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Regulation of Histone Deacetylase 3 by Metal Cations and 10-Hydroxy-2E-Decenoic Acid: Possible  
Molecular Epigenetic Mechanisms of Queen-Worker Bee Differentiation

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## 30 Abstract

31 Histone deacetylases (HDACs) catalyze the hydrolysis of  $\epsilon$ -acetyl-lysine residues of histones.  
32 Removal of acetyl groups results in condensation of chromatin structure and repression of gene  
33 expression. Human class I, II, and IV HDACs are said to be zinc-dependent in that they require divalent  
34 zinc ions to catalyze the deacetylase reaction. HDACs are considered potential targets for the treatment  
35 of cancer due to their role in regulating transcription. They are also thought to play important roles in  
36 the development of organisms such as honey bees. The fatty acid, 10-hydroxy-2E-decenoic acid (10-  
37 HDA), which can account for up to 5% of royal jelly composition has been reported as an HDAC inhibitor.  
38 The crystal structure of the HDAC3:SMRT complex possesses two monovalent cations (MVCs) labeled as  
39 potassium with one MVC binding site near the active site Zn(II) and the second MVC binding site  $\geq 20$  Å  
40 from the active site Zn(II). We report here the inhibitory effects of excess Zn(II) on the catalytic activity  
41 of histone deacetylase 3 (HDAC3) bound to the deacetylase activating domain of nuclear receptor  
42 corepressor 2 (NCOR2). We also report the effects of varying concentrations of potassium ions where  
43  $[K^+]$  up to 10 mM increase HDAC3 activity with a maximum  $k_{cat}/K_M$  of approximately  $80,000 \text{ M}^{-1}\text{s}^{-1}$  while  
44  $[K^+]$  above 10 mM inhibit HDAC3 activity. The inhibition constant ( $K_i$ ) of 10-HDA was determined to be  
45 5.32 mM. The regulatory effects of zinc, potassium, and 10-HDA concentration on HDAC3 activity  
46 suggest a strong correlation between these chemical species and epigenetic control over *Apis mellifera*  
47 caste differentiation among other control mechanisms.

## 48 Keywords

49 Histone deacetylase, epigenetics, enzyme kinetics, zinc hydrolase, zinc biochemistry, *Apis mellifera* caste  
50 differentiation

## 51 **1. Introduction**

52           The different morphological, reproductive, and behavioral phenotypes observed in the *Apis*  
53 *mellifera* queen and worker bee is interesting considering they are genetically identical. Because they  
54 possess the same genome yet display these substantial differences, nutritional control mechanisms are  
55 thought to be involved in queen-worker differentiation [1]. These mechanisms are related, in part, to  
56 nutritional differences present during development. Queen larvae are fed royal jelly throughout  
57 development while worker larvae are fed royal jelly for only the first 1-2 days followed by feeding of  
58 worker jelly. Both jellies are a mixture of sugars, amino acids, proteins, fatty acids and minerals. The  
59 two jellies have significant quantitative differences [2, 3]. Epigenetic control mechanisms are thought to  
60 be modulated by nutritional differences present during queen and worker bee development. DNA  
61 methylation appears to play an important role in honey bee caste differentiation and its role appears to  
62 be tied to nutrition [4-7]. Another study found that a fatty acid, 10-hydroxy-2E-decenoic acid (10-HDA),  
63 present in royal jelly at higher concentrations than in worker jelly reactivated the expression of  
64 epigenetically silenced genes in mammalian cells without inhibiting DNA methylation, suggesting 10-  
65 HDA is a histone deacetylase (HDAC) inhibitor [8].

66           HDACs comprise an ancient enzyme family found in plants, animals, fungi, archaeobacteria and  
67 eubacteria [9]. Histone deacetylases (HDACs) catalyze the removal of acetyl groups from  $\epsilon$ -acetyl-lysine  
68 residues of histones. Histone acetyltransferases (HATs) acetylate lysine residues of histones thereby  
69 activating gene expression. Decreased histone acetylation downregulates affected genes and is  
70 associated with cancer development [10]. HDAC inhibitors increase histone acetylation and serve as  
71 potential cancer therapeutics [11]. There are at least two FDA-approved drugs, vorinostat  
72 (suberoylanilide hydroxamic acid or SAHA) and romidepsin, for treatment of cutaneous T-cell lymphoma  
73 with several others in clinical trials [12].

