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# *In silico* identification of off-target pesticidal dsRNA binding in honey bees (*Apis mellifera*)

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**Background.** Pesticidal RNAs silencing critical gene function have great potential in pest management, but the benefits of this technology must be weighed against non-target organism risks. **Methods.** Published studies that developed pesticidal dsRNAs were collated into a database. The target gene sequences for these pesticidal RNAs were determined, and the degree of sequence homology with the honey bee genome were evaluated statistically for each. **Results.** We identified 101 insecticidal dsRNAs sharing high sequence homology with genomic regions in honey bees. The likelihood of off-target sequence homology increased with the parent dsRNA length. Non-target gene binding was unaffected by taxonomic relatedness of the target insect to honey bees, contrary to previous assertions. Gene groups active during honey bee development had disproportionately high sequence homology with pesticidal RNAs relative to other areas of the genome. **Discussion.** Although sequence homology does not itself guarantee a significant phenotypic effect in honey bees, *in silico* screening may help to identify appropriate experimental endpoints within a risk assessment framework for pesticidal RNAi.

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1 *In silico* identification of off-target pesticidal dsRNA binding in honey bees (*Apis mellifera*)

2

3 Short title: Identification of off-target RNAi in honey bees

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29 **Abstract**

30 **Background.** Pesticidal RNAs silencing critical gene function have great potential in pest
31 management, but the benefits of this technology must be weighed against non-target organism
32 risks.

33 **Methods.** Published studies that developed pesticidal dsRNAs were collated into a database. The
34 target gene sequences for these pesticidal RNAs were determined, and the degree of sequence
35 homologies with the honey bee genome were evaluated statistically.

36 **Results.** We identified 101 insecticidal dsRNAs sharing high sequence homology with genomic
37 regions in honey bees. The likelihood of off-target sequence homology increased with the parent
38 dsRNA length. Non-target gene binding was unaffected by taxonomic relatedness of the target
39 insect to honey bees, contrary to previous assertions. Gene groups active during honey bee
40 development had disproportionately high sequence homology with pesticidal RNAs relative to
41 other areas of the genome.

42 **Discussion.** Although sequence homology does not itself guarantee a significant phenotypic
43 effect in honey bees, *in silico* screening may help to identify appropriate experimental endpoints
44 within a risk assessment framework for pesticidal RNAi.

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51 **Keywords:** RNAi, non-target, risk assessment, transgenic crops

52 **Introduction**

53 The potential to silence critical gene function in pest species has led to the proposed
54 application of RNA interference (RNAi) as a novel class of agricultural products (Price and
55 Gatehouse 2008; Gu and Knipple 2013) that target several species of economically important
56 pests (Baum et al. 2007; Maori et al. 2009; Desai et al. 2012; Hajeri et al. 2014; Marr et al.
57 2014). These RNAi-based pesticides may be delivered to the target pest via a number of
58 methods, including transgenic plants and sprays of naked or encapsulated small RNAs, which
59 elicit post-transcriptional gene silencing. Once ingested, the insect's cellular machinery cleaves
60 the double stranded RNA (dsRNA) molecule into small-interfering RNAs (siRNAs) that are 19-
61 25 nucleotides in length; these serve as the functional unit of RNAi and govern the location of
62 gene suppression through the degradation of complementary messenger RNA molecules (Fire et
63 al. 1998; Martinez et al. 2002; Vermeulen et al. 2005). To date, this process has been
64 investigated in the control of a number of pest groups, including parasites of medical importance,
65 urban pests, pests and pathogens of honey bees, and agricultural pests of economic importance.

