

24

25 **Abstract**

26 Sociality has brought many advantages to various hymenoptera species, including their ability of
27 regulating physical factors in their nest (e.g., temperature). Although less studied, humidity is
28 known to be important for egg, larval and pupal development. Two subspecies of *Apis mellifera*
29 of the M branch, also called black bees, were used as models to test the “hygroregulation
30 hypothesis”, by means of monitoring hygrometry in hives during one year in four conservation
31 centers: two in France (*A. m. mellifera*) and two in Portugal (*A. m. iberiensis*). We investigated the
32 ability of both subspecies to regulate the hygrometry daily, but also during the seasons and one
33 complete year. Our data and statistical analysis probed the capacity of the bees to regulate
34 humidity in their hive, regardless of the day, season or subspecies. Furthermore, the study showed
35 that humidity in beehives is very stable even during winter, when brood is absent, and when
36 temperature is known to be less stable in the beehives. These results would support that
37 hygrometry could prevail over temperature in maintaining nest homeostasis, maybe because of a
38 bigger importance of hygrometry for all the population during a year, or because of the ‘imprint’
39 of the evolutionary history of this hymenopteran lineage.

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41

42 **Keywords:**

43 Honeybees, *Apis mellifera*, black bee, subspecies, humidity, hygroregulation

44

45 **Introduction**

46 Terrestrial insects have high sensitivity to temperature and humidity. Most of them cannot
47 control their body temperature (i.e., ectotherm species) and are, thereby, very dependent to
48 their environment [1–3]. Indeed, temperature has an important impact on foraging [1] and it is
49 also crucial for reproduction, larval and pupal development, and the success of the offspring
50 [4]. Eusocial insects have also evolved many strategies to maintain their nest temperature
51 stable and controlled [5,6], especially in order to protect the eggs from extreme temperature
52 variations [6]. Nest homeostasis provides not only an incubator for the brood, but also a
53 thermal refuge for individuals to use temperature gradients to regulate their own body
54 temperature [7].

55 The eusocial bees use the thermoregulation to ensure their survival and health [3,6,8]; for
56 instance *A. mellifera* larvae can only survive in an environment with slight temperature
57 fluctuation (i.e., from 32 to 36 °C) [9]. The nest thermoregulation strategies of eusocial
58 insects can be passive (e.g., nest orientation, architecture) or active (e.g., clustering,
59 incubation), depending on the species [6]. Those thermoregulation strategies could have an
60 impact on hygrometry levels [10], a less studied phenomenon, but also vital for terrestrial
61 insects [11]. Indeed, environmental humidity is an essential factor to control for the survival
62 of adults and eggs [11]. While eusocial insects exhibit a range of humidity preferences in the
63 nest regarding their activity or the presence of brood [12], they generally require a relative
64 humidity (RH) of above 55% to hatch successfully, with the highest survival between 90 and
65 95% RH [4].

66 Here, we chose the honeybee, *Apis mellifera* (Linnaeus 1758) (Hymenoptera, Apidae), as
67 model organism. This insect is worldwide present and it has been increasingly studied since
68 the decline of its colonies in many occidental countries, due to its key role in pollination of

69 many crops and in sustaining the wild biodiversity of ecosystems [13]. While several
70 experiments have been conducted (i) to disentangle the mechanisms that lead to creation and
71 maintenance of microclimate in a beehive [9,14], and (ii) to determine the impact of
72 parasitism on thermoregulating social behavior [8], little is known about hygrometry in
73 beehives. As for temperature, the eggs are very sensitive to humidity fluctuations within the
74 nest [11]: a dry atmosphere can lead to the eggs death, either because the embryos die or
75 because the dry egg envelopes become too hard for the larvae to hatch [11]. After hatching,
76 humidity is still essential for young bees, and considered at least as important as temperature
77 for larval and pupal development [4,15,16].

78 In this study, we investigated for the first time humidity for one year in beehives of native
79 European honeybee subspecies belonging to the M evolutionary branch (*mellifera* subspecies
80 of Western Europe, also called black bees) [17]. Two subspecies of M lineage were studied in
81 two European countries: *A. m. mellifera* in France, and *A. m iberiensis* in Portugal. Our
82 experimental setup includes daily, seasonal and annual measures of hygrometry in the
83 beehives with the main objective of assessing the capacity of bees to hygroregulate their nests
84 (i.e. “hygroregulation hypothesis”), and in order to determine if geographic, seasonal or sub-
85 species parameters could impact this capacity.

