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## Distance interaction between marine cave-dwelling sponges and crustaceans

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1 **TITLE: Distance interaction between marine cave-dwelling sponges and crustaceans**

2

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5

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15

16 **ABSTRACT**

17           Sponges are benthic organisms that are dominant in several ecosystems and known to  
18 produce a huge chemical diversity. The putative release of some specialized metabolites in the  
19 surrounding seawater is still a matter of debate, but the presence of such compounds in the  
20 environment of sponges is thought to influence the behaviour of various mobile organisms and  
21 may, thus, contribute to benthic ecosystem structuring and functioning. Underwater  
22 Mediterranean caves are characterized by stable environmental conditions and sessile species  
23 assemblages dominated by sponges. A two-choice test system was developed to assess the  
24 response of two cave-dwelling crustaceans (*Hemimysis margalefi* and *Palaemon serratus*) and  
25 two other species living in shallow water environments (*Leptomysis* sp. and *Palaemon elegans*)  
26 to various seawater treatments: control- seawater from an exposed coastline, control+ seawater,  
27 coming from an underwater cave, and seawater conditioned with four Mediterranean sponges  
28 commonly found at the entrance of underwater caves (*Aplysina cavernicola*, *Haliclona fulva*,  
29 *Oscarella tuberculata* and *Spongia officinalis*) or their chemical extracts. We tested the  
30 swimming behaviour of these crustacean species in three complementary experiments: (i)  
31 control seawater vs. cave seawater; (ii) control seawater vs. seawater conditioned with the  
32 sponge community, (iii) control seawater vs. seawater containing chemical extracts of the same  
33 sponge community.

34           Both cave-dwelling crustaceans were attracted by the seawater conditioned with the  
35 sponge community, while *Leptomysis* sp. spent more time in the control seawater and  
36 *P. elegans* exhibited indifferent responses. All four crustacean species avoided the seawater  
37 containing the sponge extracts. Interestingly, the responses shown by the crustaceans was  
38 affected by the time of day. A comparative and untargeted metabolomic approach was applied  
39 to the surrounding seawater to identify putative chemomarkers that could explain the  
40 crustaceans' behaviours. Among other compounds found in the seawater, a family of

41 metabolites with molecular formulae in accordance with those of oxylipin derivatives is  
42 released by sponges and may, therefore, serve as chemical cues acting as kairomones in the  
43 homing behaviour of cave-dwelling crustaceans.

44

#### 45 **KEYWORDS**

46 Marine Ecology; Chemical mediation; Porifera; Oxylipins; Mediterranean Sea

47

#### 48 **INTRODUCTION**

49 Distance interactions regulate the structure and functioning of marine ecosystems and  
50 species distribution within a complex mosaic of habitats. There is a need for better  
51 understanding of these interactions, in particular when attempting to assess the putative effects  
52 of human disturbances on system functioning ([van de Koppel et al. 2015](#)). Chemical mediation  
53 plays a key role in such distance interactions by influencing the behavioural ecology of mobile  
54 organisms ([Hay 2009](#)). Seawater can also be seen as a chemical “seascape” composed of  
55 dissolved and/or adsorbed molecules of diverse origins, a good number of which are released  
56 by marine organisms and carry crucial signals for the whole ecosystem ([Cassier et al. 2000](#)).  
57 Surrounded by this complex chemical environment, mobile animals must decipher a complex  
58 cocktail of chemical cues, some of which act as kairomones, and adjust their behavioural  
59 response by navigating, selecting habitat, locating food or prey, detecting the presence of  
60 conspecifics, or defending against competitors and/or predators ([Heuschele and Selander 2014](#)).  
61 This complexity also challenges animals’ abilities to extract the information required to identify  
62 the sources of chemical cues which are crucial for their survival ([Derby and Sorensen 2008](#)).  
63 For example, fishes can detect natural chemical cues at very low concentrations (parts per  
64 billion) and, therefore, need to be able to identify the relevant cues from the higher levels of  
65 background noise ([Belanger et al. 2006](#)).