66 While the technology promises to be target specific (Whyard et al. 2009; Bachman et al.
67 2013), there is concern that the current risk assessment framework for genetically modified crops
68 is not adequate to proactively assess the risks to non-target organisms (Lundgren and Duan 2013;
69 FIFRA-SAP 2014). The risks associated with RNAi to non-target organisms include immune
70 stimulation (Lu and Liston 2009), saturation of an organism's RNAi machinery that could
71 interfere with normal cellular processes (Grimm 2011; Flenniken and Andino 2013), and
72 unintentional gene silencing. Unintentional gene silencing in non-target organisms is the primary
73 risk posed by pesticidal RNAi; within a non-target species, this unintentional gene silencing can
74 be of the targeted gene sequence (non-target binding) or occur elsewhere in the genome with

75 high sequence homology to the target gene (off-target binding) (Lundgren and Duan 2013;
76 FIFRA-SAP 2014). Because pesticidal RNAi poses risks to non-target organisms that are unique
77 from other pesticides, a risk assessment framework has been proposed to proactively assess these
78 risks using a series of steps (FIFRA-SAP 2014; Roberts et al. 2015).

79 The hazard to non-target organisms should be predictable if the functional genome of a
80 non-target organism is known, recognizing that numerous circumstances influence gene
81 silencing even when sequence homology is identical between a small RNA and the non-target
82 genome (Kerschen et al. 2004). Bioinformatic analyses have thus been advocated as an initial
83 screen of potential risks posed by RNAi (FIFRA-SAP 2014; Roberts et al. 2015). In the present
84 study, we used *in silico* searches to determine whether putative pesticidal dsRNAs share
85 sequence homologies with off-target regions of the honey bee (*Apis mellifera* L.), a model non-
86 target organism. We were specifically interested in testing the hypotheses that 1) longer dsRNAs
87 increase the potential for off-target binding, 2) non-target silencing of the target gene is
88 dependent on relatedness of the target and non-target species, and 3) certain gene groups in the
89 honey bee are more prone to off-target sequence homologies with pesticidal dsRNAs.

90 **Materials and Methods**

91 *Literature review*

92 Published studies evaluating the effects of *pesticidal* dsRNAs were searched using the ISI
93 Web of Knowledge database, using combinations of the search terms “pesticidal,” “insecticidal,”
94 “siRNA,” “dsRNA,” “RNAi,” and “RNA interference.” Studies were included if they evaluated
95 the pesticidal effects of a dsRNA and provided either the dsRNA sequence or primer sets that
96 allowed the dsRNA sequences to be determined from the target species’ genome using the NCBI
97 genome database (http://www.ncbi.nlm.nih.gov/genome/). A total of 24 studies were included,

98 with pesticidal qualities being evaluated for 74 dsRNAs and 21 siRNAs targeting 57 genes
99 (Supplemental Data 1). These included species of medical importance (Hajdusek et al. 2009;
100 Kwon et al. 2013), urban pests (Zhou et al. 2008; Itakura et al. 2009), parasites and pathogens of
101 honey bees (Maori et al. 2009; Campbell et al. 2010; Desai et al. 2012), agricultural pests (Mutti
102 et al. 2006; Baum et al. 2007; Whyard et al. 2009; Tang et al. 2010; Choudhary and Sahi 2011;
103 Wuriyanghan et al. 2011; Gong et al. 2013; Ochoa-Campuzano et al. 2013; Yao et al. 2013;
104 Christiaens et al. 2014; Chu et al. 2014; Han et al. 2014; Meng et al. 2014; Miyata et al. 2014;
105 Yu et al. 2014), and others (Whyard et al. 2009; Kelkenberg et al. 2015; Petrick et al. 2015).
106 In silico *sequence homology identification*
107 Published pesticidal dsRNAs ranged from 19 to 2500+ nucleotides in length. These were
108 queried against the annotated honey bee genome accessed through GenBank
109 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLAST nucleotide algorithm for somewhat
110 similar sequences (blastn). Homologous regions were mostly less than 25 nt long, the length
111 expected for resultant siRNAs randomly generated from the parent dsRNA molecule. Sequence
112 homologies of 19/21, 20/21, and 21/21 nt were tallied for each dsRNA against the honey bee
113 genome, and the off-target gene name was recorded. Each off-target gene was only tallied once
114 per dsRNA, even when that dsRNA targeted multiple locations along that gene. Sequence
115 similarity for the target gene (non-target binding) was also recorded. Low quality proteins (as
116 defined by NCBI) and genes of unknown function were excluded from the analysis, as were any
117 homologous regions that did not return any protein or gene information, such that the resultant
118 database represents a conservative estimate of putative binding.
119 *Statistical analysis*

