

1 **Expression biomarkers used for the selective breeding of complex polygenic**
2 **traits**

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27 **Abstract**

28 We present a novel way to select for highly polygenic traits. For millennia,
29 humans have used observable phenotypes to selectively breed stronger or more
30 productive livestock and crops. Selection on genotype, using single-nucleotide
31 polymorphisms (SNPs) and quantitative trait loci (QTLs), is also now applied broadly in
32 livestock breeding programs; however, selection on protein or mRNA expression
33 markers have not been proved useful yet. Here we demonstrate the utility of protein
34 markers to select for disease-resistant behaviour in the European honey bees (*Apis*
35 *mellifera* L.). Robust, mechanistically-linked protein expression markers, by integrating
36 cis and trans effects from many genomic loci, may overcome limitations of genomic
37 markers to allow for selection. After three generations of selection, the resulting stock
38 performed as well or better than bees selected using phenotype-based assessment of
39 this trait, when challenged with disease. This is the first demonstration of the efficacy of
40 protein markers for selective breeding in any agricultural species, plant or animal.

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42 **Significance statement**

43 The honey bee has been in the news a lot recently, largely because of world-
44 wide die-offs due to the parasitic Varroa mite, which is becoming resistant to the
45 chemical controls the bee industry uses. In this study, we show that robust expression
46 biomarkers of a disease-resistance trait can be used, in an out-bred population, to select
47 for that trait. After three generations of selection, the resulting stock performed as well or
48 better than bees selected using the phenotypic best method for assessing this trait when
49 challenged with disease. This is the first demonstration of an expression marker for
50 selective breeding in any agricultural species, plant or animal. This also represents a
51 completely novel way to select for highly polygenic traits.

52

53 Introduction

54 European honey bees are a keystone species in agriculture as many crops
55 depend on them for pollination and increased yield⁴. Honey bee colonies have been
56 dying at increased rates over the past decade, largely due to increased pressure from
57 diseases and pests⁵. Since these pests and pathogens are continually evolving
58 resistance to the synthetic chemicals used to treat them, the most sustainable, long-term
59 solution for bee health is the development of selective breeding programs that can
60 enrich natural disease resistance mechanisms. However, selective breeding in *A.*
61 *mellifera* is particularly challenging because most traits are expressed at the colony
62 level⁶, and due to the haplo-diploid sex determination system in bees, challenges in
63 storing germplasm⁷, the requirement for heterozygosity at the complementary sex
64 determination locus⁸ that severely limits in-breeding, and the tendency for queens to
65 mate with up to two dozen different drones. These factors mean that continual selection
66 is required to maintain stock.

67 Bees do, however, have some effective disease-resistance traits, which also
68 happen to be highly polygenic: one example is the social immunity function known as
69 hygienic behaviour. Bees exhibiting hygienic behaviour are more efficient at removing
70 dead, diseased, or dying brood from the hive^{9, 10}, enabling them to resist or at least co-
71 exist with pathogens such as American Foulbrood (*Paenibacillus larvae*) or parasites
72 such as *Varroa* mites (*V. destructor*) that would otherwise kill the colony. A closely
73 related but distinct trait known as *Varroa*-sensitive hygiene enables bees to detect and
74 disrupt the life cycle of reproductive female *Varroa* mites¹¹. Both hygienic behaviour and
75 *Varroa*-sensitive hygiene are heritable^{12, 13} and can therefore be selectively bred for;
76 likewise, QTLs and SNPs have been linked to each behaviour^{14, 15}, opening the door for
77 their use in marker-assisted selective (MAS) breeding. Historically, genomic markers
78 have been favoured for MAS because of their stability and reliability, whereas

79 expression markers such as the levels of transcripts or proteins, are typically thought to
80 be too variable and dependent on environment for use in MAS.

81 However, even a closely linked DNA feature may not be sufficient for MAS in
82 honey bees; *A. mellifera* has one of the fastest recombination rates (~32 cM/Mb) known
83 among animals, and this will rapidly break down inter-allele linkage through repeated
84 rounds of meiosis¹⁶. On the other hand, causally-linked expression markers should be
85 more robust to recombination since their presence is required for the trait, even though
86 they have historically been thought to be too dependent on environment. Here we use a
87 panel of protein markers identified through a multi-generational study³ to guide selective
88 breeding of disease-resistance traits in honey bees through three generations. By the
89 third generation, bee stocks selected through MAS were able to resist disease as
90 effectively as bees raised through conventional selective breeding using standard field
91 tests, with no detectable loss of other desirable traits such as honey production. This is
92 the first successful use of expression markers for MAS that we are aware of.