86

87 **Materials and Methods**

88 **European study sites**

89 The study was conducted in four conservation centers created to preserve *A. m. mellifera* in
90 France and *A. m. iberiensis* in Portugal. These conservation centers, of an approximate
91 extension of 350km², are organized in a sanctuary zone encircled by a buffer zone, designed
92 to prevent queens from mating with males from outside the conservatory area. The French
93 conservation centers are located at Rochefort (48°35'47"N; 1°57'57"E) and Pontaumur

94 (45°51'51"N; 2°40'24"E), in the regions of "Ile-de-France" and "Auvergne-Rhône-Alpes",
95 respectively. The landscape in Rochefort corresponds to that of "plain beekeeping", and the
96 landscape in Pontaurmur corresponds to that of "semi-mountain beekeeping". The Portuguese
97 conservation centers are located at two latitudinal extremes in Gimonde (41°48'31"N;
98 6°42'41"W) and Zavial (37°03'14"N; 8°52'40"W), in the regions of "Trás-os-Montes" and
99 "Algarve", respectively. The landscape in Gimonde corresponds to that of "semi-mountain
100 beekeeping", and the landscape in Zavial corresponds to that of "plain beekeeping".

101

102 **Experimental design to monitor the hygrometry in hives**

103 In each conservation center, six healthy beehives from the sanctuary zone were randomly
104 chosen to be monitored. relative humidity (RH) was measured by three thermo-hygro button
105 data loggers (also named iButtons). The iButtons were placed in the nest of each of the six
106 monitored colonies, at the two outermost brood frames (iButton A and C) and at the central
107 brood frame (iButton B), each of them hanging between two frames by an iron thread. An
108 external thermo-hygro button was placed in each apiary at approximately 200-cm high, near
109 the monitored beehives, to register environmental RH variation. Each iButton was
110 programmed to measure RH hourly with a precision of $\pm 1\%$. Data from iButtons were
111 collected every four months by a reader (Plug & Track, Progues PLUS) and exported to Excel
112 format using the Thermotrack PC V.7 software. The pluviometry data was daily collected at 2
113 a.m., 8 a.m., 2 p.m. and 8 p.m. by a weather station (Micro El d.o.o., Zagreb) placed in each
114 sanctuary.

115

116 **Statistical Analysis**

117 To test the "hygroregulation hypothesis" at daily scale, first, RH (%) measured for each
118 hive was plot to give an overview of the humidity evolution within the hives. Then, the days

119 with the most similar external RH daily profile between the four geographic locations and for
120 two contrasting seasons in beekeeping: summer (high colony activity) and winter (low colony
121 activity) were selected using a similarity index (S) for days without rain in the four
122 conservatories. A similarity matrix $S=1-D$, where S denotes similarity and D denotes distance
123 ($D(x,y) = (\sum_i (x_i - y_i)^2)^{1/2}$), with x_i and y_i being the peers of conservatories to compare, was
124 calculated using Statistica 8.0 software (Stat Soft Inc., Arizona, USA). This index allowed to
125 select one day in winter and one day in summer to compare in-hive RH and external RH in
126 each conservatory. Then, both days were divided into two parts: the part of the day with
127 gradual decrease in external RH (i.e., from maximum RH, at 6 a.m., to minimum RH, at 5
128 p.m.), and the part of the day with a gradual increase of external RH (i.e., from the minimum
129 RH of the day, at 6 p.m., until next day's morning, when RH was maximum, at 5 a.m.). For
130 each selected day, linear regression was calculated using in-hive from iButton B and external
131 data, for each day part, to test the daily "hygroregulation hypothesis" (i.e. weak positive
132 correlation ($0 < r \leq 0,6$) linked with a $R^2 \leq 0,40$ (weak coefficient of determination), or
133 negative or null correlation ($r \leq 0$). An index $((RH_i - RHe)/RHe)$ with RH_i being the in-hive
134 data of iButton B and RHe being the external RH, was calculated for each hour of each
135 selected day. A clustering analysis was performed with this index in order to classify the hives
136 from each conservatory in a hierarchical way. This analysis was performed using the
137 PermutMatrix 1.9.4 seriation software (SupAgro, Montpellier, France).

138 An analysis of RH was also performed for one complete year, from September 2015 to
139 August 2016. In-hive data from all iButtons and beehives were put together for each
140 conservation center to obtain a global mean and standard error and compare them with the
141 external data, using Statistica 8.0. A statistical analysis was also conducted at the season level,
142 by considering September to November for autumn, December to February for winter, March
143 to May for spring and June to August for summer.