120 Because data violated parametric assumptions, the number of off-target homologies were
121 log(x+1) transformed and dsRNA length were log transformed to uphold assumptions for
122 analysis with linear regression (Systat v.13.1, San Jose, CA, USA). A chi-square test of
123 independence was used to determine whether there was a significant effect of target taxa on the
124 incidence of non-target binding in honey bees, and whether certain functional gene groups were
125 targeted more frequently.
126
127 **Results and Discussion**
128 *dsRNA length-suppression*
129 Each of the 74 pesticidal dsRNAs shared at least one region of perfect or high sequence
130 homology with the honey bee genome (average 28.6 ± 3.32 off-target homologies per dsRNA)
131 (Supplemental Data 1). However, none of the published pesticidal siRNAs (21 total, 19-23 nt in
132 length) found sequence homology within the honey bee genome at our specified level (19/21,
133 20/21, 21/21 nt matches), indicating that these much smaller sequences were more specific when
134 focusing on a single non-target organism. This result was mirrored by Li et al. (2015), though
135 siRNAs are not always this benign: Qiu et al. (2005) demonstrated that 5-80% of tested siRNAs
136 resulted in off-target binding among diverse organisms.
137 Off-target sequence homology increased significantly as the parent dsRNA increased in
138 length (linear regression: $F_{1,100} = 623$, $P < 0.001$) (Figure 1a), with every increase of 100 nt in
139 the dsRNA resulting in 6 more predicted hits. This strong relationship between dsRNA length
140 and potential off-target binding can be further demonstrated using only the genes described in
141 Miyata et al. (2014), in which the authors evaluated the effects of dsRNA length on RNAi
142 activity *in vivo* in western corn rootworms. Although the gene targets in this study were not

143 pesticidal specifically, and thus excluded from our overall analysis, the authors evaluated
144 silencing of the same gene targets (*laccase 2* and *ebony*) using different sized dsRNAs to
145 evaluate efficacy. When we examined this suite of genes from a risk assessment perspective
146 using the same methodology as for the pesticidal RNAs, the longer dsRNAs returned
147 significantly more regions of off-target sequence homology in the honey bee genome (*laccase 2*:
148 $F_{1,5} = 181$, $P < 0.001$; *ebony*: $F_{1,2} = 103$, $P = 0.01$) (Figure 1b). While intuitive (Bolognesi et al.
149 2012), this is the first demonstration of the possibility for increased length-suppression in a non-
150 target organism. Thus, optimizing dsRNA length to have maximum gene suppression efficacy in
151 the target pest needs to be balanced against the non-target risks posed by longer molecules.
152 *Target-species specificity*
153 Taxonomic relatedness of the target organism to honey bees had no effect on potential
154 binding of siRNAs on the original gene target (non-target binding) ($\chi^2 = 9.4$, df = 7, $P = 0.23$)
155 (Figure 2). Contrary to assertions of pesticidal specificity (Bachman et al. 2013), this implies that
156 silencing of the target gene in a non-target organism may be more likely to occur from random
157 sequence similarities than based on evolutionary relatedness to the target organism. Although the
158 pool of available literature is limited to date with regards to targeted applications of RNAi
159 against pest species, with certain species being more frequently researched (e.g. *Diabrotica*
160 *virgifera*), our results suggest that non-target hazard assessments should focus on species of
161 ecological relevance rather than strictly on phylogenetic relatedness to the target species.
162 Unfortunately, when conducting bioinformatics analyses for the purposes of a risk
163 assessment, the availability of sequenced genomes from representative species becomes a
164 limiting factor. Further, the potential non-target community will differ depending on the specific
165 pest being targeted, making it difficult to have a standard suite of species to evaluate for non-

166 target effects. Bioinventories are crucial for identifying appropriate non-target species for each
167 target pest. Supporting initiatives such as i5K (i5K Consortium 2013), which strives to sequence
168 the genomes of 5000 representative invertebrates, and making these genomes freely available,
169 will bolster the applicability of future *in silico* analyses aimed at identifying potential risks of
170 gene-oriented pest control.

