes are complex [17,18]. The scant information  
87 available regarding the molecular mechanisms of heavy metal toxicity is limited mainly to fish  
88 and crustaceans [19–22].

89 While cobalt is a trace element indispensable for various physiological processes  
90 [21,23,24] it is toxic at high intracellular concentrations [25]. In humans, excessive exposure to  
91 cobalt results in complex health deficits involving heme oxidation, cytotoxicity, oxidative  
92 stress, apoptosis, altered membrane permeability, calcium channel blockage and DNA  
93 damage [26–28]. Active  $\text{Ca}^{2+}$  transport is inhibited in freshwater fish gills [22] while  
94 metabolic pathways are affected in freshwater algae [29].

95 Although cobalt concentrations in open oceanic waters are less than  $7 \text{ ng L}^{-1}$  [30],  
96 anthropogenic activities have led to progressive contamination of estuarine and coastal  
97 environments [see 25 for review]. To illustrate, mean cobalt levels measured in surface waters  
98 near an industrial plant in an Iberian Mediterranean estuarine system are  $\approx 700 \text{ } \mu\text{g Co}^{2+} \text{ L}^{-1}$  and  
99 reach up to  $\approx 2,800 \text{ } \mu\text{g Co}^{2+} \text{ L}^{-1}$  [31]. Cobalt titers in brachyuran crabs from marine/estuarine  
100 ecosystems range from  $0.26$  to  $0.43 \text{ } \mu\text{g Co}^{2+} \text{ g}^{-1}$  soft tissue dry mass in *Callinectes sapidus*  
101 [32];  $0.91$  to  $1.2 \text{ } \mu\text{g Co}^{2+} \text{ g}^{-1}$  hepatopancreas dry mass in male and female *Portunus segnis*  
102 [33]; and  $0.72$  to  $0.86 \text{ } \mu\text{g Co}^{2+} \text{ g}^{-1}$  dry mass in whole *Carcinus maenas* [34]. The lack of

103 molecular information regarding harm to aquatic organisms, including the effects of  $\text{Co}^{2+}$ , has  
104 impaired our overall comprehension of environmental damage and particularly of toxicity  
105 owing to bioaccumulation in marine organisms [16,23,25,35].

106 Estuarine and coastal organisms are powerful indicators of the health of the marine  
107 environment [36,37]. The portunid blue crab *Callinectes danae* is a crustacean species  
108 recommended as an environmental monitor of biological responses in contaminated estuarine  
109 and coastal areas [13]. The crab is a euryhaline osmoregulator, tolerant of exposure to wide  
110 range of salinities [38], and of great commercial value. It occurs from Florida (USA) to the  
111 southern Brazilian coast [39,40] and can be found in muddy estuaries and mangroves, on  
112 sandy and muddy shores, and in coastal waters up to 75 meters depth, including biotopes in  
113 which salinity varies from brackish to sea water [40,41].

114 In crustaceans, the gills provide a selective interface between the external environment  
115 and internal fluids, contributing to osmotic, ionic, excretory, and acid-base homeostasis. They  
116 are also an important route of entry of heavy metal ions [1,2,42]. Metal toxicity can thus vary  
117 depending on osmoregulatory strategy while alterations in salinity can affect the availability  
118 of metal ion species in the water column [36,43]. In brachyuran crabs, the three posterior gill  
119 pairs are specialized in ion transport [42], exhibiting a thickened epithelium [42,44] and  
120 increased expression and activity of ion transporters, including the  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$   
121 [1,45,46]. The presence of the  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$  in the gill tissue and its role in physiological  
122 homeostasis renders this enzyme a suitable bioindicator to evaluate the kinetic effects of  
123 heavy metal ions like  $\text{Co}^{2+}$ .

124 The  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$  is a transmembrane enzyme that mediates the coupled transport  
125 of three  $\text{Na}^+$  from the cytosol into the extracellular fluid and of two  $\text{K}^+$  into the cytosol per  
126 ATP molecule hydrolyzed. The enzyme consists of two main subunits: a catalytic  $\alpha$ -subunit,  
127 responsible for ion transport driven by ATP hydrolysis, and a highly glycosylated, non-  
128 catalytic  $\beta$ -subunit that modulates the transport properties of the enzyme [47,48]. The  
129 subunits are often associated with an FXYP peptide or  $\gamma$ -subunit that modulates  $(\text{Na}^+, \text{K}^+)\text{-}$   
130  $\text{ATPase}$  activity by altering the enzyme's apparent affinity for  $\text{Na}^+$ ,  $\text{K}^+$  and ATP [49]. Briefly,  
131 the catalytic cycle involves alternation between the phosphorylated E1 and E2 conformations  
132 that show high affinity for  $\text{Na}^+$  and  $\text{K}^+$ , respectively [47,48]. Magnesium is also essential  
133 although the ion is not transported during the catalytic cycle. Rather,  $\text{Mg}^{2+}$  participates as the  
134 true substrate (a  $\text{Mg}\cdot\text{ATP}$  complex) and plays a regulatory role, interfering with the  
135 conformational transitions of the enzyme [50,51]. At high concentrations,  $\text{Mg}^{2+}$  can inhibit  
136  $\text{Na}^+$  and  $\text{K}^+$  binding by occupying a second specific inhibitory site outside the  $\alpha$ -subunit

137 membrane domains [50–52] or binding to the protein surface near the access channel of the  
138 ion binding sites [51,53,54]. Various divalent cations like  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  can  
139 substitute for  $\text{Mg}^{2+}$  by binding to the  $\text{Mg}^{2+}$  regulatory site, activating the enzyme [55–57]. The  
140 presence of both ATP and  $\text{Na}^+$  induces immediate enzyme phosphorylation such that  $\text{Mg}^{2+}$   
141 binding and the phosphorylation reaction cannot be examined separately [51].

142 While the  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$  exhibits high specificity for ATP it also catalyzes the  
143 ouabain-sensitive hydrolysis of other nucleoside triphosphates [58] and various non-  
144 nucleotide substrates such as *p*-nitrophenyl phosphate, acetyl phosphate, 2,4-dinitrophenyl  
145 phosphate,  $\beta$ -(2-furyl)-acryloyl phosphate, O-methyl fluorescein phosphate and 4-azido-2-  
146 nitrophenylphosphate [59–64]. The activity corresponding to the hydrolysis of non-nucleotide  
147 substrates is known as the  $\text{K}^+$ -phosphatase activity and requires  $\text{Mg}^{2+}$  and  $\text{K}^+$  but is inhibited  
148 by  $\text{Na}^+$ . Such activity represents a partial reaction of the  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$  in which the E2  
149 form is the main conformational state involved in *p*NPP hydrolysis [58]. The use of such non-  
150 nucleotide substrates has disclosed important kinetic characteristics of the  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$   
151 and, under most experimental conditions, these are better substrates than ATP itself [59,61–  
152 63,65]. Differently from ATP, *p*-nitrophenyl phosphate hydrolysis by the gill enzyme  
153 involves only a single substrate binding site [61,66]. Likewise, the  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$  is also  
154 phosphorylated by *p*-nitrophenyl phosphate and other acyl phosphates [56,60,67,68].  $\text{K}^+$   
155 occlusion does not participate in phosphatase turnover [69] although, in the absence of  $\text{Na}^+$ ,  
156  $\text{K}^+$  stimulates  $\text{K}^+$ -phosphatase activity [70]. The use of *p*-nitrophenyl phosphate as a substrate  
157 thus facilitates the study of  $\text{Mg}^{2+}$  binding [51].

158 Given the paucity of information on the molecular effects of  $\text{Co}^{2+}$  on aquatic  
159 crustaceans from marine environments contaminated by heavy metals, in this study we  
160 provide a comprehensive analysis of the differential effects of  $\text{Co}^{2+}$  *in vitro* on the steady state  
161 kinetic properties of the  $\text{K}^+$ -phosphatase activity of a gill  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$  from the blue  
162 crab *Callinectes danae*.