144

145 **Results**

146 **Daily hygroregulation**

147 The external RH from the two selected days for the four conservation centers are given in S1
148 Fig: December 11th, 2015, a winter day without rain or snow, had the most similar external
149 RH profile among the four conservation centers (50% similarity), whereas July 21st, 2016,
150 was the most similar not-rainy summery day (40% similarity). Fig 1 shows the regression
151 lines calculated for July 21, 2016, for the central iButton (B) of hive 1 in each conservation
152 center. These are representative of the regression lines for the remaining five hives and
153 iButtons (i.e., linear equations, r and R^2), that are shown in supplementary material S1 Table.
154 The cluster analysis was only performed for iButton B because it assessed RH in the main
155 part of the brood (i.e. eggs, larvae and pupae) (Fig 2). During summer, the results show
156 globally a positive regulation (in-hive RH > external RH) in the afternoon, especially in
157 Gimonde. In Gimonde, the beehives are mostly maintained with low humidity inside,
158 compared to the other apiaries. During winter, results show a similarity between all
159 conservation centers, since RH is maintained along the whole day at lower level inside than
160 outside the hives (S3 Fig).

161

162 **Fig 1. Linear modeling of the relationship between RH observed in the summer in the**
163 **beehives 1, iButton B (in-hive) with those recorded in their habitat (external) in each**
164 **conservation center.** The modeling was done for one day (July 21, 2016) and separated in
165 two parts: downward RH from 6 a.m. to 5 p.m. and upward RH from 6 p.m. to 5 a.m. the
166 next day. Linear modeling for *A. m. mellifera* is represented in A (downward RH) and C
167 (upward RH) for Pontaudur (▲) and Rochefort (●). Linear modeling for *A. m. iberiensis* is
168 represented in B (downward RH) and D (upward RH) for Zavial (◆) and Gimonde (■).

169

170 **Seasonal and annual hygroregulation**

171 Average annual in-hive RHs were significantly different from average annual external RHs
172 (Mann-Whitney, $p < 0.05$) for the two honeybee subspecies deployed in the four conservation
173 centers (*A. m. mellifera* in Rochefort and Pontaumur, *A. m. iberiensis* in Gimonde and Zavial)
174 (Fig 3). In contrast, the comparison of the average in-hive RH shows that, while Gimonde is
175 similar to Pontaumur, the other conservation centers are statistically different from each other
176 (Multiple Comparison, $p < 0.05$).

177

178 **Fig 3. Mean and standard errors of RH levels over a complete year (September 2015 -**
179 **October 2016) for the four conservation centers.** In-hive RH (grey) were calculated using
180 the mean of the three iButtons (A, B and C) and the six beehives for each conservation center,
181 and external RH (black) was calculated using only the external iButton for each conservation
182 center. “*” = in-hive and external RH are different (Multiple Comparisons, $p < 0.05$). Detailed
183 mean and standard errors for the four seasons are shown in supplementary material Fig S4.

184

185 The seasonal analysis of RH showed significantly differences of RH between the in-hive
186 and the external data (Mann-Whitney, $p < 0.05$), for each conservation center in each season
187 (supplementary material, Fig S4). The average RH inside the beehives was maximum at 61.0
188 $\pm 1.3\%$ (Zavial, winter) and minimum at $50.4 \pm 3.0\%$ (Gimonde, autumn). Furthermore, the
189 statistical analysis shows that, for each season, the four conservation centers had significantly
190 different in-hive RH (Multiple Comparisons, $p < 0.05$), except in spring, when Rochefort
191 ($60.2 \pm 3.2\%$) was similar to Zavial ($59.5 \pm 2.0\%$) (Multiple Comparison, $p = 1.00$).

192

193 **Discussion**

194 Eusociality brought advantages to many insect species, especially the ability of living in
195 community and, thus, to regulate some parameters of their immediate environment, such as
196 temperature and humidity. While temperature regulation in eusocial insects has been studied
197 extensively [1,5,6,14,18] providing insights about the factors that influences temperature
198 management [1,19,20], such as climate [21] or genetics [18], there is little information on the
199 humidity regulation inside nests and hives. In this study we tested the “hygroregulation
200 hypothesis” by means of using as model two native honeybee subspecies from the M branch,
201 *A. m. mellifera* and *A. m. iberiensis*, and the assessment of the temporal hygrometry variation
202 inside and outside beehives. Thereby, we sought to better understand the ability of eusocial
203 insects to regulate the humidity of their nest (hive).