171 *Targeted gene groups*

172 The homeobox genes and other genes involved in embryonic and developmental
173 pathways in honey bees frequently shared sequence homology with the pesticidal dsRNAs,
174 particularly when vATPase subunits were the pesticidal targets ($\chi^2 = 10$, df = 4, $P = 0.03$). 67%
175 of all tested dsRNAs had off-target binding with developmental genes in honey bees, and 33% of
176 these shared homology with homeobox genes specifically (Supplemental Data 1). Although we
177 have an incomplete picture of which genes are expressed in most genomes at any given time,
178 many of these genes, while important during embryogenesis and development, perform
179 additional critical functions such as cell proliferation and apoptosis, and are highly conserved
180 across metazoans. In this instance, *in silico* analysis identified potential gene targets that could
181 present a hazard requiring unique assessments across life stages to properly identify a phenotypic
182 effect. If validated in future *in vivo* assessments, this screening method may prove useful in
183 identifying appropriate experimental endpoints in non-target risk assessments.

184

185 **Conclusions**

186 Our bioinformatics-based *in silico* analysis provides a conservative assessment of
187 potential off-target binding of pesticidal dsRNAs in the honey bee genome; the actual binding
188 affinity of RISC is more nuanced than 100% or similar sequence homology for subsequent

189 mRNA degradation. While some have documented off-target gene knockdown with 20/21 nt
190 similarity (Jarosch and Moritz 2012), others have found silencing with even less sequence
191 similarity in certain study systems, particularly in the 2-8 nt seed region of the siRNA. For
192 example, in experiments with cultured human cells, Saxena et al. (2003) found gene silencing
193 with as many as 3-4 bp mismatches in addition to G.U wobbles (guanine and uracil have a slight
194 affinity for each other), while Jackson et al. (2003) found mRNA degradation with only 11/21
195 contiguous nt. The locations of the mismatches along the siRNA are also important; perfect
196 sequence homology of the seed region is particularly crucial for mRNA recognition (Jackson et
197 al. 2006; Chu et al. 2014).

198 However, *in silico* identification of sequence homology between a pesticidal dsRNA and
199 non-target organism's genome does not imply that RNAi will occur in the non-target organism.
200 Unintended gene silencing will depend on a number of factors. The organism would need to
201 possess behavioral characteristics that would put it into contact with contaminated materials, e.g.
202 leaf tissue *versus* pollen *versus* nectar feeding at a contaminated location. Other factors include
203 the length of the dsRNA and whether the organism is exposed to siRNA or dsRNA, the identity
204 of the target or off-target mRNA, the size of a non-target organism's genome (more off-target
205 binding would be expected when there are more potential gene targets), the necessary binding
206 affinity of a particular siRNA, exposure concentration of the dsRNA, and the physiological state
207 of the insect (Qiu et al. 2005; Baum et al. 2007; Huvenne and Smagghe 2010; Gu et al. 2014).

208 Ecological risk assessment is a complex and multi-stepped process, and no single piece of
209 work is sufficient to fully quantify the risk of a toxicological event. We have demonstrated that
210 an *in silico* analysis may be used as a first step in establishing whether off-target binding could
211 pose a significant threat for a particular pesticidal dsRNA in a non-target organism such as the

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212 honey bee. Future experiments to evaluate the usefulness of this tool are planned that would
213 quantify up/down gene regulation of honey bees exposed to pesticidal dsRNA. Taken together,
214 these data may provide a basis for designing biologically appropriate experiments to optimize
215 hazard assessments for applications of this novel pesticidal technology in field settings where
216 honey bees and other non-target organisms may be exposed.

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219 Mention of trade names or commercial products is solely for the purpose of providing specific
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368 **Supplemental Data 1.** Database of putative off-target gene silencing.

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370 **Figure 1.** The relationship between pesticidal dsRNA length and potential off-target binding in
371 honey bees for pesticidal dsRNAs (a) and the non-pesticidal *laccase 2* and *ebony* genes (data
372 from Miyata et al. (2014)) (b).