163

## 164 2. MATERIAL AND METHODS

### 165 Material

166 Millipore MilliQ (Merck KGaA, Darmstadt, Germany) ultrapure, apyrogenic  
167 deionized water was used to prepare all solutions. Chemicals of the highest purity  
168 commercially available were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or  
169 Merck (Darmstadt, Germany). All salts were used as chlorides. The homogenization buffer  
170 consisted of 20 mmol L<sup>-1</sup> imidazole (pH 6.8), 250 mmol L<sup>-1</sup> sucrose and a proteinase inhibitor

171 cocktail (1 mmol L<sup>-1</sup> benzamidine, 5 μmol L<sup>-1</sup> antipain, 5 μmol L<sup>-1</sup> leupeptin, 5 μmol L<sup>-1</sup>  
172 phenyl-methane-sulfonyl-fluoride, and 1 μmol L<sup>-1</sup> pepstatin A). Analytical estimation of the  
173 stock CoCl<sub>2</sub> solution concentration (100 mmol L<sup>-1</sup>) was performed employing inductively  
174 coupled mass spectrometry (Perkin Elmer Avio 200 optical emission spectrometer, Boston  
175 MA, USA). NH<sub>4</sub><sup>+</sup> was removed from the crystalline ammonium sulfate suspensions of LDH  
176 and PK according to [Fabri et al. \[71\]](#). When necessary, enzyme solutions were concentrated  
177 on YM-10 Amicon Ultra filters.

178

## 179 **2.1. Crab collection**

180 Adult specimens of *Callinectes danae* of ≈9 cm carapace width were collected at low  
181 tide using seine nets or baited hand nets from Barra Seca beach (23° 25' 01.5" S, 45° 03'  
182 01.0" W), Ubatuba, São Paulo State, Brazil (SISBIO/ICMBio/IBAMA authorization  
183 02027.002342/98-04, permit #29594-18 to John C. McNamara). The crabs were held briefly  
184 in plastic boxes containing 30 L aerated seawater from the collection site during transport to  
185 the laboratory where they were immediately anesthetized by chilling in crushed ice. After  
186 bisecting and removal of the carapace, all three posterior gill pairs (120 gills/preparation, ≈5 g  
187 wet mass) were rapidly dissected out and frozen in liquid nitrogen in homogenization buffer  
188 in Falcon tubes.

189

## 190 **2.2. Preparation of the gill microsomal fraction**

191 For each of the three (N= 3) gill homogenates prepared, the gills frozen in the  
192 homogenization buffer were thawed, diced and homogenized (20 mL homogenization  
193 buffer/g wet tissue) at 600 rpm in a Potter homogenizer in a crushed ice bath. The (Na<sup>+</sup>, K<sup>+</sup>)-  
194 ATPase-rich microsomal fraction was prepared by stepwise differential centrifugation (20,000  
195 and 100,000 ×g) of the gill homogenate [\[38\]](#). The resulting pellet was resuspended in 20  
196 mmol L<sup>-1</sup> imidazole buffer (pH 6.8) containing 250 mmol L<sup>-1</sup> sucrose (15 mL buffer/g wet  
197 tissue). Aliquots (0.5 mL) were frozen in liquid nitrogen, stored at -20 °C and used within  
198 three-month's storage provided that at least 95% (Na<sup>+</sup>, K<sup>+</sup>)-ATPase activity was present.

199

## 200 **2.3. Estimation of *p*-nitrophenyl phosphatase activity**

201 *p*-Nitrophenyl phosphate (*p*NPP) was used as the enzyme substrate. The *p*-nitrophenyl  
202 phosphatase activity (*p*NPPase) of the microsomal fraction was estimated continuously at 25  
203 °C, following the release of the *p*-nitrophenolate ion (*p*NP<sup>-</sup>) at 410 nm (ε 410nm, pH 7.5=  
204 13,160 M<sup>-1</sup> cm<sup>-1</sup>) in a Shimadzu UV-1800 spectrophotometer (Vernon Hills IL, USA)



205 equipped with thermostatted cells. The standard incubation medium contained 50 mmol L<sup>-1</sup>  
206 HEPES buffer, pH 7.5, and appropriate *p*NPP, Mg<sup>2+</sup>, K<sup>+</sup> or NH<sub>4</sub><sup>+</sup> concentrations (see Results  
207 and Figure legends for substrate and specific ionic concentrations) and 9 μg alamethicin, in  
208 final volume of 1 mL. Activity was estimated with or without 7 mmol L<sup>-1</sup> ouabain, the  
209 difference corresponding to the K<sup>+</sup>-phosphatase activity of the gill (Na<sup>+</sup>, K<sup>+</sup>)-ATPase. The  
210 reaction was always initiated by the addition of the enzyme.

211 Because tissue homogenization usually results in the formation of small vesicles in the  
212 microsomal preparation that can occlude the catalytic site of the enzyme, *p*NPPase activity  
213 also was estimated after 10 min pre-incubation with 9 μg alamethicin, a membrane pore-  
214 forming antibiotic, to verify the presence of vesicles showing ATPase activity.

215 Controls without added enzyme were used to estimate the spontaneous hydrolysis of  
216 the substrate under the assay conditions. The kinetic measurements were carried out in  
217 duplicate, and substrate hydrolysis was accompanied over the shortest possible period to  
218 guarantee initial velocity measurements (<5% of substrate hydrolyzed during the reaction  
219 period). One unit (U) of enzyme activity was defined as the amount of enzyme that  
220 hydrolyses 1.0 nmol of *p*NPP per minute at 25 °C.

221

## 222 **2.4. Protein**

223 Protein concentration was measured in duplicate aliquots of the microsomal  
224 preparations using the Coomassie Blue G dye-binding assay employing bovine serum  
225 albumin as a standard. Assays were read at 595 nm using a Shimadzu UV-1800  
226 spectrophotometer [71].

227

## 228 **2.5. Estimation K<sup>+</sup>-phosphatase activity with cobalt ions**

229 The effect of cobalt ions on *p*NPPase activity was estimated as above using cobalt  
230 concentrations between 10<sup>-5</sup> and 2×10<sup>-2</sup> mmol L<sup>-1</sup>. Our study on the effect of Co<sup>2+</sup> on the  
231 interaction of the different ligands with the (Na<sup>+</sup>,K<sup>+</sup>)-ATPase was performed using 3 mmol L<sup>-1</sup>  
232 <sup>1</sup>Co<sup>2+</sup> (50.8 μg L<sup>-1</sup> Co<sup>2+</sup>) which inhibits K<sup>+</sup>-phosphatase activity by ≈50%.

233

## 234 **2.6. Measurement of ATP hydrolysis**

235 To provide a direct comparison with previous microsomal preparations, initial rates of  
236 ATP hydrolysis also were estimated continuously at 25 °C, using a pyruvate kinase/lactic  
237 dehydrogenase coupling system in which ATP hydrolysis is coupled to NADH oxidation [72].  
238 The specific activity of (Na<sup>+</sup>, K<sup>+</sup>)-ATPase for ATP of 296.5 ± 10.2 nmol Pi min<sup>-1</sup> mg<sup>-1</sup> protein



239 corresponding to  $125.5 \pm 2.3$  nmol  $pNP^-$  min<sup>-1</sup> mg<sup>-1</sup> protein of K<sup>+</sup>-phosphatase activity is  
240 comparable to our previous findings [73].

241

## 242 2.7. Estimation of kinetic parameters

243 The kinetic parameters  $V_M$  (maximum velocity),  $K_{0.5}$  (apparent dissociation constant)  
244 and the  $n_H$  value (Hill coefficient) for  $pNPP$  hydrolysis were calculated using SigrafW  
245 software ([74]; freely available from <http://portal.ffclrp.usp.br/sites/fdaleone/downloads>). The  
246 apparent dissociation constant of the enzyme-inhibitor complex ( $K_I$ ) was estimated using the  
247 Dixon plot in which the reaction rate corresponding to the K<sup>+</sup>-phosphatase activity was  
248 corrected for residual activity at high inhibitor concentrations [75]. Data points (mean  $\pm$  SD)  
249 in the figures representing each substrate/ligand concentration are mean values of duplicate  
250 aliquots from the same preparation and were used to fit the saturation curves that were  
251 repeated using three different microsomal preparations (N= 3). The kinetic parameters ( $V_M$ ,  
252  $K_M$ ,  $K_I$  or  $K_{0.5}$ ) shown in the tables are calculated values and represent the mean ( $\pm$  SD) derived  
253 from values estimated for each of three (N= 3) microsomal preparations.