93

94 **Results & Discussion**

95 We have previously identified seven proteins in adult worker bees' antennae
96 whose expression is tightly linked to hygienic behaviour³ (HB). To complete a
97 comprehensive panel of markers for use in selective breeding, we added six more
98 proteins derived from the same data, four that showed tight correlation with *Varroa*-
99 sensitive hygiene and grooming behaviour, and two more also linked to hygienic
100 behaviour. Of the latter, one was missing in an initial dataset and therefore failed to meet
101 the inclusion criteria we used, and the other (Fig. 1a) had been 'lost' due to an
102 unrealized change in accession number between protein database versions. These
103 thirteen biomarkers were complemented by two 'housekeeping' proteins, α -spectrin and
104 β -tubulin, that showed zero correlation with any behaviours (Supplemental Table 1) to

105 serve as loading controls. The biomarker panel had originally been discovered through
106 untargeted, data-dependent liquid chromatography-tandem mass spectrometry but a
107 less stochastic detection method was required for scanning hundreds of samples.
108 Therefore, multiple reaction monitoring (MRM) assays¹⁷ were developed for up to five
109 peptides from each protein (Supplemental Table 1, Fig. 1b), with priority given to
110 peptides we had observed in the discovery of these biomarkers³. The use of stable
111 isotope-labelled standard peptides of known concentrations allowed quantitation of each
112 peptide in protein extracts from worker bee antennae (Fig. 1c).

113 To identify a robust initial breeding population of honey bees that was not already
114 enriched in disease resistance behaviours, we surveyed the hygienic behaviour (HB) of
115 635 colonies from thirty-eight commercial beekeeping operations across western
116 Canada in 2011. For this initial survey, we gave priority to beekeeping operations that
117 bred their own bees so that the bees were well-adapted to the local climate¹⁸ and
118 representative of stock being bred and used in Western Canada. All beekeepers
119 donated or sold a subset of the tested queens to incorporate into the selection program.
120 The hygienic behaviour scores in this initial survey varied regionally and ranged from
121 9.8% to 100%, with a median of 64% (Fig. 2a), matching levels of trait expression
122 observed previously among unselected populations in a smaller survey within the same
123 region¹⁹.

124 For selection using markers, nurse bees from 468 of these colonies were
125 dissected for quantitation of peptide markers in their antennae (Supplemental Table 2).
126 From the colonies surveyed, two to four colonies were randomly selected from each
127 beekeeping operation (for a total of 100 queens) to serve as benchmark, unselected
128 stock (Fig. 2a): these (BEN) were statistically indistinguishable from the wider surveyed
129 population (ALL). In addition, we selected queens from an additional 100 colonies, each
130 with the highest HB scores in their apiaries. Their HB scores ranged from 38% to 100%

131 with a mean score of 85%. We moved these queens to two breeding locations in
132 Southern British Columbia and introduced them into colonies.

133 Over the next two years we reared three successive generations (F1 and F2 in
134 2012, F3 in 2013) from this initial population using the response of parental colonies in
135 each of two ways: (1) the classic freeze-killed brood assay⁹ to quantify hygienic
136 behaviour as a positive control (FAS, field-assisted selection), and (2) the levels of the
137 best-performing subset of the peptide markers in Supplemental Table 3 (MAS, marker-
138 assisted selection). For the selective breeding, the F1 and F2 queens were generated by
139 instrumental insemination of virgin queens reared from the selected colonies using
140 semen pooled from a random collection of drones from all the selected colonies in the
141 appropriate stock. The same pooling of semen among selected was accomplished for F3
142 queens by closed breeding in isolated mountain valleys in southeastern British Columbia
143 where no known drone sources existed. In addition to these selective breedings,
144 benchmark stock (BEN) was maintained through unselected open mating, as a control.