74           There are four classes of HDACs. Class III HDACs are NAD(+)-dependent and are referred to as  
75   sirtuins [13]. This class of HDAC share no sequence similarity with class I and II HDACs and use a  
76   different catalytic mechanism [14]. Class II HDACs are subclassified as class IIa (HDAC4, -5, -7, and -9)  
77   and class IIb (HDAC6 and -10) and are homologs of yeast HDA1 protein [15, 16]. All members of class IIa  
78   can shuttle between the nucleus and cytoplasm. The only class IV deacetylase is HDAC11 [17]. It is a  
79   homolog of yeast HOS3. Class I HDACs include HDAC1, -2, -3, and -8 and are homologs of yeast RPD3  
80   [15, 18]. HDACs 1-3 require association with large multisubunit corepressor complexes and are  
81   considered inactive by themselves. HDAC8 is fully active in and of itself and is the only extensively  
82   kinetically characterized HDAC [19-22].

83           HDAC3 is unique in that it has a unique domain structure with both nuclear localization and  
84   nuclear export sequences [23]. Recombinant HDAC3 cannot be expressed in bacterial cell culture as it is  
85   inactive due to improper folding. HDAC3 requires HSC70, TRiC, and most likely HSP90 for proper folding  
86   [24, 25]. HDAC3 also requires complex formation with silencing mediator for retinoid or thyroid-  
87   hormone receptor (SMRT or NCOR2) or nuclear receptor corepressor 1 (NCOR1) in order to be fully  
88   active [26, 27]. The crystal structure of the HDAC3/SMRT shows a channel leading to the active site  
89   Zn(II) that is likely obstructed in the absence of SMRT or NCOR1 [28]. Based on the crystal structure of  
90   HDAC8, this channel is open offering an explanation for HDAC8 being fully active by itself [29]. It has  
91   also been shown that the Zn(II) of HDAC8 can be chelated using EDTA forming apo-HDAC8 and that  
92   activity can be recovered by the addition of Zn(II), Co(II), and Fe(II) [19]. It was also shown in the same  
93   study that activity was greater for Co(II)-HDAC8 and Fe(II)-HDAC8 and that excess Zn(II) inhibits the  
94   Zn(II)-HDAC8, Fe(II)-HDAC8, and Co(II)-HDAC forms.

95           The catalytic mechanism for HDAC3 and other class I HDACs is based on the crystal structure of  
96   histone deacetylase-like protein (HDLP) from *Aquifax aeolicus* [30]. His-142 functions as a general base

97 that deprotonates the metal-activated catalytic water molecule for attack on the substrate amide. A  
98 second histidine (His-143) in the active site serves as the acid and protonates the leaving group.

99         The crystal structures of HDAC3 and -8 show that each bind two monovalent cations (MVCs),  
100 likely Na<sup>+</sup> or K<sup>+</sup>, in addition to the catalytic divalent metal ion [22, 28, 29, 31]. The two MVC binding sites  
101 have been designated as site 1 and site 2 with site 1 located approximately 7 Å from the divalent  
102 catalytic center and site 2 is ≥ 20 Å from the divalent metal center [28, 29]. The crystal structures of  
103 other class I and II human HDACs also bind K<sup>+</sup> at these same sites in addition to bacterial histone  
104 deacetylase-like amidohydrolase [32-34]. A study on the effects of varying concentrations of Na<sup>+</sup> or K<sup>+</sup>  
105 on catalysis of Co(II)-HDAC8 have been reported [20]. Catalytic activity of Co(II)-HDAC8 was nominally  
106 affected by Na<sup>+</sup> concentration and this MVC was bound to sites 1 and 2 with lower affinity. This study  
107 also showed that catalytic activity of Co(II)-HDAC8 is affected by K<sup>+</sup> concentration to a greater extent  
108 with activation of activity up to 1 mM KCl. Higher concentrations of KCl inhibited activity of Co(II)-  
109 HDAC8. It was determined that site 1 of HDAC8 is the inhibitory MVC binding site and binding of MVC to  
110 site 2 increases activity. KCl concentration was also shown to affect SAHA inhibition of Co(II)-HDAC8  
111 [20].

112         The present study seeks to determine the effects of excess Zn(II) on catalytic activity of HDAC3  
113 in complex with the deacetylase activating domain (amino acids 395-489) of NCOR2 (or SMRT). We have  
114 also examined the effects of varying concentrations of KCl on catalytic activity of HDAC3:NCOR2. The  
115 results from these studies confirm and demonstrate that both Zn(II) and K<sup>+</sup> ion concentrations modulate  
116 the activity of HDAC3. We also report the inhibition constant ( $K_i$ ) of 10-HDA for HDAC3:NCOR2. In the  
117 context of honey bee development and caste differentiation, these results suggest a possible link  
118 between Zn(II), K<sup>+</sup>, and 10-HDA composition of royal and worker jelly and HDAC activity.