254

## 255 3. RESULTS

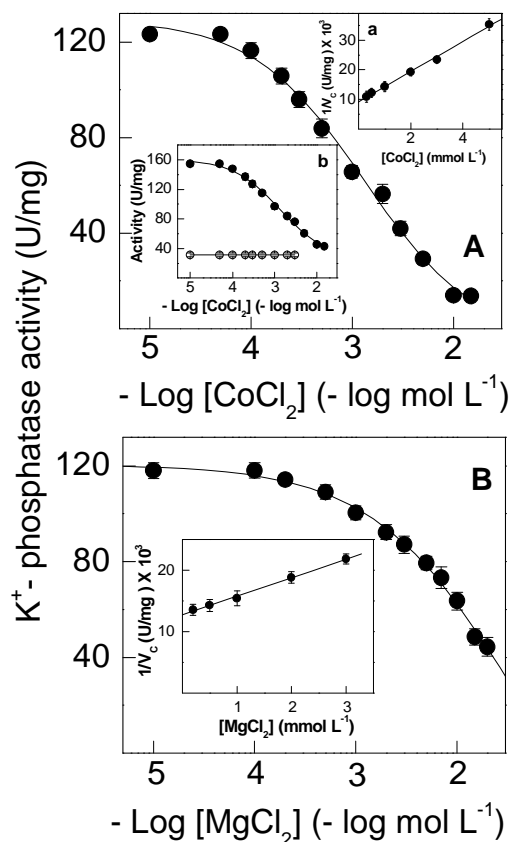
256 Estimation of  $pNPPase$  activity with alamethicin revealed that the gill microsomal  
257 preparation from fresh caught *C. danae* includes  $\approx 25\%$  sealed,  $pNPPase$ -containing vesicles  
258 ( $164.5 \pm 3.8$  and  $122.5 \pm 1.8$  nmol  $pNP^-$  min<sup>-1</sup> mg<sup>-1</sup> protein with or without alamethicin,  
259 respectively). Thus,  $pNPPase$  activity was always estimated using 9  $\mu$ g alamethicin. Seven  
260 mmol L<sup>-1</sup> ouabain decreased  $pNPPase$  activity from  $164.5 \pm 3.8$  to  $39.2 \pm 1.9$  nmol  $pNP^-$  min<sup>-1</sup>  
261 mg<sup>-1</sup> protein, indicating that  $\approx 75\%$  of the total phosphohydrolyzing activity ( $125.5 \pm 2.3$   
262 nmol  $pNP^-$  min<sup>-1</sup> mg<sup>-1</sup> protein) corresponds to the K<sup>+</sup>-phosphatase activity of the gill (Na<sup>+</sup>,  
263 K<sup>+</sup>)-ATPase. Measurements using ATP as a substrate showed a (Na<sup>+</sup>, K<sup>+</sup>)-ATPase activity of  
264  $296.5 \pm 10.2$  nmol Pi min<sup>-1</sup> mg<sup>-1</sup> protein.

265

### 266 3.1. Effect of Co<sup>2+</sup> on K<sup>+</sup>-phosphatase activity

267 Under optimal assay conditions (see legends to Fig. 1A and 1B), increasing Co<sup>2+</sup>  
268 concentrations from 10<sup>-5</sup> to 2 $\times$ 10<sup>-2</sup> mol L<sup>-1</sup> in the incubation medium inhibited K<sup>+</sup>-  
269 phosphatase activity by 90% (Fig. 1A and Table 1) with  $K_I = 2.77 \pm 0.33$  mmol L<sup>-1</sup> (inset a to  
270 Fig. 1). K<sup>+</sup>-phosphatase activity decreased following a single titration curve to  $13.7 \pm 3.6$   
271 nmol  $pNP^-$  min<sup>-1</sup> mg<sup>-1</sup> protein. The ouabain-insensitive  $pNPPase$  activity of  $\approx 38$  nmol  $pNP^-$   
272 min<sup>-1</sup> mg<sup>-1</sup> protein was unaffected by increasing Co<sup>2+</sup> concentrations (inset b to Fig. 1). On

273 fixing  $\text{Co}^{2+}$  at  $3 \text{ mmol L}^{-1}$ , increasing  $\text{Mg}^{2+}$  concentrations ( $10^{-5}$  to  $2 \times 10^{-2} \text{ mol L}^{-1}$ ) inhibited  
 274  $\text{K}^{+}$ -phosphatase activity by  $\approx 60\%$  (Fig. 1B and Table 1). A  $K_I = 4.81 \pm 0.71 \text{ mmol L}^{-1}$  was  
 275 calculated for inhibition of  $\text{K}^{+}$ -phosphatase activity by  $\text{Mg}^{2+}$  in the presence of  $\text{Co}^{2+}$  (inset to  
 276 Fig. 1B).  
 277

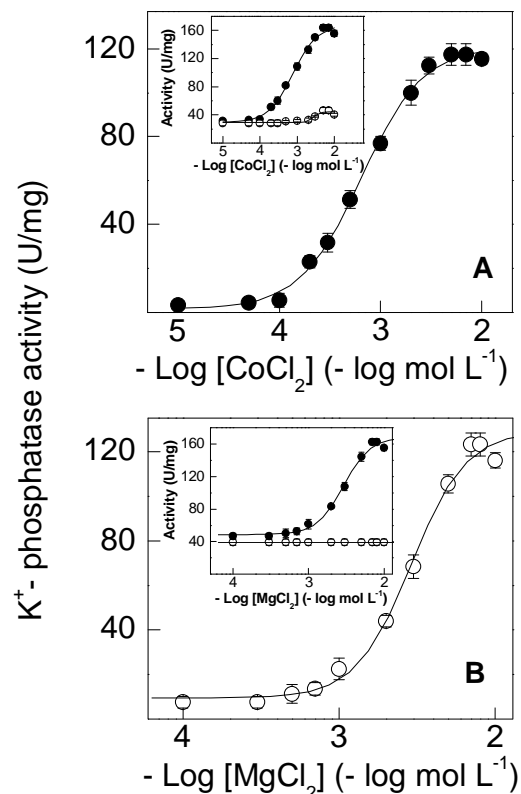


278

279 **Figure 1. Effect of cobalt or magnesium ions in the presence of each other on the  $\text{K}^{+}$ -**  
 280 **phosphatase activity of *C. danae* gill ( $\text{Na}^{+}$ ,  $\text{K}^{+}$ )-ATPase**

281 Activity was assayed continuously at  $25^{\circ}\text{C}$  in  $50 \text{ mmol L}^{-1}$  HEPES buffer, pH 7.5, containing  
 282  $10 \text{ mmol L}^{-1}$  pNPP,  $15 \text{ mmol L}^{-1}$  KCl,  $9 \mu\text{g}$  alamethicin and the metal ions in a final volume  
 283 of 1 mL. The mean activity of duplicate aliquots of the same microsomal preparation ( $\approx 15 \mu\text{g}$   
 284 protein) was used to fit the saturation curve which was repeated using three different  
 285 microsomal preparations ( $\pm$  SD,  $N = 3$ ). Where lacking, error bars are smaller than the  
 286 symbols used. **A-** with  $7 \text{ mmol L}^{-1}$   $\text{MgCl}_2$ . Inset a- Dixon plot for estimation of  $K_I$  in which  $v_c$   
 287 is the  $\text{K}^{+}$ -phosphatase activity corrected for residual pNPPase activity found at high  $\text{Co}^{2+}$   
 288 concentration. Inset b- total pNPPase activity ( $\bullet$ ) and ouabain-insensitive pNPPase activity  
 289 ( $\circ$ ). **B-** with  $3 \text{ mmol L}^{-1}$   $\text{CoCl}_2$ . Inset- Dixon plot for estimation of  $K_I$  in which  $v_c$  is the  $\text{K}^{+}$ -  
 290 phosphatase activity corrected for residual  $\text{K}^{+}$ -phosphatase activity found at high  $\text{Mg}^{2+}$   
 291 concentrations.  
 292

293 Cobalt ions can substitute for  $Mg^{2+}$ , stimulating gill  $K^+$ -phosphatase activity (Fig. 2A  
294 and Table 1). Under optimal assay conditions (see legends to Fig. 2A and 2B) without  $Mg^{2+}$ ,  
295  $Co^{2+}$  stimulated  $K^+$ -phosphatase activity to a maximum rate of  $V_M = 122.5 \pm 3.1$  nmol  $pNP^-$   
296  $min^{-1} mg^{-1}$  protein with  $K_{0.5} = 0.69 \pm 0.23$  mmol  $L^{-1}$ , showing a single saturation curve  
297 obeying cooperative kinetics ( $n_H = 1.4$ ). Ouabain-insensitive  $pNPPase$  activity was stimulated  
298 to  $46.4 \pm 3.7$  nmol  $pNP^- min^{-1} mg^{-1}$  protein over the same  $Co^{2+}$  concentration range (inset to  
299 Fig. 2A).  $K^+$ -phosphatase activity was inhibited by  $Co^{2+}$  concentrations above  $10^{-2}$  mol  $L^{-1}$ . In  
300 the absence of  $Co^{2+}$ ,  $Mg^{2+}$  ( $10^{-4}$  to  $2 \times 10^{-2}$  mol  $L^{-1}$ ) stimulated  $K^+$ -phosphatase activity to a  
301 maximum rate of  $V_M = 135.1 \pm 5.0$  nmol  $pNP^- min^{-1} mg^{-1}$  protein with  $K_{0.5} = 2.98 \pm 0.59$   
302 mmol  $L^{-1}$  and cooperative kinetics ( $n_H = 2.2$ ), following a single saturation curve (Fig. 2B and  
303 Table 1). Stimulation by  $Mg^{2+}$  of ouabain-insensitive  $pNPPase$  activity was negligible over  
304 the concentration range used (inset to Fig. 2B). The  $\approx 4.5$ -fold lower  $K_{0.5}$  suggests that  $Co^{2+}$   
305 binds more tightly to the  $Mg^{2+}$ -binding sites than does  $Mg^{2+}$  itself.  
306



