Different combinations of insect Na,K-ATPase α- and β-subunit paralogs enable fine tuning of toxin resistance and enzyme kinetics

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Summary statement

Activity and cardiac glycoside resistance of the *Oncopeltus fasciatus*' Na,K-ATPase is determined by amino acid substitutions in the α-subunit binding pocket, the α/β-subunit combination and the cardiac glycoside identity.

Abstract

Cardiac glycosides are known to fatally inhibit the Na,K-ATPase throughout the animal kingdom. Several animals, however, evolved target-site insensitivity by substitution in the otherwise highly conserved cardiac glycoside binding pocket located on the Na,K-ATPase α-subunit. The minimal functional enzyme consists of an α- and a β-subunit, considered mainly as a chaperone responsible for correct folding and membrane integration. We here analyze resistance to cardiac glycosides and kinetic properties of different Na,K-ATPase α-β-combinations of the large milkweed bug, *Oncopeltus fasciatus.* These insects have adapted to high concentrations of cardiac glycosides in their food plants via several rounds of ATPase α1 gene duplications followed by subfunctionalization of the enzymes.

To investigate their characteristics we expressed nine combinations of Na,K-ATPase α/β-sunbunits (three each) in *Sf*9 cells and tested them with two structurally distinct cardiac glycosides, calotropin, a host plant compound, and ouabain, a commonly used toxin. Differences in the number and identity of amino acid substitutions in the cardiac glycoside binding site resulted in large differences in activity and toxin resistance of the three α-

47 subunits. The enzymes' kinetics were also influenced by the β-subunits leading to
48 increased activities (αCβ3) or altered resistances. Calotropin proved to be a much more
49 potent inhibitor than ouabain. Our results show that the possession of multiple paralogs
50 enables adaptation to plant toxins and mitigates pleiotropic effects by a compromise
51 between ion pumping activity and resistance.

52 Introduction

53 The transmembrane Na,K-ATPase, is ubiquitously expressed in animal cells and is essential
54 for maintaining membrane potentials, controlling cell volume and the overall functionality
55 of cells (Horisberger, 2004). The functional enzyme consists of two subunits, the catalytic
56 α-subunit and an associated β-subunit serving as chaperone for correct folding and
57 membrane integration (Geering, 2001). In vertebrates several paralogs are present for
58 both genes and the enzyme's characteristics differ depending on the specific combination
59 of α- and β-subunits (Blanco, 2005; Blanco and Mercer, 1998). An additional modulatory
60 and tissue specific γ-subunit is present in vertebrate tissues (Geering, 2005), but has not
61 been detected in invertebrates. Much less is known about the importance of different α-
62 β-subunit combinations in invertebrates (Okamura et al., 2002). Only in *Drosophila* the
63 tissue distribution and functional roles of some of these combinations have been explored
64 to some extent (Baumann et al., 2010; Paul et al., 2007; Yasuhara et al., 2000). We here
65 explore the functional role of different Na,K-ATPase complexes in the large milkweed bug,
66 *Oncopeltus fasciatus*, that have undergone three rounds of duplications of the Na,K-
67 ATPase (Yang et al., 2019; Zhen et al., 2012) and display high insensitivity against cardiac

68 glycosides (Bramer et al., 2015; Moore and Scudder, 1986), which are universal inhibitors
69 of the Na,K-ATPase (Horisberger, 2004).

70 Inhibition of the Na,K-ATPase by cardiac glycosides, such as the cardenolides ouabain and
71 calotropin (Fig. 1), depends on a specific binding site located on the extracellular side of
72 the catalytic α-subunit. Upon binding of cardiac glycosides the enzyme is arrested in the
73 phosphorylated state and ion pumping is disrupted (Horisberger, 2004). Inhibition of the
74 Na,K-ATPase is harmful and can be lethal to almost all animals. There are, however, a few
75 notable exceptions to this: various mammals, frogs and reptiles prey unharmed on cardiac
76 glycoside-containing toads and a number of phytophagous insects feed - often exclusively
77 - on cardiac glycoside-containing host plants (reviewed in Agrawal et al., 2012; Malcolm,
78 1991). Most of these species avoid the toxic effect of cardiac glycosides and exhibit target
79 site insensitivity, by drastically lowering binding affinity of cardiac glycosides to their Na,K-
80 ATPase α-subunit (reviewed in Petschenka et al., 2017; Ujvari et al., 2015). Strikingly,
81 identical amino acid substitutions at homologous positions convergently evolved in
82 various, distantly related insect groups and even resemble the substitutions observed in
83 cardiac glycoside-resistant vertebrates (Dobler et al., 2012; Mohammadi et al., 2016;
84 Ujvari et al., 2015; Zhen et al., 2012).

85 While insect species usually express a single gene copy of the Na,K-ATPase α-subunit
86 (ATPα1) throughout their body, this gene underwent one to three rounds of gene
87 duplications in several cardiac glycoside-exposed species (Dobler et al., 2019; Petschenka
88 et al., 2017; Yang et al., 2019; Zhen et al., 2012). These duplicated ATPα1 copies differ in
89 the number and identity of presumably resistance-causing amino acid substitutions and

90 show tissue specific expression patterns (Dobler et al., 2019; Lohr et al., 2017; Yang et al.,
91 2019; Zhen et al., 2012).

92 The large milkweed bug *Oncopeltus fasciatus* (Hemiptera, Lygaeidae) possesses four
93 ATPα1 paralogs (A, B, C, D) that differ in the number and identity of amino acid
94 substitutions (Yang et al., 2019; Zhen et al., 2012). We previously introduced substitutions
95 observed in these paralogs at four usually highly conserved amino acid sites (Q111, N122,
96 F786 and T797) known to be important for high affinity to cardiac glycosides (Croyle et al.,
97 1997; Laursen et al., 2015; Palasis et al., 1996; Price and Lingrel, 1988) into the sensitive
98 ATPα1 gene of *Drosophila melanogaster* (Dalla and Dobler, 2016; Dalla et al., 2017). The
99 N122H (as observed in ATPα1D of *O. fasciatus*), N122H+T797S (as observed in ATPα1C),
100 Q111T+N122H+F786N (as in ATPα1B) or Q111T+N122H+F786N+T797A (as in ATPα1A)
101 substitutions yielded proteins with stepwise increasing resistance to the cardenolide
102 ouabain. At the same time, enzyme activity decreased with increasing numbers of
103 substitutions (Dalla and Dobler, 2016).

104 Additional untested substitutions are present in the *O. fasciatus* ATPα1 paralogs and these
105 might influence the characteristics of the enzymes, potentially further increasing
106 resistance but possibly also serving as compensatory mutations mitigating deleterious
107 intra-molecular epistatic effects of previously characterized mutations (Karageorgi et al.,
108 2019; Taverner et al., 2019). In addition, it has been shown that the β-subunit of the Na,K-
109 ATPase not only acts as a chaperone, guiding the heterodimeric enzyme to the cell
110 membrane, but also influences the behavior of the Na,K-ATPase including its sensitivity to
111 ouabain (Blanco, 2005; Dalla et al., 2017; Geering, 2001).