## 119 **2. Materials and Methods**

120           2.1. *Materials* – 10-HDA ( $\geq 98\%$ ) was purchased from Cayman Chemical. All other chemicals  
121 were purchased from Sigma Aldrich. Chelex 100 resin was purchased from BIO-RAD.  $>90\%$  purity (by  
122 SDS-PAGE) human HDAC3:NCOR2 in 25 mM HEPES pH 7.5, 300 mM NaCl, 5% glycerol, 0.04% triton X-  
123 100, and 0.2 mM TCEP was purchased from Active Motif. All enzyme came from the same lot number.  
124 The recombinant complex consists of full length human HDAC3 (accession number NP\_003874.2) with a  
125 C-terminal FLAG tag and human NCOR2 amino acids 395-489 (accession number NP\_006303.4) with an  
126 N-terminal 6xHis tag expressed in Sf9 cells. All buffers used in this study were treated with chelex resin  
127 prior to use in the enzyme assays.

128           2.2. *HDAC3:NCOR2 Activity Assay* – The deacetylase activity of the HDAC3:NCOR2 complex was  
129 measured using the commercially available Fluor de Lys HDAC3/NCOR1 assay kit from Enzo Life  
130 Sciences. Before assaying, HDAC3:NCOR2 was incubated with varying stoichiometric concentrations of  
131 KCl. Fluor de Lys-SIRT1 (p53 379-382) substrate (Cat. # BML-KI177) used in the assays comprises an  
132 acetylated lysine side chain. Enzyme assays were performed in 96-well plates and reactions were  
133 stopped at varying time points using Developer II solution containing 1  $\mu\text{M}$  TSA (an HDAC inhibitor).  
134 Fluorescence was measured using a SpectraMax i3x 96-well plate reader with excitation and emission  
135 wavelengths of 360nm and 460nm, respectively. Read height was set to 1 mm, 6 flashes per read, and  
136 PMT gain set to medium. The concentration of product at each time point was calculated from a  
137 standard curve prepared using solutions containing known concentrations of the product (0-40  $\mu\text{M}$ ).  
138 Except for the zinc inhibition study, all assays were performed in 25 mM Tris pH 8.0 with 500  $\mu\text{M}$  EDTA  
139 (free acid) at room temperature.

140           For Zn(II) inhibition assays, 2  $\mu\text{M}$  HDAC3:NCOR2 was incubated on ice for 1 hour with varying  
141 concentrations of Zn(II). The mixture was then diluted to 0.2  $\mu\text{M}$  by the addition of assay buffer and  
142 substrate (50  $\mu\text{M}$ ) and assayed as above. For assays of HDAC3:NCOR2 dependency on [KCl], varying

143 concentrations of KCl in Tris base, pH 8.0, were incubated with 0.1  $\mu$ M HDAC3:NCOR2 for 1 hour on ice.

144 In all assays, the final NaCl concentration contributed by the enzyme storage buffer is  $\leq$  6 mM.

145 The bell-shaped MVC dependence of HDAC3:NCOR2 on [KCl] was fit by Equation 1 based on

146 Scheme 1 [20]. The concentration of KCl is represented by [MCL],  $E_{tot}$  is the total enzyme concentration,

147 and the apparent binding affinities for activation and inhibition are represented by  $K_{1/2,act}$  and  $K_{1/2,inhib}$ .

148 The present study does not analyze concentration effects of NaCl due to its low affinity for MCL binding

149 sites 1 and 2 of HDAC8 accounting for the 100 mM concentration of NaCl needed for full activation of

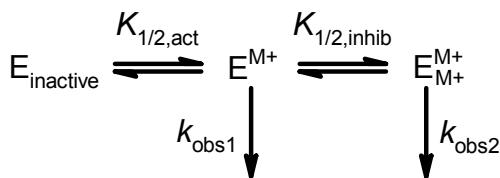
150 HDAC8 [20]. This study also uses a maximum KCl concentration of 50 mM as it has been shown that the

151 HDAC3:SMRT complex dissociates at salt concentrations exceeding 50 mM [28].

152

$$v = \frac{k_{obs1}E_{tot}}{\left(1 + \frac{K_{1/2,act}}{[MCL]} + \frac{[MCL]}{K_{1/2,inhib}}\right)} + \frac{k_{obs2}E_{tot}}{\left(1 + \frac{K_{1/2,act}K_{1/2,inhib}}{[MCL]^2} + \frac{K_{1/2,inhib}}{[MCL]}\right)}$$