66           During the past decades, special attention has been paid to the functioning of tropical  
67 coral reefs. One of the greatest challenges has been to better understand the life cycles of littoral  
68 populations of marine species living in fragmented habitats in a vast and complex ocean matrix,  
69 species which may have dispersal and larval development phases offshore, followed by the  
70 recruitment of juveniles on randomly distributed reefs (Myrberg and Fuiman 2002). Several  
71 pioneer studies demonstrated that marine fish and crustacean larvae could distinguish between  
72 oceanic and reef waters (Atema et al. 2002; Lecchini et al. 2005; Gerlach et al. 2007; Dixon et  
73 al. 2008). These larvae were shown to be capable of selecting a given habitat among the great  
74 variety of substrates found in tropical waters (Huijbers et al. 2008; Munday et al. 2009; Lecchini  
75 et al. 2010; Devine et al. 2012; Igulu et al. 2013). For example, Huijbers et al. (2008) reported  
76 that fish recruits discriminate chemical cues from mangroves, seagrass beds and coral reefs and  
77 that a given reef fish actively prefers the odour of its home coral reef. Lecchini et al. (2014)  
78 also demonstrated that fish, crustacean and cephalopod larvae were significantly more attracted  
79 by chemical cues from living corals than by dead colonies. Further studies have highlighted  
80 how environmental disturbances leading to coral reef destructions, such as ocean acidification  
81 (Munday et al. 2009; Devine et al. 2012; Leduc et al. 2013), seawater warming (McCormick et  
82 al. 2010) or pollution (O'Connor et al. 2016), may affect marine chemical mediation.  
83 Additionally, these changes in abiotic conditions may also alter the organism's metabolism and,  
84 thus, its ability to produce specialized metabolites and may consequently impact chemical  
85 mediation between organisms (Fisher et al. 2006; Munday et al. 2009; Leduc et al. 2013).  
86 Surprisingly, even though chemicals are involved in these biotic interactions and in overall  
87 ecosystem functioning, few studies have attempted to identify the chemical nature of distance  
88 marine chemical cues (Lecchini et al. 2005; Lecchini et al. 2010; Lecchini et al. 2014; Rasher  
89 et al. 2015).

90 In comparison to this knowledge gained in tropical reefs, chemical mediation in  
91 temperate benthic ecosystems has been poorly studied. Sponges can dominate in terms of  
92 biomass and diversity in numerous benthic ecosystems, both in tropical and temperate regions  
93 (Diaz and Rutzler 2001; Gerovasileiou and Voultsiadou 2012; Wulff 2012). They are also well  
94 known for their ability to produce a great chemical diversity (e.g. Paul et al. 2011). However,  
95 there is a lack of knowledge concerning the ecological functions of these chemical compounds  
96 which can be released into the surrounding water (Thompson 1985; Walker et al. 1985; Ternon  
97 et al. 2016). Today, sponge chemical ecology is mainly based on the assessment of repulsive  
98 properties, the so-called “chemical defences”, against predators or competitors for space (Paul  
99 et al. 2011; Pawlik 2011). Even though sponges are often considered to be dominant  
100 components of benthic ecosystems, the putative role of the portion of their specialized  
101 metabolites released into the water column remains unclear and few studies have demonstrated  
102 a positive influence of these cues on the recruitment of invertebrate larvae (e.g. Bingham and  
103 Young 1991). We hypothesize that the great chemical diversity produced by sponges may  
104 construct a true chemical seascape which may be the basis of a still underestimated network of  
105 interactions shaping biodiversity.

106 We surmise that underwater caves constitute good ecosystem models in which to test  
107 this hypothesis. Sponges are predominant from the semi-dark zone where they coexist with  
108 cnidarians and bryozoans to the darkest parts of the cave where they can represent 100 % of the  
109 sessile biodiversity (Gerovasileiou and Voultsiadou 2012). In low or no light conditions, other  
110 putative producers of chemicals such as macroalgae (Amsler 2008) are absent or are restricted  
111 to the entrance of this habitat. Experimenting in underwater caves also avoids, or at least  
112 attenuates, variations of abiotic factors present in the open sea such as hydrodynamics, nutrient  
113 and resource bioavailability and, in some cases, seawater temperature (Rastorgueff et al. 2011).  
114 Underwater caves can thus be considered as *in situ* mesocosms where many ecological

115 processes and interactions are simplified. Finally, considering their very low resilience facing  
116 major environmental threats (Gunn et al. 2000), a better understanding of this ecosystem  
117 functioning is needed to propose suitable management and conservation plans (Gunn et al.  
118 2000; Bussotti et al. 2006; Gerovasileiou and Voultsiadou 2012; Rastorgueff et al. 2015).

119 This study focuses on two dominant components of cave ecosystem functioning:  
120 sponges, which are active suspension feeders and which compose the vast majority of the sessile  
121 fauna, and swimming crustaceans which act on benthic-pelagic couplings through their  
122 migration in and out of caves (Rastorgueff et al. 2015). Our aim was to assess the influence of  
123 the chemical seascape of a cave which is most likely primarily determined by sponges on the  
124 swimming behaviour of crustaceans (*i.e.* attraction or repulsion). We combined the use of (i)  
125 an experimental device to assess the behavioural response of the crustaceans, and (ii) a  
126 metabolomics fingerprinting approach to decipher the chemical composition of the various  
127 seawater treatments. To conduct our experiments, we selected representatives of the sponge  
128 community found near the entrance in semi-dark conditions of Mediterranean underwater  
129 caves, and four crustacean species with contrasting ecological habits: two cave-dwelling  
130 crustaceans and two crustaceans inhabiting shallow water environments.