204 Our study has the advantage to present an approach at different scales, namely the day, the
205 season and the year, in geographic regions with contrasting climates and landscapes. Our
206 results overall show low or even no correlation between humidity inside and outside the hive
207 for the two honeybee subspecies, in summer (Fig 1, supplementary material S1 Table) and in
208 winter (S2 Fig, S1 Table). Thus, the black honeybee populations in French and in Portuguese
209 conservation centers maintain the nest humidity constant over the day, despite the extreme
210 variations that take place outside (Fig 2). In addition, the results show the ability of the two
211 honeybee subspecies to regulate their nest humidity regardless the season, even in the heart of
212 the summer or in the winter, when brood is absent [22]. Furthermore, no differences were
213 found over the year between the two honeybee subspecies, since the in-hive hygrometry in
214 Pontaumur (*A. m. mellifera*) and Gimonde (*A. m. iberiensis*) are at the same level, and those
215 from Zavial and Rochefort are higher and close to each other, with Rochefort having always a
216 higher hygrometry level ($58.1 \pm 3.2\%$ for Zavial and $59.9 \pm 4.1\%$ for Rochefort over the
217 year). The similar in-hive hygroregulation in Pontaumur and Gimonde on the one hand, and
218 of Zavial and Rochefort on the other hand, could be due to the environment, Pontaumur and

219 Gimonde being in a semi-mountain place, and Rochefort and Zavial being both in a plain
220 landscape.

221

222 **Fig 2. Hygroregulation observed in the central brood frame (iButton B) of each of the 6**
223 **hives during a 24h-period in the summer, from 6 a.m. (July 21, 2016) to 5 a.m. of the**
224 **next day (July 22, 2016), in the four conservation centers (Rochefort, R; Pontaumur,**
225 **P; Gimonde, G; and Zavial, Z).** Green means that the in-hive RH is lower than the
226 external RH (negative regulation), red means the opposite (positive regulation).

227

228 It has been shown that temperature is better regulated in the beehives in the summer than in
229 the winter, when there is brood in the hive, [23]. This is likely due to the important role of
230 temperature in egg development; it is known that at too low (i.e. $< 33^{\circ}\text{C}$) or too high (i.e. $>$
231 36°C) temperature eggs, larvae and pupae die [9,24]. Similarly, maintenance of the humidity
232 level is crucial for social insects; it directly affects the proper development of eggs, larvae and
233 pupae [4,11], which die when the ambient environment is too dry [11]. Therefore, a minimum
234 of 55% humidity is required for honeybee eggs to hatch, with a maximum survival rate
235 between 90 and 95% [4]. In addition, Ellis *et al.* (2008) have shown that honeybee workers
236 have a marked preference for approximately 75% humidity in the absence of brood [16]. In
237 this study, we obtained lower humidity levels in the beehives, with seasonal RH ranging from
238 $50.4 \pm 4.4\%$ (Gimonde, autumn) to $65.2 \pm 7.8\%$ (Rochefort, winter) (supplemental material
239 Fig S4). Seasons when brood is present in the hive (spring and summer) do not differ
240 significantly from the rest of the year (supplemental material Fig S4). However, in those two
241 seasons, RH does not drop below 50%, threshold below which eggs cannot hatch [4].

242 The fact that humidity is stable and, therefore, regulated in the winter, unlike temperature
243 [14,23] suggests two hypotheses: (i) the humidity is more easily maintained in the hive than

244 the temperature, despite the fact that the two honeybee subspecies must reduce their energy
245 efforts because food reserves are limited [25], and (ii) moisture is a more important factor for
246 adult health than previously suggested in the literature [4,11,15,16], especially for adult bees
247 that keep it constant even in the absence of brood. The first hypothesis involves a link
248 between temperature and humidity. Indeed, honeybee subspecies, such as *A. m. mellifera* and
249 *A. m. iberiensis*, mainly use active regulation systems to manage the temperature of their nest
250 [6]. This strategy includes ventilation, which has also an impact on ambient humidity [22,23].
251 The second hypothesis is based on studies by Buxton, who showed in 1932 that insects only
252 drink very rarely and need a moist environment to avoid desiccation, whether they are in the
253 larval or adult stages [11,16].

254 It is important to note that the evolutionary history of honeybees goes back several million
255 years [17], whereas their encounter with humans dates back only 15,000 years ago [22], when
256 bees moved from nesting in various natural cavities to beehives. Their ability to maintain
257 stable moisture and temperature within the colony may have facilitated the migration of
258 different subspecies to geographical areas with a climate that often varies greatly with the
259 seasons, or particularly arid countries, such as in many parts of Africa [26].