Equation 1



153

154 **2.3. Inhibition by 10-HDA** – Inhibition of HDAC3:NCOR2 (5nM final concentration) by 10-HDA (1-

155 10 mM final concentration) was studied by mixing SirT1 substrate (1  $\mu$ M final concentration) with 10

156 mM KCl solution and adding this mix to the enzyme on a 96 well plate to initiate the assay. The

157 inhibition constant ( $K_i$ ) was determined by plotting fractional activity versus [10-HDA] and fitting the

158 data using equation 2. Since the 175mM 10-HDA stock solution was dissolved in 100% DMSO,

159 uninhibited control assays containing the equivalent percentage of DMSO were performed at each

160 inhibitor concentration for fractional activity calculation.

161 
$$\frac{v_i}{v_o} = \frac{V_{max}}{1 + \frac{[I]}{K_i}} \quad \text{Equation 2}$$

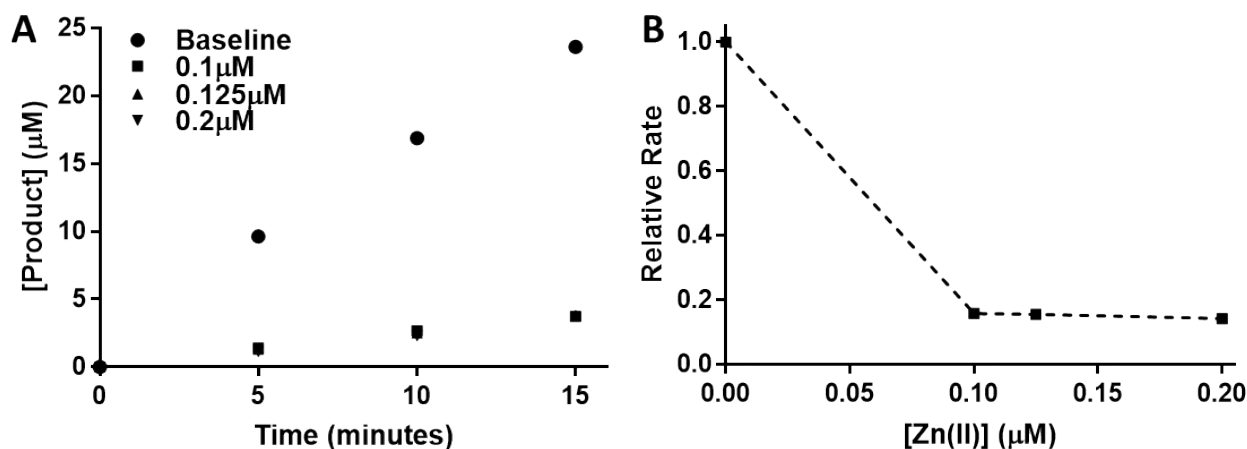
## 162 **3. Results**

163 *3.1. Zinc Inhibition of HDAC3:NCOR2 Activity.* Exhaustive attempts at producing apo-  
164 HDAC3:NCOR2 complex via dialysis against the chelators EDTA, dipicolinic acid, and/or 1,10-  
165 phenanthroline at various concentrations were unsuccessful. EDTA and dipicolinic acid were  
166 successfully used for the preparation of apo-HDAC8 and development of a metal-switching model for  
167 the regulation of HDACs [19]. In the present study, dialyzing against EDTA concentrations  $\leq 1$  mM did  
168 not affect HDAC3:NCOR2 activity. At higher concentrations ( $>1$  mM) of EDTA and 1,10-phenanthroline,  
169 a decrease in activity was observed (data not shown). However, this activity was unrecoverable upon  
170 attempted reconstitution of the treated enzyme using Zn(II) solution suggesting denaturation of  
171 HDAC3:NCOR2 and/or dissociation of the complex itself. These observations are supported by refolding  
172 studies illustrating the importance of Zn(II) as well as KCl in proper folding of the enzyme [35]. These  
173 observations also support the possible role the metal center may play in maintaining a properly folded  
174 HDAC3. Based on active site tunnel residues, HDAC3 likely possesses a more hydrophobic environment  
175 than HDAC8 preventing access of EDTA to the active site metal.