112 Although ouabain is a commonly used cardiac glycoside in studies of the Na,K-ATPase, it
113 does not occur in host plants of *O. fasciatus*. The *Asclepias* species (Apocynaceae) that the
114 bugs feed on typically contain different types of cardenolides characterized by a *trans-*
115 *trans-cis* conformation of the steroidal skeleton and a single sugar moiety attached by a
116 cyclic bridge as in the abundant calotropin (Malcolm, 1991) (Fig. 1). The most biologically
117 relevant test of the bugs' resistance against cardiac glycosides should thus involve a plant-
118 derived compound like calotropin. To address these issues, we identified and
119 heterologously expressed nine possible *O. fasciatus* Na,K-ATPase α/β combinations and
120 characterized their activities and sensitivities to ouabain and calotropin.

121 Since activity and resistance of the Na,K-ATPase is determined by the number and identity
122 of amino acid substitutions in the cardenolide binding site located in the α-subunit, (1) we
123 hypothesized that α-subunit paralogs – associated with different β-subunits – differ
124 significantly in their activities and insensitivities to the two cardenolides we tested. (2) We
125 further hypothesized that the different β-subunits modulate the Na,K-ATPase α-subunit
126 paralog behavior influencing their response to the cardenolides. Additionally, although
127 ouabain and calotropin both seem to be strong inhibitors of the Na,K-ATPase, (3) we
128 expected the two cardenolides to differ significantly in their inhibitory potency against the
129 Na,K-ATPase of *O. fasciatus* due to differences in structure, steric conformation and
130 lipophilicity.

131 Material and methods

132 **Identification of sequences**

133 Two of the three previously published gene copies of the Na,K-ATPase α1 gene (Zhen et
134 al., 2012) (α1B Acc.No. JQ771519, α1C Acc.No. JQ771518) were incomplete at the 3' end.
135 Both missing ends could be recovered by RACE PCR (SMARTer RACE kit, Takara Bio,
136 Mountain View, CA, USA).

137 Previously published transcriptomic data of *O. fasciatus* (Zhen et al., 2012) were used to
138 identify the full-length coding sequences of the Na,K-ATPase β-subunits. The raw data
139 were trimmed and assembled with Trinity (Grabherr et al., 2011) as described in Meinzer
140 et al. (2019). *O. fasciatus* β-subunit sequences were recovered using published
141 orthologous sequences from *Locusta migratoria* (Orthoptera; Acc.No. KF813098-
142 KF813100) and *Blatta germanica* (Blattodea; Acc.No. HE819405) in tblastn searches
143 against the transcriptome. *De novo* assembly of the raw reads and quality control was
144 performed with the program CLC-Genomics Workbench (Qiagen, Hilden, Germany). The
145 resulting *contigs* were screened for open reading frames (ORFs) with a length of roughly
146 900 bp (the consensus length of the highly conserved Na,K-ATPase β-subunit) using the
147 program VectorNTI (Thermo Fisher, Darmstadt, Germany). Homology of the *contigs* were
148 assigned by a further round of tblastx searches against published sequences in genbank.

149 **Construction of recombinant baculoviruses**

150 The coding sequences of the Na,K-ATPase α- and β-subunits genes (α1A, α1B, α1C, β1, β2
151 and β3) were amplified from cDNA transcribed with Superscript III (Thermo Fisher) from
152 RNA extracted from nervous tissue, gut, testes or muscles with the RNAmagic-kit (Bio-
153 Budget Technologies GmbH, Krefeld, Germany). For downstream cloning all subunits were

154 amplified with the Pfu-Phusion Polymerase (Thermo Scientific) and primers including the
155 full coding sequence and incorporating suitable restriction sites at the 5' end.

156 PCR products were restricted with the chosen enzymes (Thermo Fisher), gel extracted
157 (QIAquick gel extraction kit, Qiagen) and ligated into pFastBac-Dual vectors (Thermo
158 Fisher). The final plasmids had an α-subunit under the control of the polyhedrin promoter
159 (PH) and a β-subunit under the p10 promoter. Recombinant baculoviruses were generated
160 as previously described (Dalla et al., 2017) and transfected into *Sf*9 cells to obtain a first
161 (P1) and second (P2) generation of recombinant virus stock.

162 **Production of recombinant proteins**

163 Production of different recombinant Na,K-ATPase α-β-subunit constructs started from
164 infection of Sf9 cells with P2 virus stock and followed our previously published protocol
165 (Dalla et al., 2017). The cells were harvested by centrifugation, the pellet resuspended and
166 sonicated and cell debris removed by centrifugation. Cell membranes were recovered by
167 ultracentrifugation of the supernatant and redissolved in pure water (ROTIPURAN® p.a.,
168 ACS water; Roth). The concentrations of the resuspended protein pellets were determined
169 by Bradford assay (Sigma-Aldrich, Taufkirchen, Germany) using bovine serum albumin
170 (BSA) as a standard.

171 **SDS-PAGE/ Western blotting**

172 Thirty µg of the heterologously expressed proteins were deglycosylated with
173 endoglycosidase H following the manufacturer's protocol (Promega GmbH, Wiesloch,
174 Germany) but instead of boiling proteins for 5 minutes at 95°C we performed the

175 denaturation step at 50°C for 30 minutes to maintain the integrity of the protein. SDS-
176 polyacrylamide gel electrophoresis and western blotting was performed as previously
177 reported (Dalla et al., 2017) with few modifications. The membrane proteins were blotted
178 on a nitrocellulose membrane for 90 minutes at 100 V with an additional ice block at 4°C.
179 To mask unspecific binding sites the membrane was blocked with BlueBlock PF (Serva,
180 Heidelberg, Germany). The split membranes were incubated with the primary monoclonal
181 antibodies α5, α-tubulin (DSHB Hybridoma Bank, University of Iowa; deposited by D.M.
182 Fambrough (Lebovitz et al., 1989), and customized specific polyclonal antibodies for the
183 subunits β1, β2 and β3 (Davids Biotechnology, Regensburg, Germany). For detection of
184 the primary antibodies the membrane was incubated for one hour with goat anti-mouse
185 (α5, α-tubulin), anti-rabbit (β2) and anti-chicken (β1, β3) secondary antibodies conjugated
186 with horseradish peroxidase (Dianova, Hamburg, Germany) and developed with 4-chloro-
187 1-naphtol as previously described (Dalla et al., 2017).

188 **Extraction and preparation of cardenolides**

189 Calotropin was extracted according to (Petschenka et al., 2018) from approximately 8 g of
190 dried larvae of the monarch butterfly *Danaus plexippus* reared on *Asclepias curassavica*.
191 The cardenolides were extracted with methanol, lipids extracted with hexane and the
192 cardenolides retrieved by chloroform extraction. The concentrated methanolic extract
193 was subjected to preparative HPLC (Agilent 1100) equipped with a photodiode array
194 detector and a semi-preparative RP-Nucleodur C18 Htec column (5 µm, 250 x 10 mm,
195 Macherey-Nagel, Düren, Germany) using a water-acetonitrile gradient. Calotropin was
196 identified by its retention time and checked for molecular weight and LS-MS spectrum by

197 LC-ESI-TOF spectrometry by Dr. Maria Riedner (Department of Chemistry, Universität
198 Hamburg) and the structure confirmed by NMR spectroscopy. The resulting calotropin
199 preparation was 80% pure.

200 Calotropin and the commercially available ouabain (Sigma-Aldrich) were first dissolved in
201 DMSO and prepared for Na,K-ATPase activity assays as dilutions in sterile H_2O reaching
202 final concentrations from 10^{-8} to 10^{-3} M with 2% DMSO. Ouabain could in addition be
203 prepared as a $5x10^{-3}$ M solution.