145 The freeze-killed brood field assay (FAS) is the gold standard for identifying
146 hygienic behaviour⁹ and colonies selectively bred using FAS showed the greatest
147 enrichment of hygienic behaviour (Fig. 2b) over three generations. Notably, selective
148 breeding based on the panel of protein markers (MAS) was also effective for enriching
149 hygienic behaviour, demonstrating the potential of this technique in selective breeding.

150 By measuring hygienic behaviour in the colonies bred using MAS we could also
151 monitor the specificity and sensitivity characteristics of the biomarker panels; while there
152 was a statistically significant improvement detectable even in F1, by F2 there was a very
153 marked improvement that was little changed in the F3 (Fig. 2c, d, e). The distribution of
154 hygienic behaviour in the unselected BEN colonies, however, was unchanged between
155 the starting group and the final population (Fig. 2b, left-most plot).

156 Hygienic behaviour can confer resistance to brood pests and pathogens that
157 contribute to honey bee colony losses. We therefore evaluated how well colonies
158 headed by selected queens performed under disease (American foulbrood (AFB), *P.*
159 *larvae*) and mite (*V. destructor*) challenge conditions. Selected and benchmark stocks,
160 as well as imported stock commonly used by Canadian beekeepers, were inoculated
161 with either parasitic mites or AFB bacterial spores at levels that would normally result in
162 high levels of colony mortality.

163 To test for the ability to survive with *Varroa* mites, in the early summer 2012 we
164 pooled a large population of worker bees from colonies that were infested with *V.*
165 *destructor* (about three hundred mites per colony, or a 3.5% infestation rate based upon
166 mites per 100 bees) and aliquoted 8,600 bees (1 kg of bees) into individual colonies.
167 Twenty-three F3 selected or unselected queens were then randomly introduced into
168 these individual colonies and the colonies were left untreated until the following spring; a
169 fall survey for mites measured infestation rates of 23.0 ± 1.4 mites per 100 bees. For
170 AFB, F3 colonies normalized for population received a frame with 225 cm² of brood
171 comb with 30 to 54% of wax cells showing visible *P. larvae* disease symptoms on each
172 side. We assessed the impact of the parasite and pathogen challenge on winter survival
173 for the varroa challenge and for overall survival of asymptomatic colonies for the AFB
174 challenge (Fig. 3).

175 To check that an independent but critical performance indicator of these stocks
176 was not being degraded by too much focused selection on another trait, we also
177 examined the ability of F3 colonies to collect honey in a separate study from the
178 disease-challenge experiments. Reassuringly, honey production was not affected by
179 selection for hygienic behaviour, regardless of which selection method was used (Fig.
180 3c).

181 The results of these experiments were subsequently used to model the economic
182 impact of integrating the use of marker-selected stock into a Canadian beekeeping
183 operation. The marker-selected colonies' increased disease resistance and greater
184 survival rates enhanced beekeeper profit. The economic modeling shows that when a
185 beekeeper replaces his conventional colonies with MAS colonies selected for hygienic
186 behavior we see up to a 5% increase in profit for a 40-colony apiary. This is likely an
187 underestimate as we have not assumed any increase in colony productivity for the
188 disease-resistant MAS colonies. The greatest economic value derived from MAS colony
189 adoption was when resistance to traditional Varroa treatments was modeled in the
190 apiary. To reduce the risk of economic loss associated with treatment resistance (or an
191 equivalent ineffective/ lack of treatment), MAS colonies replaced 25%, 50% and 100% of
192 traditional colonies within the apiary showing profit increases of over 300%, 600% and
193 800% respectively compared to the no-MAS apiary.

194 The first genetic modification of bees has been reported²¹ but industrial use of
195 genetically modified bees is unlikely to be accepted by the public at this time. Therefore,
196 tools that enable accelerated stock enrichment without resorting to genetic modification
197 are highly desirable. While genetic markers for hygienic behaviour and *Varroa*-sensitive
198 hygiene have been identified^{14, 15}, they are unlikely to be linked tightly enough to be
199 robust to the high recombination rate bees exhibit for more than a few generations. In
200 addition, there may be several loci missing since both traits are highly polygenic²².
201 Expression markers integrate many different *cis*- (e.g., transcriptional enhancer
202 elements) and *trans*- (e.g., transcription factors) effects so if they are functionally linked
203 to the trait in question then they should be robust to recombination. We have not yet
204 shown that the markers we use here are functionally linked to hygienic behaviour,
205 *Varroa*-sensitive hygiene, or grooming but they are tightly linked enough to enrich the
206 trait as quickly as the best conventional methods available.