176 The addition of 0.1  $\mu$ M Zn(II) to 50 nM HDAC3:NCOR2 reduced the observed rate 5-fold (Figure  
177 1), indicating a second Zn(II) binding site that is inhibitory of HDAC3:NCOR2 as observed for HDAC8 [19]  
178 and other metalloenzymes [36-38]. The lack of linearity observed (Figure 1B) is likely caused by the  
179 presence of Zn(II) in the Fluor de Lys SIRT1 substrate. The baseline sample buffer contained 500  $\mu$ M  
180 EDTA which would have chelated contaminating Zn(II) present in substrate and/or elsewhere. Assays  
181 with final concentrations of Zn(II) at 0.1  $\mu$ M, 0.125  $\mu$ M, and 0.2  $\mu$ M did not contain EDTA resulting in a  
182 greater decrease in activity than expected. An attempt was made to treat the substrate by washing with



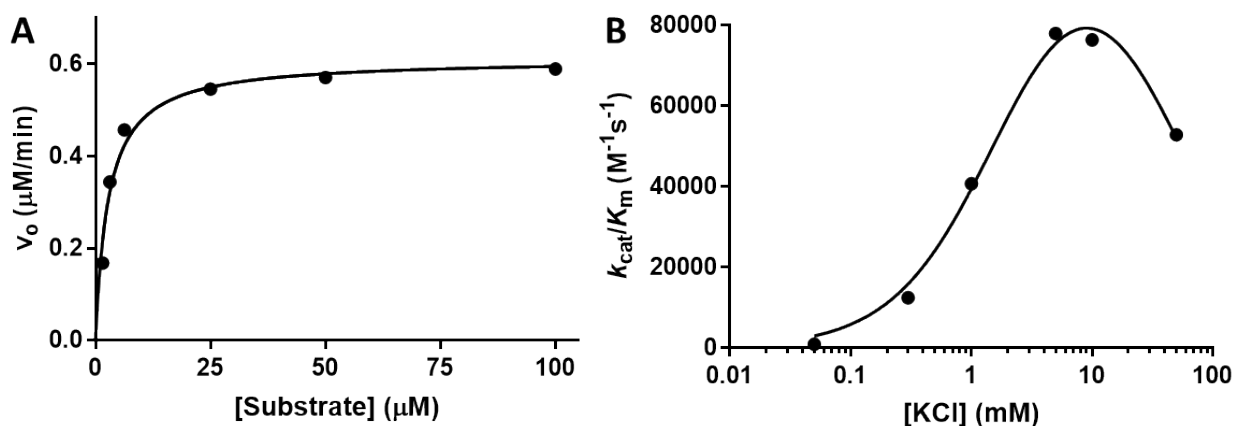
183 chelex 100 resin but this resulted in a substantial decrease in fluorescent signal from the treated  
184 substrate suggesting the it was bound and removed from solution by chelex resin.



185  
186 **Figure 1: HDAC3:NCOR2 Inhibition by Zn(II).** HDAC3:NCOR2 (2 μM final concentration) was incubated  
187 with various concentrations of Zn(II) for one hour on ice and then diluted to 50 nM and assayed using  
188 the Fluor de Lys assay (panel A). Assay buffer was composed of 25 mM Tris, pH 8, and 10 mM KCl. The  
189 baseline sample buffer contained 500 μM EDTA and no added Zn(II). Observed rates at each  
190 concentration of Zn(II) were normalized to the baseline rate (panel B).

191  
192 *3.2. Potassium Ions Modulate HDAC3:NCOR2 Activity.* The roles of the two monovalent cations  
193 observed in the HDAC8 crystal structures have been previously studied [20]. However, such a study of  
194 HDAC3 has yet to be completed. The activity of HDAC3:NCOR2 and its dependency on KCl concentration  
195 was determined by producing Michaelis-Menten plots (Figure 2A) at five different concentrations of KCl  
196 to a maximum of 50 mM. The kinetic parameters  $K_M$ ,  $k_{cat}$ , and  $k_{cat}/K_M$  were determined by fitting the  
197 data to the Michaelis-Menten equation at each concentration of KCl (Table 1). The KCl dependence of  
198 HDAC3:NCOR2 is bell-shaped with maximal deacetylase activity at approximately 10 mM KCl (Figure 2B).  
199 The data in Figure 2B were fit to equation 1 derived from a two-state sequential binding model (scheme  
200 1) [20]. In this model applied to HDAC3:NCOR2, the enzyme is inactive until the binding of one ion of  $K^+$   
201 at the activating site. The binding of a second ion of  $K^+$  results in a decrease in activity. Potassium ion  
202 was bound to the higher affinity activation site with a  $K_{1/2,act}$  of 1.78 mM and to the lower affinity  
203 inhibitory site with a  $K_{1/2,inhib}$  of 46.1 mM. Comparing these values with those determined for HDAC8