204

205 **Na,K-ATPase activity and inhibition assay**

206 Activities of the nine different Na,K-ATPase α/β-subunit combinations of *O. fasciatus* were
207 determined by photometric measurements of inorganic phosphate released from
208 enzymatic ATP hydrolysis while subtracting background ATPase activity following
209 previously described methods (Dalla et al., 2017; Petschenka et al., 2013). Based on
210 previous results (Dalla et al., 2017) the buffer regime used was 50 mM NaCl, 4 mM $MgCl_2$,
211 50 mM imidazol, 20 mM KCl, pH 7.4. The inhibition of the different Na,K-ATPases was
212 tested under increasing ouabain and calotropin concentrations and expressed as
213 percentage activity of a non-inhibited control. For each α/β-subunit construct duplicates
214 of at least three biological replicates of recombinant proteins isolated from independently
215 transfected *Sf*9 cells were tested.

216 **Analysis of data/ Statistics**

217 After checking for normal distribution (Shapiro Wilk's test) and variance homogeneity
218 (Levene's test on squared deviations), differences in total Na,K-ATPase activities were
219 tested and compared between each α/β-subunit construct by one-way ANOVA followed
220 by Tukey's HSD posthoc test. The data of each Na,K-ATPase α/β-subunit construct was
221 subjected to nonlinear curve fitting with dose response function and Levenberg
222 Marquardt iteration algorithm in Origin-Pro 9.1G (OriginLab Corporation, Northampton,
223 USA) using a four parameter logistic function, top asymptote was set to 100, bottom
224 asymptote set to 0 and a variable Hill slope. For each construct IC_{50} values were calculated
225 and differences were analyzed separately for constructs affected by ouabain and
226 calotropin by one-way ANOVA followed by Tukey's HSD posthoc tests. A pairwise, F-test
227 based comparison of similarity between the fitted curves was performed using the
228 command "compare datasets". All calculations were performed with Origin-Pro 9.1G.

229

230

231 # Results

232 *Bioinformatic analysis*

233 Four potential β-subunits were identified from the *O. fasciatus* transcriptome and
234 according to their similarity to published sequences named β1, β2, β3 and βx (Genbank
235 AccNo xxx-xxx). While β1-3 conform to the standard length of β-subunits of around 900
236 bp, βx is much shorter and missing most of the conserved N-terminal and half of the
237 transmembrane region (Fig. S1A). This β-subunit was therefore not further considered

238 here. The 5' end of α1B and α1C could successfully be recovered by RACE protocols (Fig.
239 S1B). The only minimally expressed α1D (Yang et al., 2019) was not further investigated
240 here.

241 *Expression of α/β-subunit constructs in* Sf*9 cells*

242 All nine α/β-subunit constructs (αAβ1, αAβ2, αAβ3, αBβ1, αBβ2, αAβ3, αCβ1, αCβ2, and
243 αCβ3) were successfully expressed in *Sf*9 cells and harvested. The western blot shows that
244 identical concentrations were loaded onto the gel (α-tubulin as positive control), all α-
245 subunits were expressed at similar levels and the deglycosylated β-subunits can be easily
246 discriminated by their specific patterns (Fig. 2).

247

248 *Na,K-ATPase activity of different α/β-subunit constructs*

249 The activities of different α/β-subunit combinations of the Na,K-ATPase were tested *in*
250 *vitro* to determine whether the amino acid substitutions in the cardenolide binding site or
251 the associated β-subunit influence the catalytic activity of the enzyme. All enzymes
252 showed catalytic activity, but the activity of the α/β-subunit constructs differed
253 significantly from one another (Fig. 3). The α-subunits A and B with their associated β-
254 subunits (1-3) showed significantly lower activity than αC combined with β1, 2 or 3. The
255 activities of αA and αB did not differ significantly from each other regardless of the β-
256 subunit, except for αAβ2. This combination was significantly less active than αB associated
257 with the three β-subunits (Fig. 3). By far the highest activity was measured for αC in

258 association with β3 that had an average turnover of 36.5 nmol Pi mg^{-1} Protein * minute
259 and differed significantly from all other constructs.

260 *Influence of α/β-subunit association on ouabain sensitivity*

261 *In vitro* tests with the cardenolide ouabain revealed large differences in sensitivity of the
262 Na,K-ATPases depending on the number of amino acid substitutions in the cardenolide
263 binding site and was modulated by the identity of the β-subunit (Fig. 4). Yet, when
264 comparing the IC_{50} values only the one of αC associated with β1 was significantly lower
265 than those for the other two αC constructs (ANOVA, $F_{2,8}$=8.035, p=0.02, Tukey's HSD,
266 p=0.035 and p=0.027, for αC β1 vs β2 and β1 vs β3, respectively; Fig. 4c, Table 1) whereas
267 the IC_{50} values of the three αA or αB constructs did not differ significantly.

268 Although analyzing differences in IC_{50} is a standard approach, the IC_{50} values only reflect
269 one point of the dose response curve. We therefore aimed to get a better insight into the
270 overall responses of the Na,K-ATPases to increasing ouabain concentrations by analyzing
271 the differences between the full dose response curves of constructs with the same α-
272 subunit as well as constructs with the same β-subunit (Table 2).

273 We found that α-subunits associated with different β-subunits behaved differently when
274 subjected to ouabain. The construct αAβ3 was significantly more sensitive to the
275 cardenolide than the other two αA complexes (Table 2, Fig. 4a), and αBβ1 was inhibited
276 more strongly by ouabain than αBβ2 (Table 2, Fig. 4b). Nevertheless, the reaction of
277 constructs with identical α-subunits was more uniform than the one of constructs with
278 identical β- but different α-subunits. Most of the latter constructs differed significantly in

279 their reactions, reflecting their varying insensitivity levels against ouabain (Fig. 4 a-c, Table
280 2).

281

282 *Influence of α/β-subunit association on calotropin sensitivity*

283 Testing the inhibitory effect of the host plant cardenolide calotropin on the recombinant
284 Na,K-ATPases resulted in total inhibition of the most calotropin-sensitive αC/β-subunit
285 constructs at the highest calotropin concentration. Furthermore, the three αB constructs
286 turned out to be most resistant against calotropin. As for the inhibition by ouabain only
287 the IC_{50} values of αC associated with the three different β-subunits differed significantly
288 from each other, αC associated with β3 was significantly more insensitive than αC
289 associated with β1 and β2 (ANOVA, $F_{2,8}$=18.554, p=0.003, Tukey's HSD, p=0.002 and
290 p=0.027, for αCβ3 versus αCβ1 and αCβ3 versus αCβ2, respectively; Fig. 4c).

291 When comparing the full dose response curves of the constructs (Table 2) neither the
292 three αA constructs differed significantly from one another nor the αB or αC constructs,
293 indicating a similar response to calotropin exposition independent of the associated β-
294 subunit (Fig. 4 a-c). In contrast, the inhibition by calotropin of the three α-subunits
295 combined with the same β-subunits was significantly different (from high to low
296 inhibition: αC> αA> αB; Fig. 4a-c). The curves of αAβ1 and αBβ1 did not differ significantly
297 from one another, since αAβ1 tends to be the most resistant combination of the three αA
298 constructs and αBβ1 the most sensitive of the three αB constructs (Fig. 4a & b).