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374 **Figure 2.** Potential non-target binding of pesticidal dsRNAs in honey bees (y-axis, shaded area)
375 versus the original target taxa (x-axis), in relation to the total number of examined pesticidal
376 dsRNAs. Taxa are ordered by increasing relative divergence time from honey bees.

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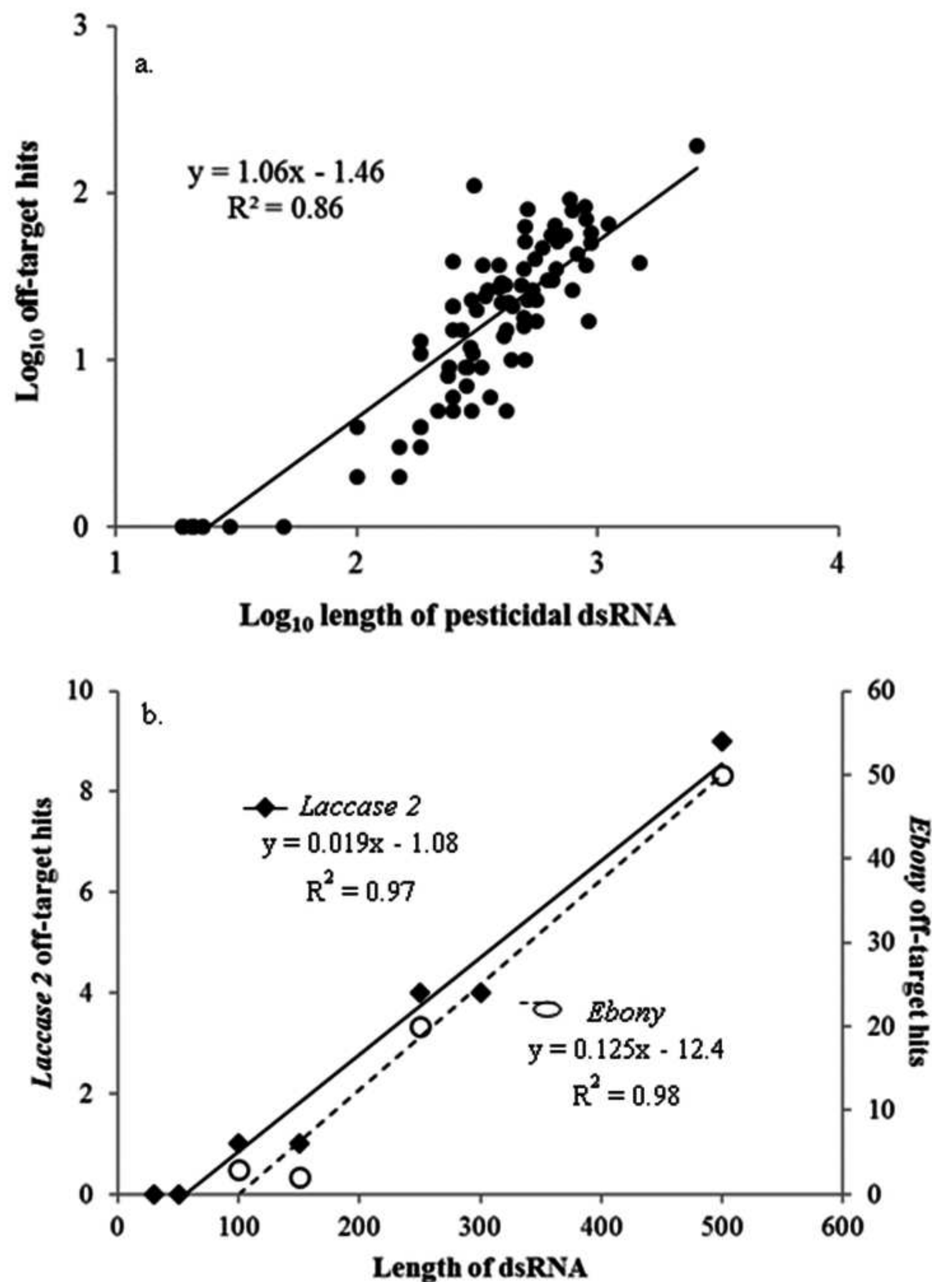
# Figure 1

Pesticidal dsRNA length and potential off-target binding in honey bees

The relationship between pesticidal dsRNA length and potential off-target binding in honey bees for pesticidal dsRNAs (a) and the non-pesticidal *laccase 2* and *ebony* genes (data from Miyata et al. (2014)) (b).