307

308 **Figure 2. Estimation by cobalt or magnesium ions of  $K^+$ -phosphatase activity of *C.***  
309 ***danae* gill ( $Na^+$ ,  $K^+$ )-ATPase**

310 Activity was assayed continuously at 25 °C in 50 mmol  $L^{-1}$  HEPES buffer, pH 7.5, containing  
311 10 mmol  $L^{-1}$   $pNPP$ , 15 mmol  $L^{-1}$  KCl, 9  $\mu g$  alamethicin in a final volume of 1 mL. The mean

312 activity of duplicate aliquots of the same microsomal preparation ( $\approx 15 \mu\text{g}$  protein) was used  
313 to fit the saturation curve which was repeated using three different microsomal preparations  
314 ( $\pm$  SD,  $N=3$ ). Where lacking, error bars are smaller than the symbols used. **A-** with cobalt  
315 ions. **B-** with magnesium ions. Inset to figures- total *p*NPPase activity ( $\bullet$ ); ouabain-  
316 insensitive *p*NPPase activity ( $\circ$ ).

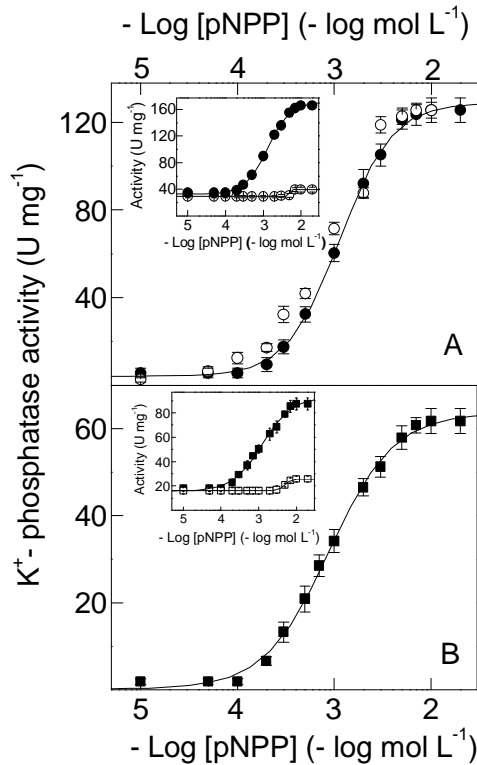
317

318

### 319 **3.2. Effect of $\text{Co}^{2+}$ on *p*NPP hydrolysis**

320 Under optimal assay conditions (see legends to Fig. 2A and 2B), increasing *p*NPP  
321 concentrations stimulated  $\text{K}^+$ -phosphatase activity to a maximum rate of  $V_M = 138.1 \pm 4.2$   
322  $\text{nmol } p\text{NP}^{-1} \text{ min}^{-1} \text{ mg}^{-1}$  protein with  $K_{0.5} = 1.76 \pm 0.49 \text{ mmol L}^{-1}$ , following cooperative  
323 kinetics ( $n_H = 1.3$ ) and showing a single saturation curve (Fig. 3A and Table 1). Substitution of  
324  $\text{Mg}^{2+}$  with  $3 \text{ mmol L}^{-1} \text{ Co}^{2+}$  also gave a single saturation curve, overlapping that for  $\text{Mg}^{2+}$ ,  
325 showing a maximum rate of  $V_M = 128.2 \pm 4.4 \text{ nmol } p\text{NP}^{-1} \text{ min}^{-1} \text{ mg}^{-1}$  protein with  $K_{0.5} = 1.15 \pm$   
326  $0.61 \text{ mmol L}^{-1}$  (Fig. 3A). With  $\text{Mg}^{2+}$ , the ouabain-insensitive *p*NPPase activity of  $\approx 30 \text{ nmol}$   
327  $p\text{NP}^{-1} \text{ min}^{-1} \text{ mg}^{-1}$  protein was not affected by increasing *p*NPP concentrations (not shown).  
328 However, with  $3 \text{ mmol L}^{-1} \text{ Co}^{2+}$  this activity was stimulated by  $\approx 35\%$  over the same *p*NPP  
329 concentration range (inset to Fig. 3A). With both  $\text{Mg}^{2+}$  and  $\text{Co}^{2+}$ , under the same saturating  
330 ionic and substrate concentrations,  $\text{K}^+$ -phosphatase activity decreased to a maximum rate of  
331  $V_M = 63.1 \pm 3.8 \text{ nmol } p\text{NP}^{-1} \text{ min}^{-1} \text{ mg}^{-1}$  protein with  $K_{0.5} = 0.92 \pm 0.28 \text{ mmol L}^{-1}$  also obeying  
332 cooperative kinetics (Fig. 3B and Table 1). This  $\approx 50\%$  inhibition of  $\text{K}^+$ -phosphatase activity  
333 was accompanied by a 2-fold decrease in  $K_{0.5}$  compared to  $\text{Mg}^{2+}$  (Table 1). The ouabain-  
334 insensitive *p*NPPase activity of  $\approx 20 \text{ nmol } p\text{NP}^{-1} \text{ min}^{-1} \text{ mg}^{-1}$  protein was stimulated  $\approx 60\%$  over  
335 the same *p*NPP concentration range (inset to Fig. 3B).

336



337

338 **Figure 3. Effect of cobalt or magnesium ions on the modulation by pNPP of the K<sup>+</sup>-**  
 339 **phosphatase activity of *C. danae* gill (Na<sup>+</sup>, K<sup>+</sup>)-ATPase**

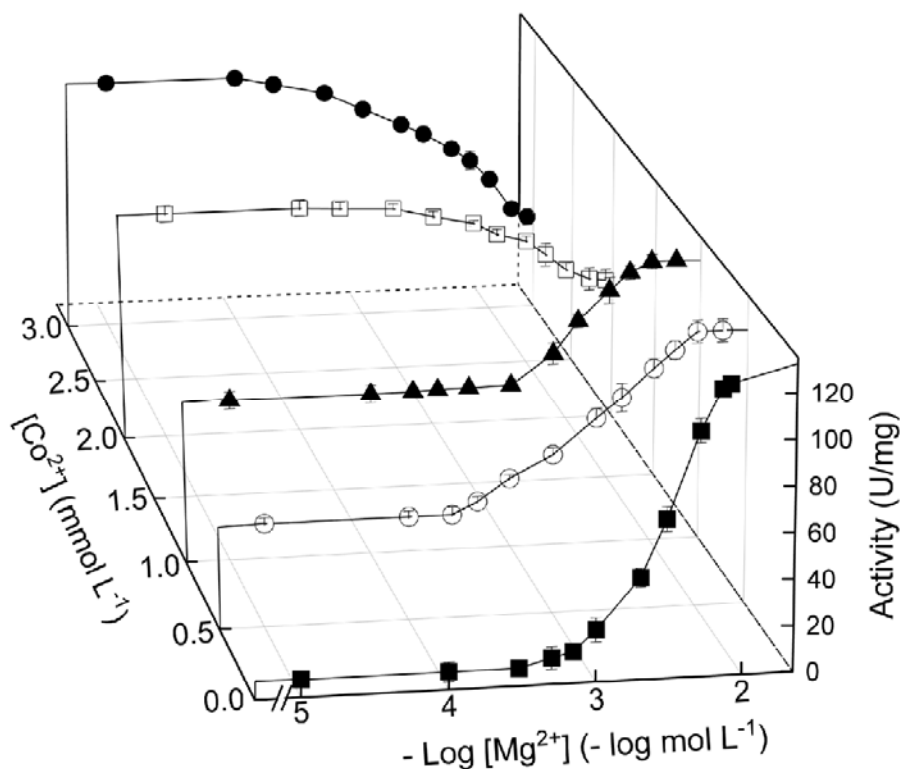
340 Activity was assayed continuously at 25 °C in 50 mmol L<sup>-1</sup> HEPES buffer, pH 7.5, containing  
 341 15 mmol L<sup>-1</sup> KCl, 9 μg alamethicin and the metal ion (7 mmol L<sup>-1</sup> Mg<sup>2+</sup> or 3 mmol L<sup>-1</sup> Co<sup>2+</sup>)  
 342 in a final volume of 1 mL. The mean activity of duplicate aliquots of the same microsomal  
 343 preparation (≈15 μg protein) was used to fit the saturation curve which was repeated using  
 344 three different microsomal preparations (± SD, N= 3). Where lacking, error bars are smaller  
 345 than the symbols used. **A-** with magnesium (○) or cobalt ions (●). **B-** with both magnesium  
 346 and cobalt ions. **Inset to figures-** total pNPPase activity (●,■); ouabain-insensitive pNPPase  
 347 activity (○,□) for Co<sup>2+</sup> only.