299

300 *Comparison of both cardenolides*

301 Calotropin had a higher inhibitory potency and inhibited especially the three αC-
302 constructs to a much higher extent than ouabain (ANOVA, $F_{5,17}$=57.25882, p<0.001,
303 Tukey's HSD, p<0.001 for αC β1, αC β2 and αC β3, respectively; Fig. 4c, Table 1).
304 Calotropin inhibited αCβ1 18-fold, αCβ2 20-fold and αCβ3 14-fold more strongly than
305 ouabain did. Similarly, αB associated with β3 was significantly more inhibited by calotropin
306 than by ouabain, resulting in an approximately 24-fold higher IC_{50} (ANOVA, $F_{5,17}$=7.54487,
307 p=0.00205, Tukey's HSD, p=0.00357; Fig. 4b, Table 1) while the IC_{50} values of the two
308 cardenolides did not differ significantly for αBβ1 and αBβ2 (Tukey's HSD, p=0.70595 and
309 p=0.51146, respectively; Fig. 4b, Table 1).

310 Additionally, we compared the dose response curves of same constructs inhibited by
311 calotropin and ouabain. Besides similar results for the αC constructs, we found that the
312 construct αAβ1 was significantly more insensitive against calotropin than against ouabain,
313 reflected by a flat descending curve at the top (Fig. 4c & 4a, Table 3).

314

315 Table 1: Mean IC_{50} values and Hill slopes of different Na,K-ATPase α-β-constructs (3 biological replicates with
316 standard deviation (± s.d.)) obtained from dose response curves upon inhibition with ouabain and calotropin
317 (Origin-Pro 9.1G).

α/β-combination	IC_{50} values [M] Ouabain (± s.d.)	Hill slope (± s.d.)	IC_{50} values [M] Calotropin (± s.d.)	Hill slope (± s.d.)

αAβ1	4.148E-04 (± 6.541E-04)	-0.221 (± 0.047)	1.211E-04 (± 4.443E-05)	-0.271 (± 0.03)
αAβ2	3.471E-04 (± 3.598E-04)	-0.234 (± 0.013)	1.449E-05 (± 5.994E-06)	-0.269 (± 0.076)
αAβ3	2.607E-05 (± 2.767E-05)	-0.260 (± 0.044)	6.959E-05 (± 7.54E-05)	-0.27 (± 0.036)
αBβ1	2.089E-03 (± 2.515E-03)	-0.213 (± 0.039)	1.108E-04 (± 3.423E-05)	-0.293 (± 0.044)
αBβ2	2.777E-03 (± 9.676E-04)	-0.462 (± 0.055)	3.281E-04 (± 2.119E-04)	-0.398 (± 0.066)
αBβ3	7.09E-03 (± 3.119E-03)	-0.261 (± 0.045)	2.944E-04 (± 8.195E-05)	-0.254 (± 0.071)
αCβ1	4.274E-04 (± 8.104E-05)	-0.869 (± 0.071)	2.402E-05 (± 4.636E-06)	-0.88 (± 0.029)
αCβ2	7.369E-04 (± 1.001E-04)	-0.869 (± 0.093)	3.699E-05 (± 5.517E-06)	-1.289 (± 0.198)
αCβ3	7.579E-04 (± 1.476E-04)	-0.988 (± 0.35)	5.594E-05 (± 8.55E-06)	-0.912 (± 0.098)

319 Table 2: Comparison between the dose response curves of different α/β constructs inhibited by ouabain and

320 calotropin analyzed with Origin-Pro 9.1G. Significant differences are highlighted in bold.

Data comparison α/β-combinations	F-statistic Ouabain	F-statistic Calotropin
αAβ1 – αAβ2	$F_{2,8}$= 0.074, p= 0.929	$F_{2,8}$= 1.319, p= 0.320
αAβ1 – αAβ3	**$F_{2,8}$= 5.218, p= 0.035**	$F_{2,8}$= 0.728, p= 0.512
αAβ2 – αAβ3	**$F_{2,8}$= 6.092, p= 0.025**	$F_{2,8}$= 0.079, p= 0.925
αBβ1 – αBβ2	**$F_{2,8}$= 10.981, p= 0.005**	$F_{2,8}$= 2.607, p= 0.134
αBβ1 – αBβ3	$F_{2,8}$= 3.781, p= 0.070	$F_{2,8}$= 0.724, p= 0.514
αBβ2 – αBβ3	$F_{2,8}$= 0.455, p= 0.650	$F_{2,8}$= 0.655, p= 0.545
αCβ1 – αCβ2	$F_{2,8}$= 0.745, p= 0.505	$F_{2,8}$= 1.887, p= 0.213
αCβ1 – αCβ3	$F_{2,8}$= 1.535, p= 0.273	$F_{2,8}$= 3.402, p= 0.085
αCβ2 – αCβ3	$F_{2,8}$= 1.080, p= 0.384	$F_{2,8}$= 2.217, p= 0.171
αAβ1 – αBβ1	$F_{2,8}$= 1.039, p= 0.397	$F_{2,8}$= 0.554, p= 0.595
αAβ1 – αCβ1	**$F_{2,8}$= 22.729, p< 0.001**	**$F_{2,8}$= 8.724, p= 0.010**
αBβ1 – αCβ1	**$F_{2,8}$= 11.975, p= 0.004**	**$F_{2,8}$= 8.795, p= 0.010**
αAβ2 – αBβ2	**$F_{2,8}$= 57.311, p< 0.001**	**$F_{2,8}$= 9.763, p= 0.007**
αAβ2 – αCβ2	**$F_{2,8}$= 4.835, p= 0.042**	**$F_{2,8}$= 6.422, p= 0.022**
αBβ2 – αCβ2	$F_{2,8}$= 2.353, p= 0.157	**$F_{2,8}$= 13.589, p= 0.003**
αAβ3 – αBβ3	**$F_{2,8}$= 32.590, p< 0.001**	**$F_{2,8}$= 37.553, p< 0.001**
αAβ3 – αCβ3	**$F_{2,8}$= 33.295, p< 0.001**	**$F_{2,8}$= 19.271, p< 0.001**

αBβ3 – αCβ3	$F_{2,8}$= 1.614, p= 0.258	**$F_{2,8}$= 8.239, p= 0.011**

321

322 Table 3: Comparison between dose response curves of same α/β constructs inhibited by ouabain and
323 calotropin analyzed with Origin-Pro 9.1G. Significant results are highlighted in bold.

Data comparison α/β-combinations	F-statistic calotropin vs. ouabain
αAβ1	**$F_{2,8}$= 11.752, p= 0.004**
αAβ2	$F_{2,8}$= 2.421, p= 0.151
αAβ3	$F_{2,8}$= 0.589, p= 0.577
αBβ1	$F_{2,8}$= 0.343, p= 0.720
αBβ2	$F_{2,8}$= 4.257, p= 0.055
αBβ3	$F_{2,8}$= 3.054, p= 0.103
αCβ1	**$F_{2,8}$= 35.226, p< 0.001**
αCβ2	**$F_{2,8}$= 100.356, p< 0.001**
αCβ3	**$F_{2,8}$= 74.665, p< 0.001**

324

325 # Discussion

326 Here we have identified the full length coding sequences of three Na,K-ATPase α- and
327 three full length β-subunits of *O. fasciatus* and functionally characterized all nine possible
328 recombinant α/β enzyme complexes. These nine functional enzymes strongly differed in
329 their activity levels. We found that enzyme complexes with αC had a significantly higher

330 activity compared to the other constructs. This perfectly fits with the fact that αC is mainly
331 expressed in the nervous tissue of *O. fasciatus* (Lohr et al., 2017; Zhen et al., 2012) where
332 the Na,K-ATPase plays a crucial role in maintaining electric potentials that guarantee the
333 excitability of neurons. Consequently, a high ionic pumping activity is indispensable. Two
334 of our nine α/β constructs clearly stand out amongst the other enzyme complexes: αAβ2
335 and αCβ3. The activity of αAβ2 is reduced in comparison to the other αA and αB
336 constructs that all show similar activity levels. Therefore, we suppose that αAβ2 is not a
337 functional enzyme *in vivo*. In contrast, the activity of αC combined with β3 is significantly
338 higher and would therefore well fit with a natural occurrence of this complex in the
339 nervous tissue. Data of an ongoing proteomic study support this assumption and show
340 that the subunits αC and β3 are most abundant in the nervous tissue of *O. fasciatus* (M.
341 Winter et al., unpublished data).