207 The most important pests and pathogens of bees are currently controlled with
208 acaricides, antibiotics and antimycotics, but emerging resistance to these treatments
209 may be partially responsible for the higher level of colony losses seen over the past
210 seven years²³. These exogenous treatments can leave residues in the hive²⁴ and honey
211 though so disease-resistant stock is seen as the best solution, simultaneously reducing
212 colony losses and the need for synthetic chemicals while ensuring food safety. Marker-
213 assisted selection of honey bees will enable more sophisticated breeding of this critical
214 agricultural service provider.

215 Selective breeding is a vital tool for improving yields and disease resistance in all
216 plants and animals used in agriculture. Marker-assisted selection has the potential to be
217 more precise and more robust to external influences; it has been widely used in certain
218 plants²⁵ and animals²⁶. To date, however, the markers used have been genomic loci
219 exclusively, starting with restriction fragment length polymorphisms and leading up to
220 single nucleotide polymorphisms. This is undoubtedly due, in part, to the availability of
221 efficient genetic approaches for finding such markers. It is also a matter of focus though:
222 researchers have spent more time looking for genetic loci than for expression markers
223 (i.e., transcripts or proteins) because the latter have been hitherto considered to be too
224 dependent on environment. Here we have shown that expression markers can be used
225 to select for a very complex, polygenic trait. Even in this proof-of-principle with a first-
226 generation panel of markers, MAS was as efficient at enriching disease-resistance as
227 FAS methods: bees bred using marker-assisted selection could resist levels of disease
228 that would typically kill 75% or more of unselected colonies. The data presented here
229 have implications beyond bees: this is the first demonstration of marker-assisted
230 selection in livestock using expression markers and it opens the door for molecular
231 diagnostic approaches for selecting complex polygenic traits that are recalcitrant to
232 genetic mapping methods²⁷.

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248

249 **Author Contributions**

250 LJF, SFP, RWC, and MMG conceived the experiments. LJF, MMG, SFP, RWC, SEH,
251 EH, AI, APM, and HH designed the experiments. EH and HH managed the selective
252 breeding. RW and MMG developed the statistical treatment of the biomarkers and
253 refined the prediction models. KMM oversaw the proteomic sample collection and
254 processing. SEH, SFP, MMG and KMM analysed data from freeze-killed brood and
255 MRM assays data to select breeding colonies. All authors except RW, MB, DD and CB
256 helped with sample collection, hygienic behaviour testing, and general beekeeping
257 activities. DD and CB developed and applied the multiple reaction monitoring assays.

258 SEH, AI, MP, SD, DM, SFP and RWC conducted the *Varroa*- and *P. larvae*-challenge
259 experiments, as well as the evaluation of honey production. MEFB developed the
260 economic model.

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264 **Figure Legends**

265 **Figure 1: Multiple reaction monitoring assays for markers of disease resistance.** a)

266 amino acid sequence of gi:110761334, Glycine-rich cell wall structural protein-like
267 protein, one of the markers of hygienic behaviour. The two peptides identified in the
268 initial discovery are highlighted in red; these same two peptides were targeted with
269 multiple reaction monitoring assays here. b) overlaid chromatograms of the three
270 selected transitions for the stable isotope-labelled forms of all fifty-five peptides listed in
271 Table 1 for the fifteen proteins comprising the biomarker panel. c) Transitions for the
272 stable isotope standard (SIS) and natural (NAT) forms of MGSIDEGVSK from Glycine-
273 rich cell wall structural protein-like protein. The primary (1°) transition of each peptide
274 was used for quantitation, while the secondary and tertiary transitions were used to
275 confirm specificity.