348

### 349 **3.3. Effect of Co<sup>2+</sup> on Mg<sup>2+</sup> stimulation**

350 Magnesium can displace Co<sup>2+</sup> bound to the gill (Na<sup>+</sup>, K<sup>+</sup>)-ATPase below 2 mmol L<sup>-1</sup>  
 351 Co<sup>2+</sup> (Fig. 4). Increased Mg<sup>2+</sup> (10<sup>-5</sup> to 5×10<sup>-2</sup> mol L<sup>-1</sup>) displaces bound Co<sup>2+</sup> (0.5 and 1 mmol  
 352 L<sup>-1</sup>), stimulating K<sup>+</sup>-phosphatase activity to a maximum rate of ≈130 nmol pNP<sup>-</sup> min<sup>-1</sup> mg<sup>-1</sup>  
 353 protein with similar K<sub>0.5</sub> (Fig. 4 and Table 1). However, at 2 mmol L<sup>-1</sup> Co<sup>2+</sup> and above no  
 354 displacement was seen and increasing Mg<sup>2+</sup> concentrations inhibited K<sup>+</sup>-phosphatase activity  
 355 with K<sub>i</sub>= 4.41 ± 0.69 and 4.81 ± 0.71 mmol L<sup>-1</sup> for 2 and 3 mmol L<sup>-1</sup> Co<sup>2+</sup>, respectively (Table  
 356 1).

357



358

359 **Figure 4. Effect of magnesium ions on the modulation of K<sup>+</sup>-phosphatase activity at**  
 360 **different cobalt ion concentrations in *C. danae* gill (Na<sup>+</sup>, K<sup>+</sup>)-ATPase.**

361 Activity was assayed continuously at 25 °C in 50 mmol L<sup>-1</sup> HEPES buffer, pH 7.5, containing  
 362 10 mmol L<sup>-1</sup> pNPP, 15 mmol L<sup>-1</sup> KCl and 9 μg alamethicin in a final volume of 1 mL. The  
 363 mean activity of duplicate aliquots of the same microsomal preparation (≈15 μg protein)  
 364 was used to fit the saturation curve which was repeated using three different microsomal  
 365 preparations (± SD, N= 3). Where lacking, error bars are smaller than the symbols used. (■)  
 366 without Co<sup>2+</sup>. (○) 0.5 mmol L<sup>-1</sup> Co<sup>2+</sup>. (▲) 1 mmol L<sup>-1</sup> Co<sup>2+</sup>. (□) 2 mmol L<sup>-1</sup> Co<sup>2+</sup>. (●)  
 367 3 mmol L<sup>-1</sup> Co<sup>2+</sup>.

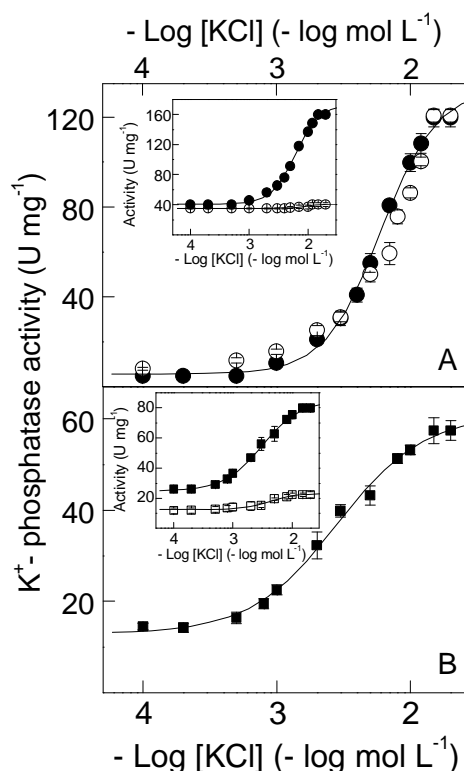
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369

### 370 3.4. Effect of Co<sup>2+</sup> on K<sup>+</sup> stimulation

371 Under saturating ionic and substrate concentrations (see legends to Fig. 5A and 5B) in  
 372 the absence of Co<sup>2+</sup>, increasing K<sup>+</sup> stimulated K<sup>+</sup>-phosphatase activity to a maximum rate of  
 373  $V_M = 134.2 \pm 4.5$  nmol pNP<sup>-</sup> min<sup>-1</sup> mg<sup>-1</sup> protein with  $K_{0.5} = 9.60 \pm 2.04$  mmol L<sup>-1</sup> (Fig. 5A and  
 374 Table 1) following cooperative kinetics ( $n_H = 2.2$ ). Substitution of Mg<sup>2+</sup> by 3 mmol L<sup>-1</sup> Co<sup>2+</sup>  
 375 also gave a single saturation curve overlapping with that for Mg<sup>2+</sup> and showing a maximum  
 376 rate of  $V_M = 133.3 \pm 3.3$  nmol pNP<sup>-</sup> min<sup>-1</sup> mg<sup>-1</sup> protein with  $K_{0.5} = 6.00 \pm 1.50$  mmol L<sup>-1</sup> (Fig.  
 377 5A and Table 1). Ouabain-insensitive pNPPase activity was not stimulated by either metal ion  
 378 over the concentration range used (inset to Fig. 5A). With Co<sup>2+</sup> plus Mg<sup>2+</sup>, K<sup>+</sup>-phosphatase  
 379 activity decreased to  $V_M = 59.5 \pm 4.0$  nmol pNP<sup>-</sup> min<sup>-1</sup> mg<sup>-1</sup> protein with  $K_{0.5} = 2.79 \pm 0.41$   
 380 mmol L<sup>-1</sup> following a single titration curve (Fig. 5B and Table 1), obeying cooperative

381 kinetics ( $n_H = 1.5$ ).  $K^+$ -phosphatase activity at  $K^+$  concentrations below  $10^{-4}$  mol L $^{-1}$  was  $\approx 15$   
 382 nmol  $pNP^-$  min $^{-1}$  mg $^{-1}$  protein. Ouabain-insensitive  $pNPPase$  activity was stimulated to  $\approx 22$   
 383 nmol  $pNP^-$  min $^{-1}$  mg $^{-1}$  protein over the same  $K^+$  concentration range (inset to Fig. 5B).  $K^+$ -  
 384 phosphatase activity was not synergically stimulated by  $K^+$  plus  $NH_4^+$  (not shown).



385

386 **Figure 5. Effect of cobalt or magnesium ions on the modulation by potassium ions of  $K^+$ -**  
 387 **phosphatase activity of *C. danae* gill ( $Na^+$ ,  $K^+$ )-ATPase**