204 [20], HDAC3 shows a two-fold greater affinity for  $K^+$  binding at the activating MVC site while showing a  
205 two-fold decrease in affinity for  $K^+$  binding at the inhibiting MVC site. From 0.05 mM KCl to 10 mM KCl,  
206  $K^+$  binding to HDAC3:NCOR2 increased enzymatic activity 140-fold. At its maximum activity,  
207 HDAC3:NCOR2 shows an approximate 3.5-fold increase in  $k_{cat}/K_M$  versus HDAC8. Due to the ionic  
208 strength limitations of maintaining the HDAC3:NCOR2 complex, a complete range of KCl concentrations  
209 cannot be performed above 50 mM KCl. Therefore, the  $k_{cat}/K_M$  of the HDAC3:NCOR2 complex with a  
210 higher percentage of the two  $K^+$  ions bound form cannot be experimentally determined. The  
211 modulation of HDAC8 activity by NaCl has been determined previously [20]. The maximum  $k_{cat}/K_M$  was  
212 found to occur at 100 mM NaCl. The same limitation prevents a similar study of HDAC3:NCOR2.



213

214 **Figure 2: KCl Regulates HDAC3:NCOR2 Catalytic Activity.** HDAC3:NCOR2 activity and its dependency on  
215 KCl concentration was determined by constructing Michaelis-Menten plots (panel A) at varying  
216 concentrations of KCl (0.05 mM-50 mM), Fluor de Lys SIRT1 substrate (1.56  $\mu\text{M}$ -100  $\mu\text{M}$ ) and 0.1  $\mu\text{M}$   
217 HDAC3:NCOR2 in 25 mM Tris pH 8.0 with 500  $\mu\text{M}$  EDTA. Initial velocities were determined from time  
218 course data based on changes in fluorescence. Catalytic parameters were determined and are  
219 summarized in Table 1. Equation 1 was used to fit the bell-shaped dependency of HDAC3:NCOR2  
220 activity with varying [KCl] (panel B) yielding  $K_{1/2,act}$  and  $K_{1/2,inhib}$  values.

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Table 1: Reactivity of HDAC3:NCOR2 and [KCl] Dependency<sup>a,b</sup>

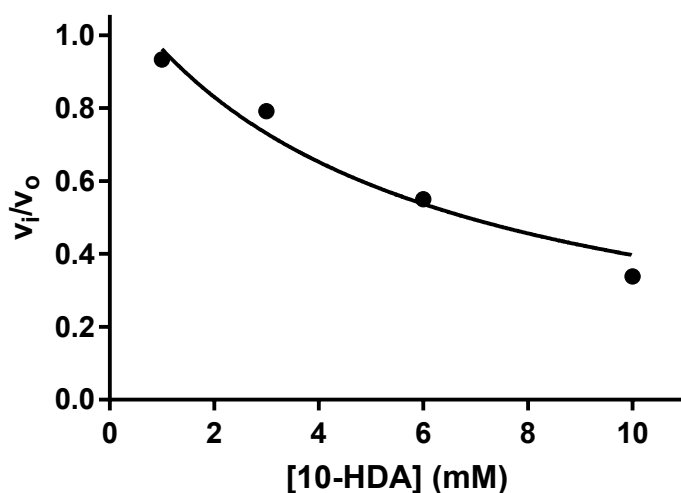
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[KCl]	$k_{cat}$	$K_M$	$k_{cat}/K_M$
(mM)	( $\text{min}^{-1}$ )	( $\mu\text{M}$ )	( $\text{M}^{-1}\text{s}^{-1}$ )
0.05	nd <sup>c</sup>	nd	nd
0.3	49.2	65.5	12500
1	6.60	2.70	41000
5	6.00	1.28	78000
10	7.14	1.56	76500
50	20.0	6.31	53000

<sup>a</sup> HDAC3:NCOR2 was assayed as described in the legend of Figure 2. <sup>b</sup> Steady-state kinetic parameters were determined from a fit of the Michaelis-Menten equation to the dependence of the initial rate on the substrate concentration at 0.1  $\mu\text{M}$  HDAC3:NCOR2. <sup>c</sup> Value not determined.