342 Regardless of the strikingly high overall resistance to inhibition by cardenolides that Na,K-
343 ATPase preparations of *O. fasciatus* display (Bramer et al., 2015; Dalla and Dobler, 2016;
344 Moore and Scudder, 1986) we found that the individual α/β-combinations strongly
345 differed in their resistance against the two cardenolides ouabain and calotropin. Overall
346 calotropin proved to be the much more potent inhibitor. This is despite the fact that *O.*
347 *fasciatus* is naturally exposed to calotropin in its host plants (Malcolm, 1991) and thus
348 could be specifically adapted to this toxin. Yet, our data demonstrate that in particular the
349 most active αC-subunit was roughly 20-times more strongly inhibited by calotropin than
350 by ouabain. A differential inhibition can also be observed for the three αB constructs while
351 the difference in inhibition between ouabain and calotropin is least pronounced for the

352 αA constructs. Overall, the αA- and αB-subunits are more insensitive to both cardenolides
353 than αC, which fits well with their abundant expression in the Malpighian tubules where
354 the enzymes are directly exposed to toxins in the hemolymph (Lohr et al., 2017). In
355 contrast, the least sensitive but most active αC is the dominant Na,K-ATPase α-subunit in
356 the nervous tissue where it is protected from intruding cardenolides by the perineurium
357 (blood-brain-barrier) which controls xenobiotica access to the nervous system by a tight
358 network of septate junctions and active efflux carriers (Dermauw and Van Leeuwen,
359 2014).

360 The difference in inhibitory potencies of the two cardenolides results from differences in
361 their structure, steric conformation and - *in vivo* - by their ability to permeate membranes,
362 as reflected by their lipophilicity. Ouabain is produced in plants of the genus *Acokanthera*
363 and in seeds of *Strophanthus gratus* (both Apocynaceae), whereas calotropin is
364 synthesized in plants of the tribe Asclepiadeae (Apocynaceae). Cardiotonic activity is
365 determined by the steroidal aglycone, specifically by the conformation of the steroid rings.
366 The A/B and C/D rings of ouabain, and many other cardenolides, are *cis*-fused and the B/C
367 ring is *trans*-fused (*cis-trans-cis*) (Katz et al., 2010; Malcolm, 1991). In contrast calotropin
368 and other cardenolides produced in the genus *Asclepias,* have a *trans-trans-cis*
369 conformation (Malcolm, 1991) that is predicted to lower the toxicity (Hoch, 1961) and the
370 binding affinity of the cardenolide (Katz et al., 2010). However, a doubly-linked sugar
371 moiety, often found in cardenolides produced in plants of the tribe Asclepiadeae,
372 enhances the binding to the Na,K-ATPase as well as the toxicity (Piacente et al., 2009;
373 Venkateswara Rao and Vaidyanathan, 1991). Despite their differences in structure and

374 steric conformation, molecular docking analyses suggest that both cardenolides fit well
375 into the cardenolide binding site due to formation of strong H-bonds between the
376 interaction partners (Zhang et al., 2014). Similar to our results a study testing various
377 cardiac glycosides against the Na,K-ATPase of the monarch butterfly, *Danaus plexippus,*
378 (Petschenka et al., 2018) found calotropin and its isomer calactin to be the strongest
379 tested inhibitors, with calotropin inhibiting 60-fold more strongly than ouabain.

380 A comparison of the present results with our previous studies (Dalla and Dobler, 2016;
381 Dalla et al., 2017) on *O. fasciatus* Na,K-ATPase mimics reveals good concordance regarding
382 our conclusions about enzyme activities. We previously introduced one, two, three or four
383 of the potentially resistance-conferring substitutions into the α-subunit of *D.*
384 *melanogaster* to mimic the known α1-gene copies of *O. fasciatus* (in this order: αD, αC,
385 αB, αA-mimic; c.f. Yang et al., 2019; Zhen et al., 2012) and combined them with two *D.*
386 *melanogaster* β-subunits, Nrv2.2 and Nrv3 (Dalla and Dobler, 2016; Dalla et al., 2017). The
387 overall behavior of these mimics strongly resembled the present data for the original *O.*
388 *fasciatus* Na,K-ATPase complexes. Most strikingly, the activity levels of the αA and αB
389 mimics were significantly lower than of the Drosophila wildtype enzyme, the αC or the αD
390 (N122H) mimics. The two Drosophila β-subunits also modulated the activity: the
391 association with Nrv3 strongly reduced the activity of all mimics and the Drosophila
392 wildtype enzyme when compared to those bearing Nrv2.2. In the present study, such a
393 large influence of the β-subunit on ion pumping activity was observed only in the αC-
394 subunit while the effect was less pronounced for the αA and αB-subunits.

395 The β-subunits also strongly modulated the resistance of the enzymes against ouabain,
396 but their effect was specific to the α-subunits they were combined with. Previous work
397 has shown that a combination with Nrv3 instead of Nrv2.2 lead to lowered resistance in
398 the αA- and αB-mimic and increased resistance in the αC-mimic (Dalla et al., 2017). In the
399 present study, resistance changed in the same direction with β1, β2, or β3 for αA
400 (decreasing) and αC (increasing IC_{50}) but also lead to increased IC_{50}-values for αB.

401 The most striking differences, however, appear when comparing the overall ouabain-
402 sensitivity of the original enzyme combinations with the previously constructed mimics
403 (Dalla et al., 2017). While the mimics of the *O. fasciatus* αA-copy (featuring the
404 substitutions Q111T,N122H,F786N,T797A) were by far the most insensitive constructs
405 followed by the αB-mimics (with Q111T,N122H,F786N) and the more sensitive αC-mimics
406 (N122H,F786S), the original αA-constructs tested here are at least five times less sensitive
407 than αB-constructs. On the other hand, the original αC-constructs are 10-times more
408 resistant than predicted from the experiments with the αC-mimics.

409 These differences in sensitivity between mimics and original enzymes strongly point to the
410 importance of additional substitutions in the *O. fasciatus* Na,K-ATPase α1-paralogs. When
411 constructing the mimics, we focused on the four most conspicuous amino acid exchanges
412 in the binding pocket of the Na,K-ATPase that were previously shown functionally to be
413 essential for cardiac glycoside binding (Croyle et al., 1997; Holzinger et al., 1992; Qiu et al.,
414 2005) and confirmed to interact with cardiac glycosides by crystal structure analysis
415 (Laursen et al., 2015). However, there are additional amino acid substitutions in the
416 cardiac glycoside binding pocket of the Na,K-ATPase α1-genes of *O. fasciatus* that may

417 further increase their resistance (Dobler et al., 2012; Zhen et al., 2012). These and further
418 additional amino acid exchanges that at first appear inconsequential may be of
419 importance in modulating the effects of the resistance conferring substitutions or
420 ameliorating their pleiotropic effects (Karageorgi et al., 2019; Taverner et al., 2019;
421 Mohammadi et al., submitted).