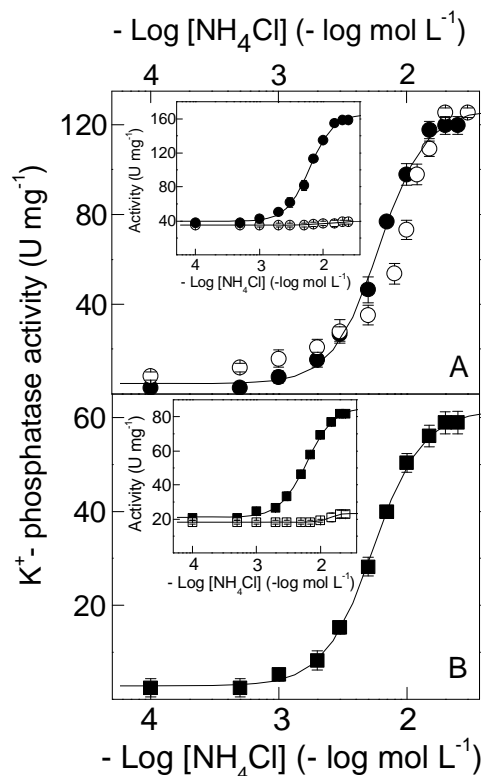
388 Activity was assayed continuously at 25 °C in 50 mmol L $^{-1}$  HEPES buffer, pH 7.5, containing  
 389 10 mmol L $^{-1}$   $pNPP$ , 9  $\mu$ g alamethicin and the metal ion (7 mmol L $^{-1}$   $Mg^{2+}$  or 3 mmol L $^{-1}$   
 390  $Co^{2+}$ ) in a final volume of 1 mL. The mean activity of duplicate aliquots of the same  
 391 microsomal preparation ( $\approx 15$   $\mu$ g protein) was used to fit the saturation curve which was  
 392 repeated using three different microsomal preparations ( $\pm$  SD, N= 3). Where lacking, error  
 393 bars are smaller than the symbols used. **A-**  $K^+$ -phosphatase activity with  $Mg^{2+}$  (O) or  $Co^{2+}$   
 394 (●). **B-**  $K^+$ -phosphatase activity with both  $Mg^{2+}$  and  $Co^{2+}$ . Insets to figures- total  $pNPPase$   
 395 activity (●) and ouabain-insensitive  $pNPPase$  activity (O)  $Co^{2+}$ .  
 396

### 397 3.5. Effect of $Co^{2+}$ on $NH_4^+$ stimulation

398 Under saturating ionic and substrate concentrations (see legends to Fig. 6A and 6B) in  
 399 the absence of  $Co^{2+}$ , a maximal rate of  $V_M = 122.2 \pm 5.2$  nmol  $pNP^-$  min $^{-1}$  mg $^{-1}$  protein with  
 400  $K_{0.5} = 9.02 \pm 2.51$  mmol L $^{-1}$  was estimated for  $NH_4^+$  concentrations increasing from  $10^{-4}$  to  
 401  $5 \times 10^{-2}$  mol L $^{-1}$  (Fig. 6A and Table 1). Substitution of  $Mg^{2+}$  by 3 mmol L $^{-1}$   $Co^{2+}$  also gave a



402 single saturation curve with a maximum rate of  $V_M = 127.9 \pm 4.2$  nmol  $pNP^-$   $min^{-1} mg^{-1}$   
 403 protein with  $K_{0.5} = 6.00 \pm 1.10$  mmol  $L^{-1}$ , overlapping with that for  $Mg^{2+}$  (Fig. 6A and Table  
 404 1). Stimulation of ouabain-insensitive  $pNPPase$  activity by  $Mg^{2+}$  and  $Co^{2+}$  was negligible over  
 405 the  $NH_4^+$  concentration range used (inset to Fig. 6A). With  $Co^{2+}$  plus  $Mg^{2+}$ ,  $K^+$ -phosphatase  
 406 activity decreased to  $V_M = 61.9 \pm 3.7$  nmol  $pNP^-$   $min^{-1} mg^{-1}$  protein with  $K_{0.5} = 5.46 \pm 0.64$   
 407 mmol  $L^{-1}$  (Fig. 6B and Table 1). Stimulation of the ouabain-insensitive  $pNPPase$  activity was  
 408  $<10\%$  with  $Co^{2+}$  plus  $Mg^{2+}$  (inset to Fig. 6B).  $K^+$ -phosphatase activity was not stimulated  
 409 synergically by  $K^+$  plus  $NH_4^+$  (not shown).



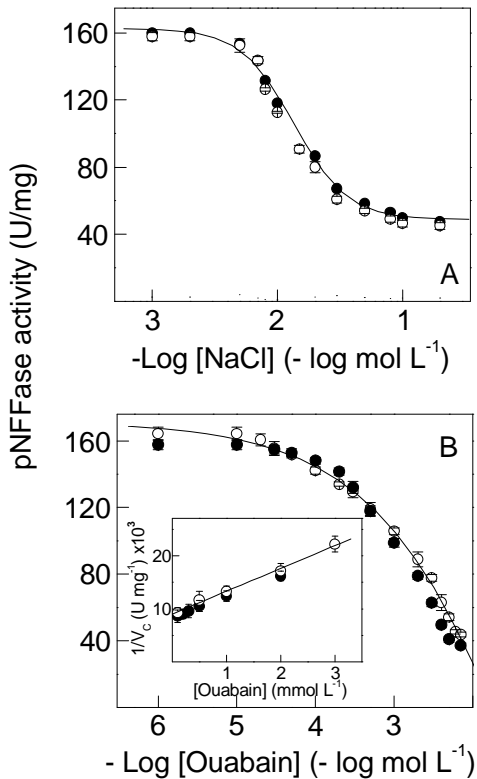
410

411 **Figure 6. Effect of cobalt or magnesium ions on the modulation by ammonium ions of**  
 412  **$K^+$ -phosphatase activity of *C. danae* gill ( $Na^+$ ,  $K^+$ )-ATPase**

413 Activity was assayed continuously at  $25^\circ C$  in  $50 \text{ mmol } L^{-1}$  HEPES buffer, pH 7.5, containing  
 414  $10 \text{ mmol } L^{-1}$   $pNPP$ ,  $9 \mu g$  alamethicin and the metal ion ( $7 \text{ mmol } L^{-1}$   $Mg^{2+}$  or  $3 \text{ mmol } L^{-1}$   
 415  $Co^{2+}$ ) in a final volume of  $1 \text{ mL}$ . The mean activity of duplicate aliquots of the same  
 416 microsomal preparation ( $\approx 15 \mu g$  protein) was used to fit the saturation curve which was  
 417 repeated using three different microsomal preparations ( $\pm$  SD,  $N=3$ ). Where lacking, error  
 418 bars are smaller than the symbols used. **A-**  $K^+$ -phosphatase activity with  $Mg^{2+}$  ( $\circ$ ) or  $Co^{2+}$   
 419 ( $\bullet$ ). **B-**  $K^+$ -phosphatase activity with both  $Mg^{2+}$  and  $Co^{2+}$ . Insets to figures- total  $pNPPase$   
 420 activity ( $\bullet$ ) and ouabain-insensitive  $pNPPase$  activity ( $\circ$ )  $Co^{2+}$ .

421 **3.6. Effect of  $Co^{2+}$  on inhibition by  $Na^+$  and ouabain**

422 Cobalt does not affect inhibition of *p*NPPase activity by Na<sup>+</sup> or ouabain (Fig. 7).  
423 Sodium concentrations from 10<sup>-3</sup> to 0.2 mol L<sup>-1</sup> inhibited *p*NPPase activity by ≈70% either  
424 with or without Co<sup>2+</sup> (Fig. 7A and Table 1). The IC<sub>50</sub> estimated for Na<sup>+</sup> inhibition of *p*NPPase  
425 activity was ≈16 mmol L<sup>-1</sup> (Table 1). Over the ouabain concentration range from 10<sup>-6</sup> to 10<sup>-2</sup>  
426 mol L<sup>-1</sup>, the Na<sup>+</sup> and ouabain inhibition curves overlapped independently of Co<sup>2+</sup> (Fig. 7B and  
427 Table 1). Their monophasic behavior with very similar inhibition constants ( $K_i = 2.27 \pm 0.62$   
428 mmol L<sup>-1</sup> and  $K_i = 2.13 \pm 0.97$  mmol L<sup>-1</sup> for Mg<sup>2+</sup> and Co<sup>2+</sup>, respectively) (inset to Fig. 7B)  
429 suggests a single ouabain binding site.



430

431 **Figure 7. Effect of cobalt or magnesium ions on the inhibition by sodium and ouabain on**  
432 ***p*NPPase activity of *C. danae* gill (Na<sup>+</sup>, K<sup>+</sup>)-ATPase**

433 Activity was assayed continuously at 25 °C in 50 mmol L<sup>-1</sup> HEPES buffer, pH 7.5, containing  
434 10 mmol L<sup>-1</sup> *p*NPP, 15 mmol L<sup>-1</sup> KCl and 9 μg alamethicin in a final volume of 1 mL. The  
435 mean activity of duplicate aliquots of the same microsomal preparation (≈15 μg protein) was  
436 used to fit the saturation curve which was repeated using three different microsomal  
437 preparations (± SD, N= 3). Where lacking, error bars are smaller than the symbols used. **A-**  
438 **sodium ion. B-** ouabain. Inset to Fig. 8B- Dixon plot for estimation of K<sub>i</sub> in which v<sub>c</sub> is the  
439 *p*NPPase activity corrected for residual *p*NPPase activity found at high ouabain  
440 concentrations. (○) with 7 mmol L<sup>-1</sup> Mg<sup>2+</sup>. (●) with 3 mmol L<sup>-1</sup> Co<sup>2+</sup>.