276

277 **Figure 2: Starting distributions and enrichment of hygienic behaviour.** BEN =

278 benchmark, MAS = Marker-assisted selection, FAS = Field-assisted selection. (a) 90/10
279 box-and-whisker plots of the hygienic behaviour scores from all colonies in initial survey
280 across Western Canada, in British Columbia (BC), Alberta (AB), Manitoba (MB)(left
281 section) (Means followed by the same letter are not different from each other Tukey
282 $P < 0.05$), all colonies together (ALL), and the randomly selected starting benchmark
283 population (BEN). 'All' is statistically identical to BEN ($p = 0.21$, Analysis of Means Test)

284 (b) The distribution of hygienic behaviour in the F1 and F3 generations of the benchmark
285 population (left section, BEN, no statistical difference between F1 and F3, $P=0.65$,
286 contrast), the colonies selected by the biomarker panel (middle section, MAS, $F3 > F1$,
287 $p=0.03$, contrast), and the freeze-killed brood assay (right section, FAS, $F3 > F1$,
288 $p=0.002$, contrast). Within each generation, means followed by the same letter are not
289 different from each other Tukey $P=0.05$). Bottom: Receiver operating characteristics
290 illustrating the performance of the F1 (c), F2 (d) and F3 (e) marker panels used for MAS.
291

292 **Figure 3: Performance of selected stock.** IMP = imported stock, BEN = benchmark,
293 MAS = Marker-assisted selection, FAS = Field-assisted selection. (a) Difference in
294 winter survival of F3 generation colonies headed by queens from each stock type that
295 were challenged with *Varroa* mites (*Varroa* challenge) (d.f. 3, Chi Sq 14.84 $p > \chi =$
296 0.002). (b) Difference in symptom-free survival when challenged with American
297 Foulbrood (*P. larvae*; AFB challenge:) (d.f. 3, Chi Sq 12.65 $p > \chi = 0.0054$). Horizontal
298 lines represent Holm-Bonferonni adjusted single degree of freedom contrasts between
299 MAS selected stock and the benchmark and imported stock controls. Siimilar results
300 were found for FAS, with FAS survival higher than the BEN and IMP stocks for both the
301 *Varroa* challenge ($p=0.05$ and $p=0.025$, respectively) and AFB challenge experiments
302 ($p=0.025$ and $p=0.013$, respectively). Error bars represent the standard error of the
303 binomial proportion. (c) Honey produced per colony for all stocks tested at three
304 experimental sites in Alberta and Manitoba. There was no significant difference in honey
305 production among the four stocks tested (d.f. 3,161; $F=2.12$, $p=0.099$).

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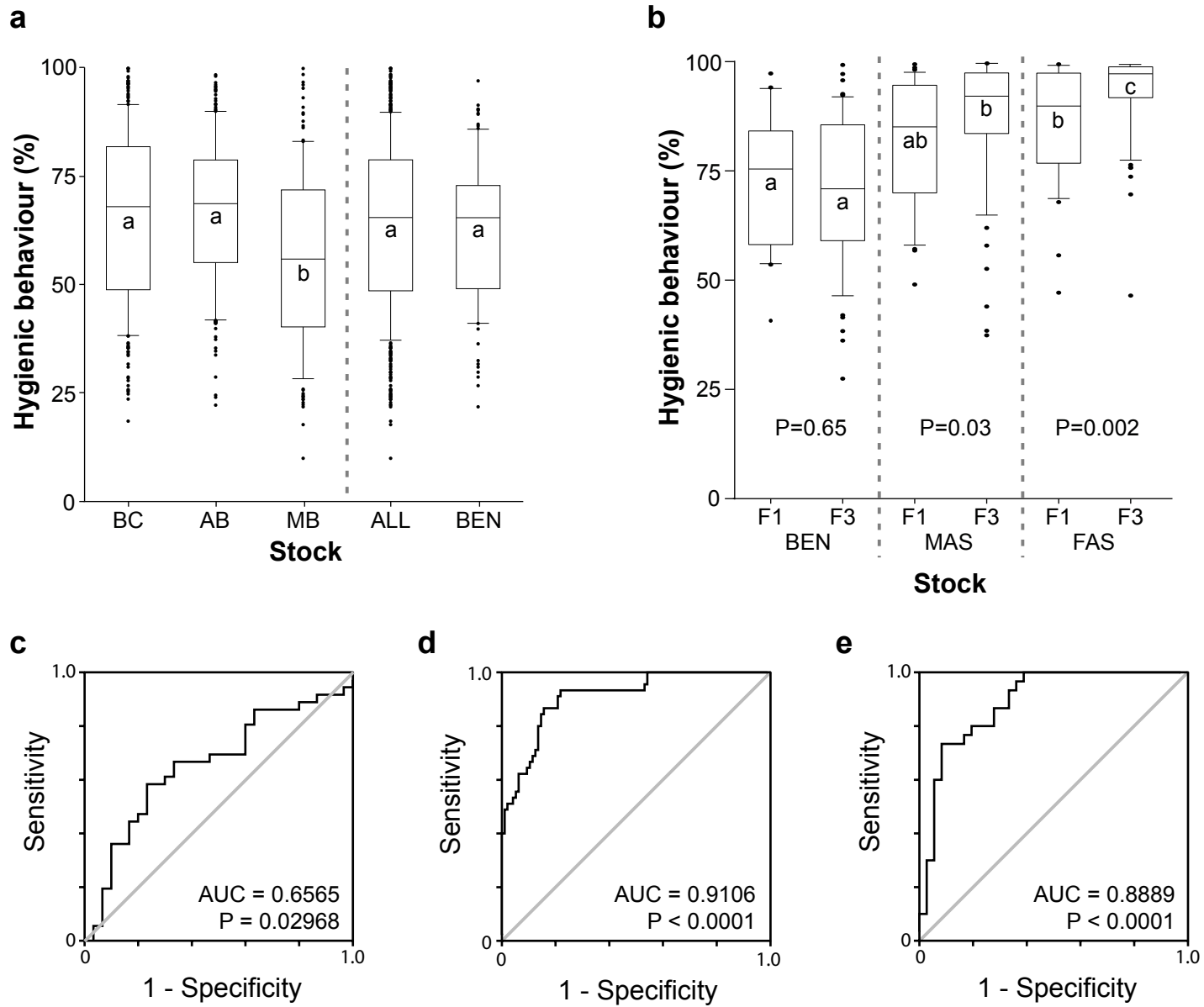