422

423

424 Author contribution - S. Da. and S. Do. conceptualization; S. Da., M. W., V. W., R. T., M. H.
425 cloning and protein expression; S. Da. and M. W. enzyme assays, data analysis and
426 visualization; M. W. and S. Do. writing-original draft; S. Da. writing-review and editing.

427

428 The authors declare that they have no conflicts of interest with the contents of this article.

429

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435

436 References

437 **Agrawal, A. A., Petschenka, G., Bingham, R. A., Weber, M. G. and Rasmann, S.** (2012).
438 Toxic cardenolides: chemical ecology and coevolution of specialized plant-herbivore
439 interactions. *New Phytol.* **194**, 28–45.

440 **Baumann, O., Salvaterra, P. M. and Takeyasu, K.** (2010). Developmental changes in β-
441 subunit composition of Na,K-ATPase in the *Drosophila* eye. *Cell Tissue Res.* 215–228.

442 **Blanco, G.** (2005). Na,K-ATPase subunit heterogeneity as a mechanism for tissue-specific
443 ion regulation. *Semin. Nephrol.* **25**, 292–303.

444 **Blanco, G. and Mercer, R. W.** (1998). Isozymes of the Na-K-ATPase: heterogeneity in
445 structure, diversity in function. *Am. J. Physiol. Ren. Physiol.* F633–F650.

446 **Bramer, C., Dobler, S., Deckert, J. and Petschenka, G.** (2015). Na+/K+-ATPase resistance
447 and cardenolide sequestration: basal adaptations to host plant toxins in the
448 milkweed bugs (Hemiptera: Lygaeidae: Lygaeinae). *Proc. R. Soc. B Biol. Sci.* 20142346.

449 **Croyle, M. L., Woo, A. L. and Lingrel, J. B.** (1997). Extensive random mutagenesis analysis
450 of the Na + / K + - ATPase a subunit identifies known and previously unidentified
451 amino acid residues that alter ouabain sensitivity implications for ouabain binding.
452 *Eur. J. Biochem.* **248**, 488–495.

453 **Dalla, S. and Dobler, S.** (2016). Gene duplications circumvent trade-offs in enzyme
454 function: insect adaptation to toxic host plants. *Evolution (N. Y).* **70**, 2767–2777.

455 **Dalla, S., Baum, M. and Dobler, S.** (2017). Substitutions in the cardenolide binding site
456 and interaction of subunits affect kinetics besides cardenolide sensitivity of insect
457 Na,K-ATPase. *Insect Biochem. Mol. Biol.* **89**, 43–50.

458 **Dermauw, W. and Van Leeuwen, T.** (2014). The ABC gene family in arthropods:
459 comparative genomics and role ininsecticide transport and resistance. *Insect*
460 *Biochem. Mol. Biol.* **45**, 89–110.

461 **Dobler, S., Dalla, S., Wagschal, V. and Agrawal, A. A.** (2012). Community-wide convergent
462 evolution in insect adaptation to toxic cardenolides by substitutions in the Na,K-
463 ATPase. *Proc. Natl. Acad. Sci.* **109**, 13040–13045.

464 **Dobler, S., Wagschal, V., Pietsch, N., Dahdouli, N., Meinzer, F., Romey-Glüsing, R. and**
465 **Schütte, K.** (2019). New ways to acquire resistance: imperfect convergence in insect
466 adaptations to a potent plant toxin. *Proc. R. Soc. B* **286**, 20190883.

467 **Geering, K.** (2001). The functional role of β subunits in oligomeric P-type ATPases. *J.*
468 *Bioenerg. Biomembr.* **33**, 425–438.

469 **Geering, K.** (2005). Function of FXYD proteins, regulators of Na,K-ATPase. *J. Bioenerg.*
470 *Biomembr.* **37**, 387–392.

471 **Grabherr, M. G. ., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I.,**
472 **Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., et al.** (2011). Trinity: reconstructing
473 a full-length transcriptome without a genome from RNA-Seq data. *Nat. Biotechnol.*
474 **29**, 644–652.

475 **Hoch, J. H.** (1961). *A survey of cardiac glycosides and genins*. Columbia, SC, USA:
476 University of South Carolina Press.

477 **Holzinger, F., Frick, C. and Wink, M.** (1992). Molecular basis for the insensitivity of the
478 Monarch (*Danaus plexippus*) to cardiac glycosides. *FEBS Lett.* **314**, 477–480.

479 **Horisberger, J.-D.** (2004). Recent Insights into the structure and mechanism of the sodium
480 pump. *Physiology* **19**, 377–387.

481 **Karageorgi, M., Groen, S. C., Sumbul, F., Pelaez, J. N., Verster, K. I., Aguilar, J. M.,**
482 **Hastings, A. P., Bernstein, S. L., Matsunaga, T., Astourian, M., et al.** (2019). Genome
483 editing retraces the evolution of toxin resistance in the monarch butterfly. *Nature*
484 **574**, 409–412.

485 **Katz, A., Lifshitz, Y., Bab-Dinitz, E., Kapri-Pardes, E., Goldshleger, R., Tal, D. M. and**
486 **Karlish, S. J. D.** (2010). Selectivity of digitalis glycosides for isoforms of human Na,K-
487 ATPase. *J. Biol. Chem.* **285**, 19582–19592.

488 **Laursen, M., Gregersen, J. L., Yatime, L., Nissen, P. and Fedosova, N. U.** (2015). Structures
489 and characterization of digoxin- and bufalin-bound Na^+,K^+-ATPase compared with
490 the ouabain-bound complex. *Proc. Natl. Acad. Sci.* **112**, 1755–1760.

491 **Lebovitz, R. M., Takeyasu, K. and Fambrough, D. M.** (1989). Molecular characterization
492 and expression of the (Na+ + K+)-ATPase alpha-subunit in *Drosophila melanogaster*.
493 *EMBO J.* **8**, 193–202.

494 **Lohr, J. N., Meinzer, F., Dalla, S., Romey-Glüsing, R. and Dobler, S.** (2017). The function
495 and evolutionary significance of a triplicated Na,K-ATPase gene in a toxin-specialized
496 insect. *BMC Evol. Biol.* **17**, 256.

497 **Malcolm, S. B.** (1991). Cardenolide-mediated interactions between plants and herbivores.
498 In *Herbivores: their Interactions with Secondary Plant Metabolites* (ed. Rosenthal, G.
499 A.) and Berenbaum, M. R.), pp. 251–296. San Diego, CA: Academic Press.

500 **Meinzer, F., Dobler, S., Donath, A. and Lohr, J. N.** (2019). Robust reference gene design
501 and validation for expression studies in the large milkweed bug, *Oncopeltus fasciatus*,
502 upon cardiac glycoside stress. *Gene* **710**, 66–75.

503 **Mohammadi, S., Gompert, Z., Gonzalez, J., Takeuchi, H., Mori, A. and Savitzky, A. H.**
504 (2016). Toxin-resistant isoforms of Na+/K+-ATPase in snakes do not closely track
505 dietary specialization on toads. *Proc. R. Soc. B* **283**, 20162111.

506 **Mohammadi, S., Yang, L., Harpak, A., Herrera-Alvarez, S., Rodriguez-Ordonez, M.d.P.,**
507 **Peng, J., Zhang, K., Storz, J.F., Dobler, S., Crawford, A. J., Andolfatto, P.** Concerted
508 evolution reveals co-adapted amino acid substitutions in frogs that prey on toxic
509 toads. submitted.