441 **Table 1. Kinetic parameters calculated for the effect of *p*NPP, Mg<sup>2+</sup>, K<sup>+</sup>, NH<sub>4</sub><sup>+</sup>, Na<sup>+</sup>, Co<sup>2+</sup> and ouabain on K<sup>+</sup>-phosphatase activity of**  
 442 **(Na<sup>+</sup>, K<sup>+</sup>)-ATPase in a gill microsomal preparation from *Callinectes danae*.**

443

Ligand	Co <sup>2+</sup> mmol L <sup>-1</sup>	<i>p</i> NPP mmol L <sup>-1</sup>	Mg <sup>2+</sup> mmol L <sup>-1</sup>	K <sup>+</sup> mmol L <sup>-1</sup>	NH <sub>4</sub> <sup>+</sup> mmol L <sup>-1</sup>	Na <sup>+</sup> mmol L <sup>-1</sup>	V <sub>M</sub> U mg <sup>-1</sup>	K <sub>0.5</sub> mmol L <sup>-1</sup>	n <sub>H</sub>	K <sub>I</sub> mmol L <sup>-1</sup>	IC <sub>50</sub> mmol L <sup>-1</sup>
Co <sup>2+</sup>	Variable	10	-	15	-	-	122.5 ± 3.1	0.69 ± 0.23	1.4	-	-
Co <sup>2+</sup>	Variable	10	7	15	-	-	-	-	-	2.77 ± 0.33	-
<i>p</i> NPP	-	Variable	7	15	-	-	138.1 ± 4.2	1.76 ± 0.49	1.3	-	-
<i>p</i> NPP	3	Variable	-	15	-	-	128.2 ± 4.4	1.15 ± 0.61	1.7	-	-
<i>p</i> NPP	3	Variable	7	15	-	-	63.1 ± 3.8	0.92 ± 0.28	1.4	-	-
K <sup>+</sup>	-	10	7	Variable	-	-	134.2 ± 4.5	9.60 ± 2.04	2.2	-	-
K <sup>+</sup>	3	10	-	Variable	-	-	133.3 ± 3.3	6.00 ± 1.50	2.0	-	-
K <sup>+</sup>	3	10	7	Variable	-	-	59.5 ± 4.0	2.79 ± 0.41	1.5	-	-
NH <sub>4</sub> <sup>+</sup>	-	10	7	-	Variable	-	122.2 ± 5.2	9.02 ± 2.51	3.1	-	-
NH <sub>4</sub> <sup>+</sup>	3	10	-	-	Variable	-	127.9 ± 4.2	6.00 ± 1.10	2.2	-	-
NH <sub>4</sub> <sup>+</sup>	3	10	7	-	Variable	-	61.9 ± 3.7	5.46 ± 0.64	2.1	-	-
Mg <sup>2+</sup>	-	10	Variable	15	-	-	135.1 ± 5.0	2.98 ± 0.59	2.2	-	-
Mg <sup>2+</sup>	0.5	10	Variable	15	-	-	129.6 ± 3.0	2.25 ± 0.85	1.2	-	-
Mg <sup>2+</sup>	1	10	Variable	15	-	-	126.4 ± 3.7	2.99 ± 0.66	2.6	-	-
Mg <sup>2+</sup>	2	10	Variable	15	-	-	-	-	-	4.41 ± 0.69	-
Mg <sup>2+</sup>	3	10	Variable	15	-	-	-	-	-	4.81 ± 0.71	-
Na <sup>+</sup>	-	10	7	15	-	Variable	-	-	-	-	16.7 ± 3.65
Na <sup>+</sup>	3	10	-	15	-	Variable	-	-	-	-	15.2 ± 3.42
Ouabain	-	10	7	15	-	-	-	-	-	2.27 ± 0.62	-
Ouabain	3	10	-	15	-	-	-	-	-	2.13 ± 0.97	-

444

445

446

447

#### 448 4. DISCUSSION

449 We provide a comprehensive analysis of the effects of  $\text{Co}^{2+}$  on the modulation *in*  
450 *vitro* of the  $\text{K}^+$ -phosphatase activity in a gill ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase from the blue crab  
451 *Callinectes danae*. Depending on  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$  serves as both stimulator and inhibitor of  
452  $\text{K}^+$ -phosphatase activity. Without  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$  stimulates activity as does  $\text{Mg}^{2+}$ , although  
453 with a  $\approx 4.5$ -fold greater affinity. With  $\text{Mg}^{2+}$ , activity is almost completely inhibited by  
454  $\text{Co}^{2+}$ , while ouabain inhibition is unaffected. Substitution of  $\text{Mg}^{2+}$  by  $\text{Co}^{2+}$  slightly  
455 increases enzyme affinity for  $\text{K}^+$  and  $\text{NH}_4^+$ .  $\text{Mg}^{2+}$  displaces bound  $\text{Co}^{2+}$  from the  $\text{Mg}^{2+}$ -  
456 binding site in a concentration dependent manner; however,  $\text{Co}^{2+}$  does not displace  
457 bound  $\text{Mg}^{2+}$  even at elevated concentrations. Saturation by  $\text{Co}^{2+}$  of the  $\text{Mg}^{2+}$ -binding  
458 site does not affect substrate recognition by the enzyme.

459  $\text{K}$ -phosphatase activities estimated with  $\text{Mg}^{2+}$  or  $\text{Co}^{2+}$  are similar and their  
460 overlapping *p*NPP saturation curves show comparable cooperative effects; their similar  
461  $K_{0.5}$  values suggest that  $\text{Co}^{2+}$  saturation of the  $\text{Mg}^{2+}$ -binding site does not affect  
462 substrate recognition. The high stability constants for the  $\text{Co}^{2+}$ -*p*NPP ( $130 \text{ mol L}^{-1}$ , [76])  
463 and  $\text{Mg}^{2+}$ -*p*NPP ( $170 \text{ mol L}^{-1}$ , [77]) complexes suggest that negligible free metal ions  
464 are present at millimolar metal ion concentrations, i.e., the metal-*p*NPP complex is the  
465 true enzyme substrate. The lower apparent dissociation constant for  $\text{Co}^{2+}$  ( $K_{0.5} = 1.15 \pm$   
466  $0.61 \text{ mmol L}^{-1}$ ), close to that for  $\text{Mg}^{2+}$  ( $K_{0.5} = 1.76 \pm 0.49 \text{ mmol L}^{-1}$ ), is comparable to  
467 the enzyme from *Cancer pagurus* axonal membranes despite its 2-fold greater  
468 maximum *p*NPP hydrolysis rate [78].

469 Millimolar  $\text{Mg}^{2+}$  concentrations are required for  $\text{K}^+$ -phosphatase activity of the  
470 *C. danae* ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase and, like the mammalian enzyme [58,79], no detectable  
471 activity can be measured without this ion. The millimolar  $\text{Mg}^{2+}$  or  $\text{Co}^{2+}$  concentrations  
472 necessary for maximum  $\text{K}^+$ -phosphatase activity exclude the likelihood of metal binding  
473 other than  $\text{Mg}^{2+}$  or  $\text{Co}^{2+}$  to the  $\text{Mg}^{2+}$ -binding site during the catalytic cycle [56]. The  
474 inhibition by free  $\text{Mg}^{2+}$  or  $\text{Co}^{2+}$  of  $\text{K}^+$ -phosphatase activity may result from competition  
475 with  $\text{K}^+$  for the  $\text{Mg}^{2+}$ -binding site [56,61,80] or to excess  $\text{Mg}^{2+}$  bound during the E2K  
476 conformation, decreasing affinity for *p*NPP [52,56,81].