225

226           3.3. HDAC3:NCOR2 Inhibition by 10-HDA. The IC<sub>50</sub> for 10-HDA and HDAC3 has been reported as  
227 6.5 mM [8]. In the present study, the fractional activity of HDAC3:NCOR2 was determined as a function  
228 of 10-HDA concentration and fit using equation 2 (Figure 3). The  $K_i$  from the fit was 5.32 mM and is in  
229 agreement with the reported IC<sub>50</sub> value. Increasing concentrations of KCl did not significantly affect the  
230  $K_i$  (data not shown). As expected, the results suggest 10-HDA inhibits HDAC3:NCOR2 activity with low  
231 affinity *in vitro*.



232

233 **Figure 3: 10-HDA Inhibition of HDAC3:NCOR2.** A solution containing Fluor de Lys SIRT1 substrate (1  $\mu$ M  
234 final concentration) and 10 mM KCl (final concentration) was added to 5 nM HDAC3:NCOR2 (final  
235 concentration) in 25 mM Tris pH 8 with 500  $\mu$ M EDTA. Initial velocities were determined from  
236 fluorescence changes over time, normalized, plotted versus 10-HDA concentration, and fit using  
237 equation 2.

238

## 239 4. Discussion

240 *4.1. Zinc Inhibition of HDAC3:NCOR2* – A detailed analysis of metal specificity of HDAC3:NCOR2  
241 was complicated by an inability of metal chelators (EDTA, dipicolinic acid, and 1,10-phenanthroline) to  
242 remove the metal center occupying the active site as isolated. HDAC3:NCOR2 activity was decreased 25-  
243 50% using higher concentrations of EDTA or 1,10-phenanthroline but this activity was not recovered  
244 following reconstitution. Several possible explanations can account for these observations. The metal  
245 center is essential to maintain structural integrity of the enzyme and removing it leads to denaturation.  
246 Second, the observation that dialysis against 1 mM EDTA and 10  $\mu$ M dipicolinic acid, conditions that  
247 successfully prepared apo-HDAC8 [19], produced no detectable decrease in activity suggest the active  
248 site metal is inaccessible to EDTA. The increased active site tunnel hydrophobicity of HDAC3 by  
249 comparison to HDAC8 leads the authors to conclude EDTA is unable to access the active site of the  
250 enzyme. The expectation was that the less polar metal chelator 1,10-phenanthroline would produce  
251 apo-HDAC3:NCOR2. Dialysis against this compound for 24 hours at its maximum solubility in water (15  
252 mM) did not produce the apo form of the enzyme. Attempts at active site metal chelation and removal  
253 are ongoing. The identity of the *in vivo* HDAC3 metal is important as the metal occupying the active site  
254 has been shown to modulate HDAC8 activity [19]. If Zn(II) of HDAC3 cannot be removed *in vitro*, is it  
255 inserted *in vivo* into HDAC3 during folding by another zinc-carrying protein? Can Zn(II) of HDAC3 be  
256 replaced via metal-switching as has been demonstrated with HDAC8 and is Zn(II)-HDAC3 the most  
257 catalytically active form?

258 Excess Zn(II) inhibits HDAC3:NCOR2 as has been demonstrated with many other  
259 metallohydrolase enzymes [38, 39] including HDAC8 [19]. HDAC8 may not necessarily exist *in vivo* as  
260 Zn(II)-HDAC8 leading to the hypothesis of a metal-switching model for HDAC regulation where HDAC8  
261 may exist *in vivo* as Zn(II)-HDAC8, Fe(II)-HDAC8, or even Co(II)-HDAC8 with the Fe(II) and Co(II) forms  
262 showing substantially larger  $k_{cat}/K_M$  [19]. It was also shown in the same study that Zn(II) can inhibit each  
263 form of HDAC8. The inhibitory metal binding site on zinc metalloenzymes has been proposed as a  
264 potential regulatory mechanism as well [40]. It is interesting to note that the royal jelly fed to queen  
265 larvae is significantly higher in zinc content than jelly fed to developing worker bees [3] and the queen is  
266 fed royal jelly throughout her life. In addition, the important zinc-binding protein vitellogenin has been  
267 positively correlated to high zinc levels, low juvenile hormone, decreased foraging, and longer lifespan in  
268 *Apis mellifera* [41-46]. Juvenile hormone has also been positively correlated to stress while vitellogenin  
269 protects cells from anti-oxidative damage [45, 46]. We hypothesize that there is a link between dietary  
270 zinc levels related to juvenile hormone/vitellogenin titer during development as well as the adult life of  
271 *Apis mellifera*. It has been previously shown Fe(II)-HDAC8 can readily be oxidized to an inactive form  
272 Fe(III)-HDAC8 [19]. The metal-switching hypothesis for HDACs is appealing based on our knowledge of  
273 vitellogenin and juvenile hormone. In the case of high oxidative stress, Fe(II)-HDACs could be readily  
274 oxidized to the Fe(III) form resulting in inactive HDAC thereby changing levels of gene expression. In this  
275 scenario, vitellogenin and zinc levels are low and therefore foraging activity is high. As oxidative stress  
276 increases, levels of juvenile hormone increase resulting in increased foraging behavior and an increase in  
277 dietary iron and zinc. The two largest quantities of divalent metals found in worker jelly and royal jelly  
278 are zinc and iron with royal jelly possessing significantly greater quantities of zinc than worker jelly [3].  
279 It is also possible that an increased level of Zn(II)-HDAC over Fe(II)-HDAC form would lead to a decrease  
280 in oxidative stress and an increase in longevity.