510 **Moore, L. V. and Scudder, G. G. E.** (1986). Ouabain-resistant Na,K-ATPases and
511 cardenolide tolerance in the large milkweed bug, *Oncopeltus fasciatus*. *J. Insect*
512 *Physiol.* **32**, 27–33.

513 **Okamura, H., Yasuhara, J. C., Fambrough, D. M. and Takeyasu, K.** (2002). P-type ATPases
514 in *Caenorhabditis* and *Drosophila*: implications for evolution of the P-type ATPase
515 subunit families with special reference to the Na,K-ATPase and H,K-ATPase subgroup.
516 *J. Membr. Biol.* **191**, 13–24.

517 **Palasis, M., Kuntzweiler, T. A., Argüello, J. M. and Lingrel, J. B.** (1996). Ouabain

518 Interactions with the H5-H6 Hairpin of the Na,K-ATPase Reveal a Possible Inhibition
519 Mechanism via the Cation Binding Domain. *J. Biol. Chem.* **271**, 14176–14182.

520 **Paul, S. M., Palladino, M. J. and Beitel, G. J.** (2007). A pump-independent function of the
521 Na,K-ATPase is required for epithelial junction function and tracheal tube-size
522 control. *Development* **134**, 147–55.

523 **Petschenka, G., Fandrich, S., Sander, N., Wagschal, V., Boppré, M. and Dobler, S.** (2013).
524 Stepwise evolution of resistance to toxic cardenolides via genetic substitutions in the
525 Na+/K+-ATPase of milkweed butterflies (lepidoptera: Danaini). *Evolution (N. Y).* **67**,
526 2753–2761.

527 **Petschenka, G., Wagscha, V., Von Tschirnhaus, M., Donath, A. and Dobler, S.** (2017).
528 Convergently evolved toxic secondary metabolites in plants drive the parallel
529 molecular evolution of insect resistance. *Am. Nat.* **190**, S29–S43.

530 **Petschenka, G., Fei, C. S., Araya, J. J., Schröder, S., Timmermann, B. N. and Agrawal, A. A.**
531 (2018). Relative selectivity of plant xardenolides for Na+/K+-ATPases from the
532 monarch butterfly and non-resistant insects. *Front. Plant Sci.* **9**, 1424.

533 **Piacente, S., Masullo, M., De Nève, N., Dewelle, J., Hamed, A., Kiss, R. and Mijatovic, T.**
534 (2009). Cardenolides from *Pergularia tomentosa* display cytotoxic activity resulting
535 from their potent inhibition of Na+/K+-ATPase. *J. Nat. Prod.* **72**, 1087–1091.

536 **Price, E. M. and Lingrel, J. B.** (1988). Structure - function relationships in the Na,K-ATPase
537 α subunit: site-directed mutagenesis of glutamine-111 to arginine and asparagine-
538 122 to aspartic acid generates a ouabain-resistant enzyme. *Biochemistry* **27**, 8400–
539 8408.

540 **Qiu, L. Y., Krieger, E., Schaftenaar, G., Swarts, H. G. P., Willems, P. H. G. M., De Pont, J. J.**
541 **H. H. M. and Koenderink, J. B.** (2005). Reconstruction of the complete ouabain-
542 binding pocket of Na,K-ATPase in gastric H,K-ATPase by substitution of only seven
543 amino acids. *J. Biol. Chem.* **280**, 32349–32355.

544 **Taverner, A. M., Yang, L., Barile, Z. J., Lin, B., Peng, J., Pinharanda, A. P., Rao, A. S.,**

Roland, B. P., Talsma, A. D., Wei, D., et al. (2019). Adaptive substitutions underlying cardiac glycoside insensitivity in insects exhibit epistasis in vivo. *Elife* 48224.

Ujvari, B., Casewell, N. R., Sunagar, K., Arbuckle, K., Wüster, W., Lo, N., O'Meally, D., Beckmann, C., King, G. F., Deplazes, E., et al. (2015). Widespread convergence in toxin resistance by predictable molecular evolution. *Proc. Natl. Acad. Sci.* **112**, 11911–11916.

Venkateswara Rao, R. and Vaidyanathan, C. S. (1991). Chemistry and biochemical pharmacology of cardiac glycosides - A review. *J. Indian Inst. Sci.* **71**, 329–364.

Yang, L., Ravikanthachari, N., Mariño-Pérez, R., Deshmukh, R., Wu, M., Rosenstein, A., Kunte, K., Song, H. and Andolfatto, P. (2019). Predictability in the evolution of Orthopteran cardenolide insensitivity. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 20180246.

Yasuhara, J. C., Baumann, O. and Takeyasu, K. (2000). Localization of Na/K-ATPase in developing and adult *Drosophila melanogaster* photoreceptors. *Cell Tissue Res.* 239–249.

Zhang, R.-R., Tian, H.-Y., Tan, Y.-F., Chung, T.-Y., Sun, X.-H., Xia, X., Ye, W.-C., Middleton, D. A., Fedosova, N., Esmann, M., et al. (2014). Structures, chemotaxonomic significance, cytotoxic and Na + ,K + -ATPase inhibitory activities of new cardenolides from *Asclepias curassavica*. *Org. Biomol. Chem.* **12**, 8919–8929.

Zhen, Y., Aardema, M. L., Medina, E. M., Schumer, M. and Andolfatto, P. (2012). Parallel molecular evolution in an herbivore community. *Science (80-.).* **337**, 1634–1637.

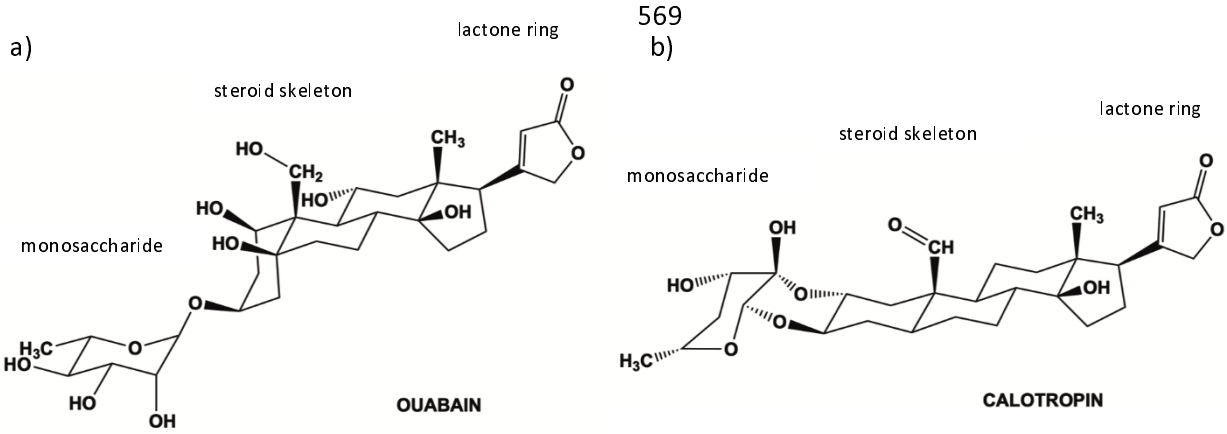

Figure 1: Structure of the cardenolides ouabain (a) and calotropin (b). Both are characterized by a monosaccharide attached to a steroid skeleton at C3 (4 steroid rings: A-D) and a five-membered unsaturated lactone ring at C-17. a) In ouabain the monosaccharide is attached by a single glycosidic link to the steroid rings that are *cis-trans-cis* fused. b) Calotropin is characterized by a doubly-linked monosaccharide as well as a *trans-trans-cis* conformation of the steroid rings (structures generated by Daniel Tal, The Weizmann Institute of Science, 2020).