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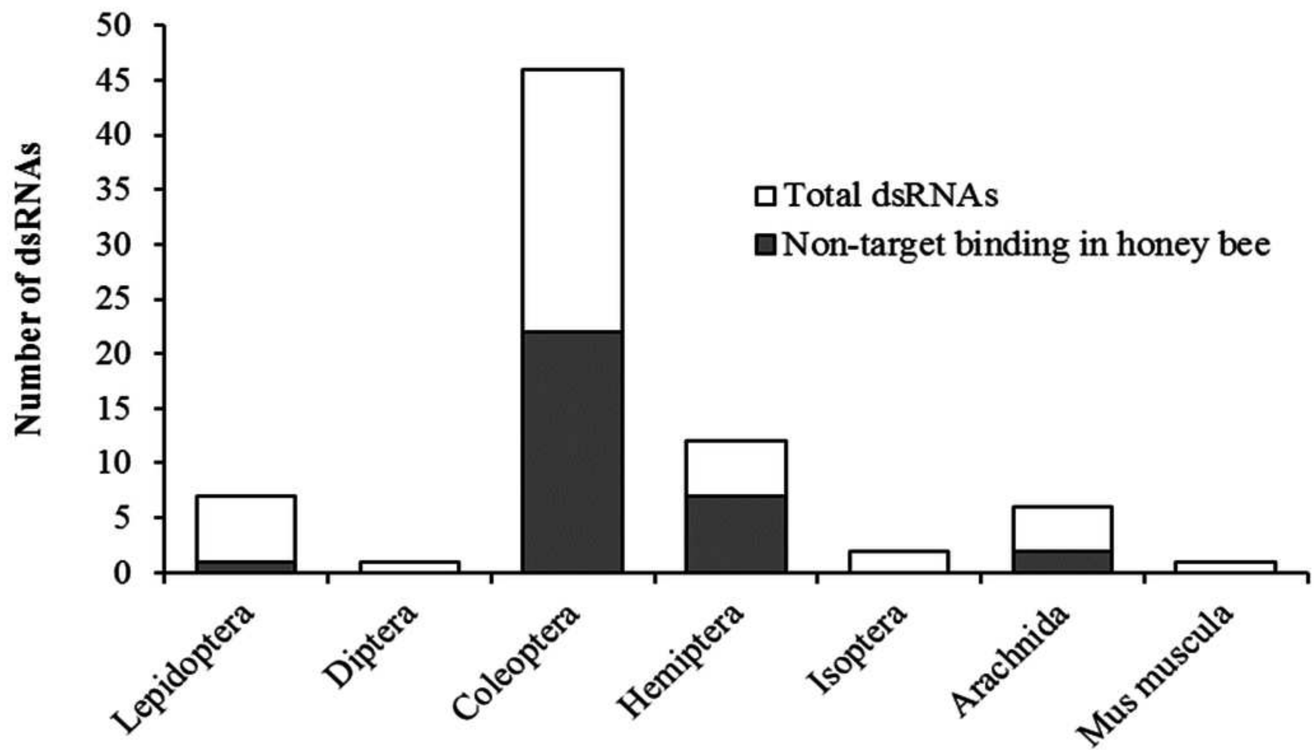
# Figure 2

Pesticidal dsRNA target organisms and the likelihood of off-target binding in the honey bee genome.

Potential non-target binding of pesticidal dsRNAs in honey bees (y-axis, shaded area) versus the original target taxa (x-axis), in relation to the total number of examined pesticidal dsRNAs. Taxa are ordered by increasing relative divergence time from honey bees.

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