477  $\text{Co}^{2+}$  can substitute for  $\text{Mg}^{2+}$ , stimulating  $\text{K}^+$ -phosphatase activity more efficiently  
478 ( $K_{0.5} \approx 4.5$ -fold lower). However,  $\text{Co}^{2+}$  does not displace  $\text{Mg}^{2+}$  from the  $\text{Mg}^{2+}$ -binding  
479 site of the *C. danae* enzyme. Inhibition by excess  $\text{Co}^{2+}$  likely results from  $\text{Co}^{2+}$  binding at  
480 a site different from the  $\text{Mg}^{2+}$ -binding site. Two distinct  $\text{Mg}^{2+}$  binding sites are known

481 for crustacean [73] and mammalian [50] ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPases, although only one is  
482 relevant for *p*NPPase and ATPase activities [82]. Like  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$  also stimulates sheep  
483 kidney ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase activity, the  $K_D=0.88 \mu\text{mol L}^{-1}$  for  $\text{Mn}^{2+}$  binding being very  
484 similar to the kinetic constant for ATP hydrolysis [83].  $\text{Co}^{2+}$  stimulates dog kidney outer  
485 medulla ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase activity by substituting for  $\text{Mg}^{2+}$  [20]. Differently from  $\text{Co}^{2+}$ ,  
486  $\text{Cu}^{2+}$  inhibits the gill ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase activity of rainbow trout *Oncorhynchus mykiss*  
487 [84], and the  $\text{K}^+$ -phosphatase and ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase activities of rabbit kidney [85] by  
488 directly interfering with  $\text{Mg}^{2+}$  binding, affecting  $\text{Mg}\cdot\text{ATP}$  hydrolysis.

489 The similar  $V_M$  and  $K_{0.5}$  values for stimulation by  $\text{K}^+$  or  $\text{NH}_4^+$  of  $\text{K}^+$ -phosphatase  
490 activity with  $\text{Mg}^{2+}$  or  $\text{Co}^{2+}$  suggests that  $\text{NH}_4^+$  binds to the same site as  $\text{K}^+$  during the  
491 catalytic cycle, independently of  $\text{Mg}^{2+}$  or  $\text{Co}^{2+}$  bound to the  $\text{Mg}^{2+}$ -binding site. While  
492  $\text{K}^+$ -phosphatase activity is inhibited to same degree by  $\text{Mg}^{2+}$  and  $\text{Co}^{2+}$ , the 2-fold greater  
493  $K_{0.5}$  for  $\text{NH}_4^+$  ( $5.46 \text{ mmol L}^{-1}$ ) compared to  $\text{K}^+$  ( $2.79 \text{ mmol L}^{-1}$ ) suggests that  $\text{Co}^{2+}$   
494 binding to a different  $\text{Mg}^{2+}$ -binding site affecting enzyme interaction with  $\text{NH}_4^+$ .  
495 Without  $\text{Na}^+$ , stimulation by  $\text{K}^+$  of  $\text{K}^+$ -phosphatase activity involves two  $\text{K}^+$  binding  
496 sites: one that regulates *p*NPP access to the phosphatase site, the other increasing  
497 catalytic activity [86]. ATP binding to the low-affinity substrate binding site induces a  
498 conformational change in the cytoplasmic domain of the enzyme attributed to the E2 to  
499 E1 transition; the subsequent binding of  $\text{Mg}^{2+}$  to the enzyme•ATP complex induces a  
500 new conformational change that facilitates the E1 to E2 transition [87]. Like  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$   
501 may induce a conformational change, stimulating the enzyme during *p*NPP hydrolysis.

502  $\text{K}^+$ -phosphatase activity can be stimulated by  $\text{Tl}^+$ ,  $\text{Rb}^+$  or  $\text{NH}_4^+$  to rates similar to  
503  $\text{K}^+$  stimulation while  $\text{Cs}^+$  and  $\text{Li}^+$  exhibit lower stimulation (5-30%) [88,89]. For 21‰  
504 (low salinity)-acclimated *C. danae*,  $\text{Rb}^+$  stimulates gill *p*NPPase activity by 1.5-fold  
505 compared to  $\text{K}^+$  [63].  $\text{NH}_4^+$  may sustain ATP hydrolysis by replacing  $\text{K}^+$  [89,90] and is  
506 actively transported by crustacean and vertebrate enzymes [91,92]. Together with  $\text{K}^+$ ,  
507  $\text{NH}_4^+$  synergically stimulates ATP hydrolysis by the *C. danae* ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase  
508 through an additional increment, strongly influenced by  $\text{Mg}^{2+}$  and  $\text{Na}^+$ , underlying  
509 ammonia excretion in crustaceans [90]. The E2 conformation is the main state  
510 responsible for *p*NPP hydrolysis [58] and is likely the reason that  $\text{K}^+$ -phosphatase  
511 activity is not synergically stimulated by  $\text{K}^+$  and  $\text{NH}_4^+$ . Synergic stimulation using  
512 *p*NPP as a substrate is known exclusively for the shelling crab *C. ornatus* [63]. When  
513 using ATP as a substrate, species-specific synergic stimulation is found in various  
514 crustaceans [73].

515 The inhibition by  $\text{Na}^+$  of *C. danae* pNPPase activity independently of  $\text{Mg}^{2+}$  or  
516  $\text{Co}^{2+}$  is seen also for  $(\text{Na}^+, \text{K}^+)\text{-ATPases}$  from various sources [73], and reflects  
517 competition by  $\text{Na}^+$  for the cytoplasmic  $\text{K}^+$ -binding sites, favoring the E1 conformation  
518 [50,56,58,93–95].  $\text{Na}^+$  inhibition also may involve events other than the simple binding  
519 of the ion. To illustrate, the synergistic stimulation by  $\text{Na}^+$  and  $\text{K}^+$  (3%) of pNPPase  
520 activity in the electric organ of *Electrophorus electricus* [86] suggests that both ions  
521 bind to different sites. Both  $10 \text{ mmol L}^{-1} \text{Na}^+$  and  $15 \text{ mol L}^{-1} \text{K}^+$  inhibit the *C. danae*  
522 pNPPase activity by  $\approx 40\%$ , as seen in the freshwater shrimp *Macrobrachium olfersii*  
523 [61] and crabs *Cancer pagurus* [95] and *C. ornatus* [63] under identical assay  
524 conditions.  $\text{Na}^+$  inhibits pNPPase activity allosterically in various mammalian and  
525 crustacean  $(\text{Na}^+, \text{K}^+)\text{-ATPases}$  [61,63,86,89,95] including *M. olfersii* [61] and *C.*  
526 *pagurus* [95]. Thus, considering a two  $\text{K}^+$ -binding site model [86], at low (10-fold less  
527 than  $\text{K}^+$ ) concentrations,  $\text{Na}^+$  competes for the high-affinity  $\text{K}^+$  binding site, leading to  
528 allosteric effects. At high concentrations (similar to  $\text{K}^+$ ),  $\text{Na}^+$  competes for the low-  
529 affinity  $\text{K}^+$ -binding site, reducing maximum hydrolysis rate.

530  $\text{Co}^{2+}$  does not affect ouabain binding to the *C. danae* gill  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ , the  
531 single inhibition curve and  $K_i$  being very similar to  $\text{Mg}^{2+}$ . Most species, except the red  
532 river crab *Dilocarcinus pagei* [96], exhibit a single ouabain inhibition curve  
533 independently of substrate [62,63,72,97,98]. The  $K_i$  for ouabain inhibition with  $\text{Co}^{2+}$   
534 lies in the range for various  $(\text{Na}^+, \text{K}^+)\text{-ATPases}$  [73]. It should be noted that only for the  
535 cerebromicrovascular  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ ,  $\text{Pb}^{2+}$  and  $\text{Al}^{3+}$  caused selective alterations in  
536 ATP hydrolysis inhibiting and stimulating ouabain binding, respectively [99].

537 Our findings reveal that without  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$  stimulates the gill  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$   
538 to levels similar to  $\text{Mg}^{2+}$ . Without  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$  stimulates  $\text{K}^+$ -phosphatase activity  
539 similarly although with a  $\approx 4.5$ -fold greater affinity than with  $\text{Mg}^{2+}$ , which almost  
540 completely inhibits  $\text{K}^+$ -phosphatase activity.  $\text{Mg}^{2+}$  displaces  $\text{Co}^{2+}$  from the  $\text{Mg}^{2+}$ -binding  
541 sites in a concentration dependent manner. Ouabain inhibition is identical with  $\text{Co}^{2+}$  or  
542  $\text{Mg}^{2+}$ . Saturation by  $\text{Co}^{2+}$  of the  $\text{Mg}^{2+}$ -binding sites does not affect substrate recognition  
543 by the enzyme. Given the complex interactions between heavy metal ion contaminants  
544 and enzymes, their toxic effects at the molecular level are poorly understood. Our  
545 findings contribute to elucidate partly the mechanism of action of  $\text{Co}^{2+}$  on a crustacean  
546 gill  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ .

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945

946 **Data availability**

947 The datasets generated and/or analyzed during this study are available from the  
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949

950 **Declaration of competing interests**

951 All authors certify that they have no affiliations with or involvement in any  
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954

955 **Ethical approval studies in animals**

956 This investigation complies with all local, state, federal and international  
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958 also complies with the ARRIVE guidelines.