281           4.2. *Potassium Modulates HDAC3:NCOR2 Activity* – As demonstrated in a previous study with  
282 HDAC8 [20], HDAC3:NCOR2 activity is regulated by potassium ions. The crystal structure of HDAC3 in  
283 complex with SMRT [28] displayed two bound potassium ions as was observed in the crystal structures  
284 of HDAC8 [29, 31]. Like HDAC8, HDAC3:NCOR2 is inactive without potassium and possesses an  
285 activation site and an inhibitory site for potassium binding. The activating site of potassium binding has  
286 a lower dissociation constant ( $K_{1/2,act}$  of 1.78 mM) than that of the inhibitory site ( $K_{1/2,inhib}$  of 46.1 mM).  
287 Royal jelly fed to queen larvae has been shown to contain significantly higher concentrations of  
288 potassium than that of jelly fed to worker bee larvae [3]. The same study also reported levels of  
289 potassium in royal jelly at 3 to 4 times the level of sodium. In the context of the present study,  
290 potassium levels in royal and worker jelly likely modulate HDAC activity and levels of gene expression.

291           4.3. *10-HDA Inhibits HDAC3:NCOR2* – The IC<sub>50</sub> of 10-HDA for several HDACs have been reported  
292 in the low mM range [8]. In the present study, the  $K_i$  was determined to be 5.32 mM confirming our  
293 expectation of 10-HDA as a weak competitive inhibitor of HDAC3:NCOR2. Royal jelly is composed of 2-  
294 5% 10-HDA and is therefore a compelling potential epigenetic regulation factor as previously proposed  
295 [8]. Though the IC<sub>50</sub> and  $K_i$  are high, the concentration of 10-HDA present in royal jelly is approximately  
296 100mM (at a minimum). A level significantly higher than in worker jelly [3]. Also, the developing queen  
297 is fed royal jelly throughout her life providing further support for its role in differentiation and  
298 maintenance of health and longevity of the queen.

## 299 **Conclusions**

300           HDAC3:NCOR2 is regulated by zinc, potassium, 10-HDA, availability of NCOR1 and NCOR2 as well  
301 as inositol phosphates which function in HDAC3:NCOR complex formation [28, 47, 48]. We propose a  
302 strong link between queen-worker differentiation, oxidative stress, longevity and dietary levels of zinc,  
303 iron, and potassium during the developmental stages and throughout the adult life of *Apis mellifera*

304 based on the modulation of histone deacetylase activity by these chemical species. Based on the  
305 complex regulation of HDACs alone, it is unlikely that a single queen determining factor exists but  
306 consists of multiple factors that are temporally and behaviorally dependent to include but not limited to  
307 the concentrations of zinc, potassium, and iron in some complex regulatory mechanism of modulating  
308 vitellogenin and juvenile hormone titer. The possible epigenetic mechanism by which vitellogenin and  
309 juvenile hormone expression is controlled requires further exploration. The present study proposes the  
310 potential important epigenetic roles of metals present at varying levels in royal and worker jelly leading  
311 to plasticity in caste differentiation and behavior.

## 312 **Abbreviations**

313	HDAC	Histone deacetylase
314	SMRT	silencing mediator for retinoid or thyroid-hormone receptor
315	NCOR	nuclear receptor corepressor
316	MVC	monovalent cation
317	DMSO	dimethyl sulfoxide
318	EDTA	Ethylenediaminetetraacetic acid
319	10-HDA	10-hydroxy-2E-decenoic acid
320	TSA	trichostatin A

321

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325

## 326 **Competing Interests**

327

328 The authors declare they have no competing interests.

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