260 Currently, global warming is causing significant changes in the environment that
261 organisms have to face [27–31]. Since 1990, the average global surface temperature, the
262 environmental factor with the greatest impact on the biosphere, has increased by around 0.9°
263 C, with a faster rise for the minimum than for the maximum [31]. This global warming
264 contributes to the destruction of several habitats and biological invasions in several
265 ecosystems [30,31]. Insects, however, show strong adaptabilities to new climates, for example
266 by modifying their range [30,32] or their period of activity [33]. However, according to our
267 results, the two honeybee subspecies included in this study require a nest with relatively
268 stable and high humidity levels. Some eusocial hymenoptera living in relatively arid areas

269 have adapted their behavior according to their unavailability of water [2,33]. For those social
270 species, the lack of water due to global warming could lead to a significant change in their
271 geographic distribution, in order to survive to those new and limiting conditions.

272 Moreover, as our data suggest a high importance of moisture for both brood and adults, it
273 is conceivable that the development of certain diseases may be manifested by a disturbance of
274 the humidity in the nest: either (i) poor moisture control that would favor the occurrence of
275 opportunistic parasites such as varroa mites whose ability to reproduce is impacted by
276 moisture in hives [34], or (ii) the presence of parasites and pathogens causing weakening of
277 colonies [35–37], which would induce a decrease in the ability of insects to properly regulate
278 the humidity of their nest. Thus, we suggest that monitoring abiotic factors such as humidity
279 and temperature in honeybee hives could be a strategy for identifying colonies having
280 disturbance in their normal functioning as a eusocial community, and help to find the eventual
281 factors leading to the decline of a honeybee colony.

282

283 **Conclusion**

284 Our data and statistical analysis sustain the validation of the “hygroregulation hypothesis”:
285 the eusocial honeybee ability to regulate the humidity of the hive (nest) at a day, but also at
286 seasonal and year scales. Thereby, humidity is constant during the year in the beehives, even
287 in winter when temperature is less regulated because of the absence of brood. Furthermore, no
288 differences were showed between the two black bee studied subspecies, *A. m. mellifera* and *A.*
289 *m. iberiensis*. However, regardless of the genetics, it seems that the landscape could have a
290 bigger impact on the regulation, the apiaries in the plain having an in-hive hygrometry
291 slightly lower than those in the semi-mountain landscape. Overall, our results help to better
292 understanding how hygrometry is regulated in eusocial insects, and its relative importance
293 compared to temperature.

294

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299

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391 **Supporting information**

392 **S1 Table. Linear equation, r and R^2 for each model of the relationship between RH observed in the**
393 **summer (A) and in the winter (B) in the beehives (in-hive) with those recorded outside (external).**
394 The empty places correspond to iButtons that were absent from the beehive at this moment. Missing
395 data are due to a breakdown for two iButtons for a couple of days. **Figure S1:** Relative humidity
396 (RH) measured by the external iButton in the four conservation centers: Pontaudum (▲),
397 Rochefort (●), Zavial (◆) and Gimonde (■), (A) in summer (July 21, 2016) and (B) in winter

398 (December 11, 2015). These two dates were chosen because they have the most similar
399 external RH among the four conservatories in the two seasons, and because it did not rain in
400 any of the four conservation centers. The data were taken from 6 a.m. to 5 a.m. the next day
401 for both dates.

402 **S2 Fig. Linear modeling of the relationship between RH observed in the winter in the**
403 **beehives (in-hive) with those recorded in their habitat (external).** The modeling was done
404 for one day (December 11, 2015) and separated in two parts: downward RH from 6 a.m. to 5
405 p.m. and upward RH from 6 p.m. to 5 a.m. the next day. Linear modeling for *A. m. mellifera*
406 is represented in A (downward RH) and C (upward RH) for Pontaurmur (▲) and Rochefort
407 (●). Linear modeling for *A. m. iberiensis* is represented in B (downward RH) and D (upward
408 RH) for Zavial (◆) and Gimonde (■).

409 **S3 Fig. RH regulations observed in winter for each colony (iButton B) regarding to the**
410 **time of the day: from 6 a.m. to 5 p.m. the next morning.** Green means the in-hive RH is
411 lower than the external hygrometry (negative regulation), red means the opposite (positive
412 regulation).

413 **S4 Fig. Mean and standard errors of in-hive (grey) and external (black) RH for autumn**
414 **(September-November, 2015), winter (December 2015-February 2016), spring (March-**
415 **May, 2016), and summer (June-August, 2016).** The mean and standard errors for in-hive
416 data were calculated over the 3 iButtons (A, B and C) for the six beehives of the four
417 conservation centers and external data were calculated using over the external iButton. “*” =
418 in-hive and external RH are different (Multiple Comparisons, $p < 0.05$).

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