Figure 2, Guarna et al.

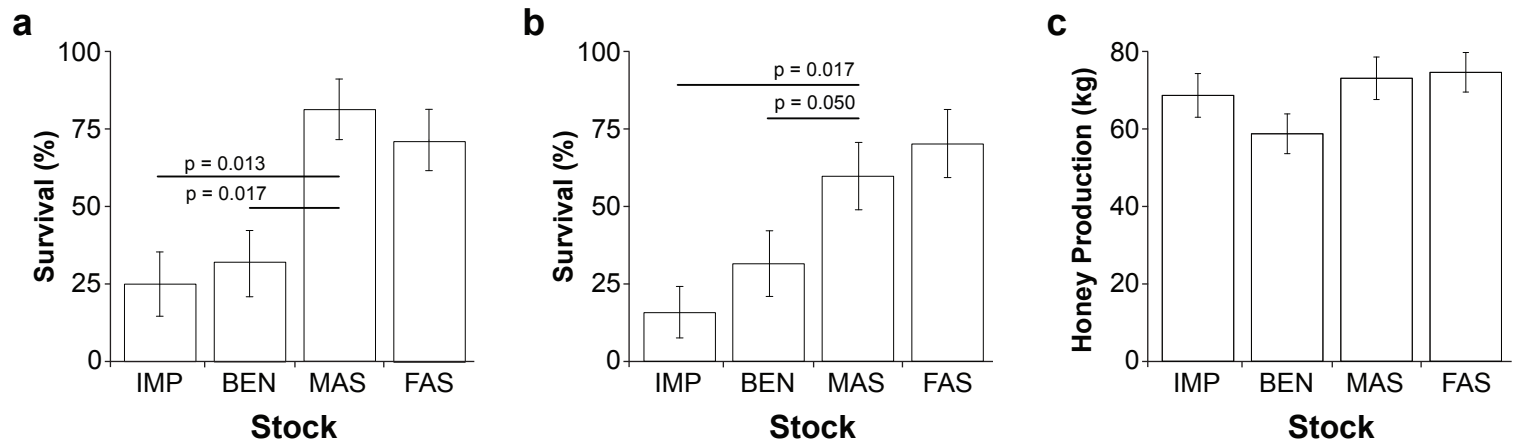


Figure 3, Guarna et al.