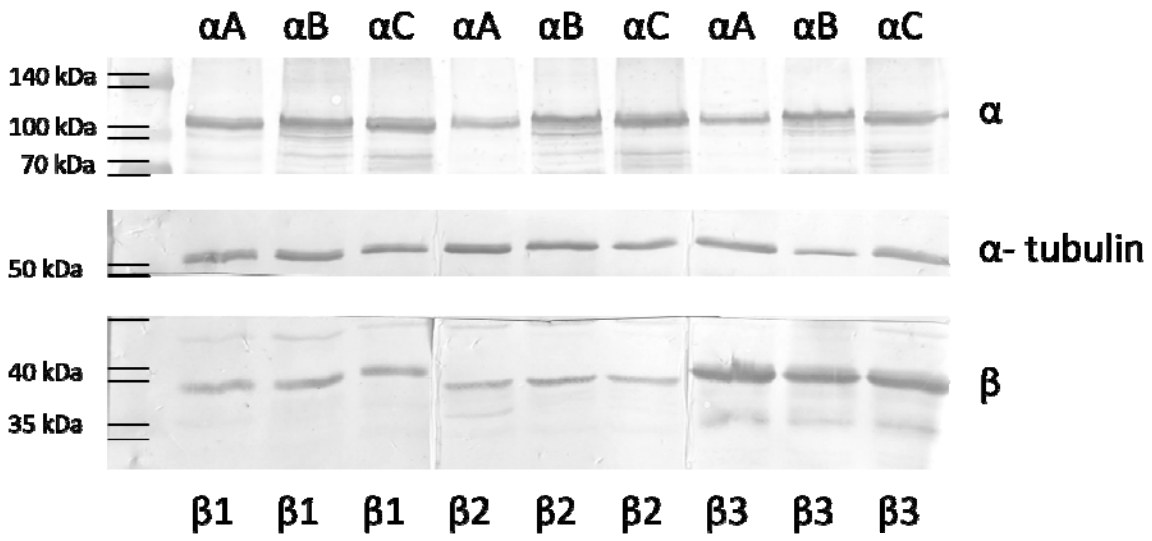

Figure 2: Western blot of nine α/β constructs of *O. fasciatus* expressed heterologously in cell culture. 30 µg of protein were separated by SDS-PAGE and blotted on a nitrocellulose membrane. The upper membrane slice shows at a size of 110 kDa αA, αB and αC in alternating order. At 55 kDa α-tubulin is shown as a positive control. Both proteins were stained with the primary antibody α5 (DSHB hybridoma bank) and the secondary HRP-conjugated antibody goat-anti mouse (Dianova). The associated β-subunits 1, 2 and 3 were deglycosylated in advance. They were stained with β-subunit specific customized primary antibodies (produced by Davids Biotechnology) and HRP-conjugated secondary antibodies goat-anti rabbit (for β2, Dianova) and goat-anti chicken (for β1 and 3, Dianova). The different β-subunits show bands at weights ranging between 35 and 40 kDa. The bands were visualized with 4-CN. Due to the grey scale some bands of the multicolor protein ladder were marked by lines.

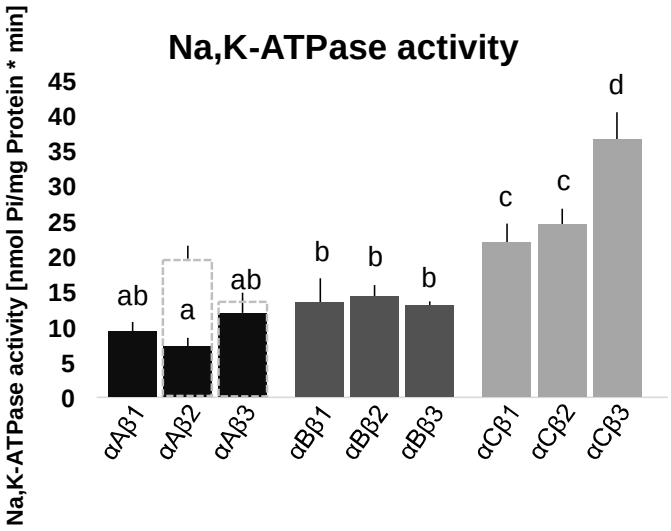

Figure 3: Na,K-ATPase activity [nmol Pi/mg protein*min] of nine α/β-constructs of *O. fasciatus.* Each bar depicts the mean and standard deviation of three biological replicates. Different letters above the bars indicate significant differences between constructs (ANOVA, $F_{8,28}$=63.932, p<0.001). Black bars: αA with three β-subunits and overall activities ranging from **7 to 12 nmol Pi/mg protein*min**. Bars with a grey dotted outline: data for the αA mimic (THNA) combined with Nrv2.2 and Nrv3 of a previous study (24). Dark gray bars: αB/β constructs ranging from **13 to 14.3 nmol Pi/mg protein*min**. Light gray bars: αC/β constructs with activity levels ranging from **22 to 36.5 nmol Pi/mg protein*min**. The activity levels of the three αC/β constructs are significantly higher than those of the other constructs, with the highest activity reached by αC combined with β3.

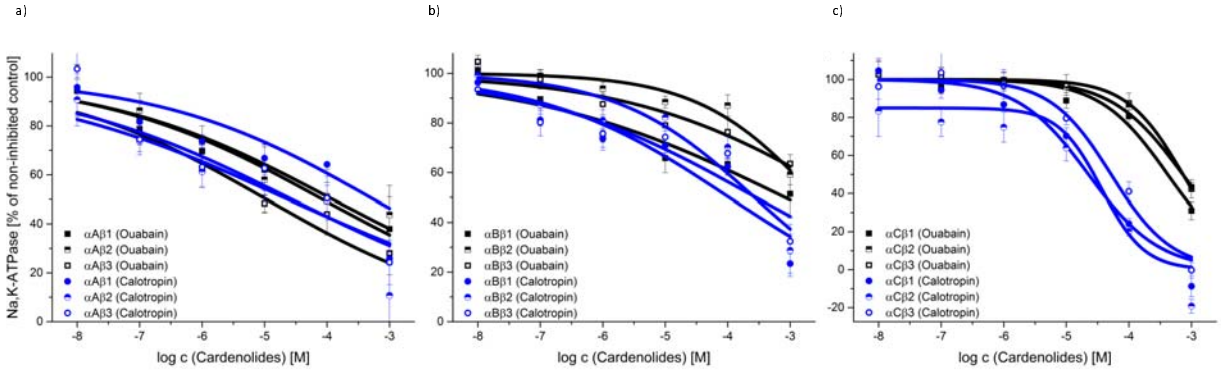

Figure 4: *In vitro* inhibition by calotropin and ouabain of nine α/β constructs of *O. fasciatus* expressed heterologously in cell culture. Dose response relationships between increasing cardenolide concentrations and residual enzyme activities were expressed in % of the non-inhibited control (calculated with Origin Pro 9.1G). Each data point represent the mean of three biological replicates and standard deviation. a) effect of calotropin (blue curves and round symbols) and ouabain (black curves and square symbols) on αA, b) on αB, and c) on αC combined each with β1, 2 and 3. Please note the different scales.