131

## 132 MATERIAL AND METHODS

### 133 *Study location and species*

134 This study was conducted using the aquaria facilities of the Marine Station of Endoume,  
135 Marseille, France. Four characteristic sponge species of the semi-dark community (*Aplysina*  
136 *cavernicola* (Vacelet, 1959), *Haliclona fulva* (Topsent, 1893), *Oscarella tuberculata* (Schmidt,  
137 1868) and *Spongia officinalis* (Linnaeus, 1759); Supplementary Fig. S1) were collected in a  
138 cave in the Riou Archipelago at an average of 20 m depth (43°12'N; 5°22'E) and were  
139 maintained alive in aquaria. These species were selected because part of their specialized

140 metabolome had already been described (Garrido et al. 1997; Ivanisevic et al. 2011; Manzo et  
141 al. 2011; Nuzzo et al. 2012; Reverter et al. 2016; personal unpublished data). In the same cave,  
142 we also collected 70 L of seawater to act as a positive control in our first experiment.

143 The four crustacean species selected to perform the chemosensory assays were two  
144 mysid species (*Hemimysis margalefi* Alcaraz, Riera and Gili, 1986 and *Leptomysis* sp.) and two  
145 palaemonid species (*Palaemon serratus* (Pennant, 1777) and *Palaemon elegans* Rathke, 1837)  
146 (Supplementary Fig. S2). *Hemimysis margalefi* (mean length  $\pm$  SD,  $2.7 \pm 0.1$  mm) was collected  
147 in the same cave as the sponges, where it usually forms dense swarms. This cave-dwelling  
148 mysid undertakes circadian migrations in order to find food outside the cave at night or early  
149 morning (Rastorgueff et al. 2011), and we hypothesized that its return towards the cave may be  
150 influenced by a chemical seascape composed primarily of sponge chemical cues. *Leptomysis*  
151 sp. ( $8.3 \pm 0.3$  mm) was collected in shallow waters (8 m depth) adjacent to the Marine Station  
152 aquarium seawater pumping site ( $43^{\circ}16.80'N$ ,  $5^{\circ}20.95'E$ ). This benthic community is  
153 dominated by macrophytes such as *Posidonia oceanica* and is, thus, a totally different chemical  
154 seascape which is devoid of any sponge influence. *Palaemon serratus* ( $13.8 \pm 0.3$  mm) and  
155 *P. elegans* ( $8.2 \pm 0.1$  mm) individuals were offspring of gravid females that were captured in  
156 the Mediterranean Sea and had been raised in aquaria, so were totally naïve regarding the  
157 experimental conditions. *Palaemon serratus* is known to live in semi-dark to dark  
158 environments, and we hypothesized that its occurrence in caves may be related to the chemical  
159 seascape, whereas *P. elegans* lives in very shallow waters among macroalgae assemblages, so  
160 they should not be influenced by a seascape conditioned by sponges. For the duration of their  
161 time spent in aquaria, all four crustacean species were fed every 2 days with *Artemia salina*  
162 nauplii.

163

164 ***Chemosensory trials***

165 A Y-shaped glass flume providing a choice of two channels was set up according to a  
166 protocol developed by [Gerlach et al. \(2007\)](#) to assess the crustacean behavioural response when  
167 faced with the choice of different seawater treatments: open flow seawater as the control  
168 seawater (CS); cave seawater (+CS), considered here as a positive control; and two types of  
169 conditioned seawater, sponge conditioned seawater (SCS) and sponge chemical extracts  
170 dissolved in seawater (SCE). Two 10 L tanks were connected to the flume by pipes, creating a  
171 constant gravity-driven flow of  $50 \text{ mL min}^{-1}$  per upstream channel. Prior to the experiments,  
172 both flows were measured by adding fluorescein to one tank to ensure that the flows were  
173 similar and mixed only in the downstream compartment. The crustaceans were introduced at  
174 the downstream end of the apparatus, where they were free to remain or to select and swim  
175 towards one of the two channels connected to the tanks ([Supplementary Fig. S3](#)). Each assay  
176 was performed with a single specimen which was maintained for a 1-min acclimation period  
177 followed by a 5-min testing period. During the selection phase of the test, the position of the  
178 crustacean in the downstream compartment or in the right or left channels was regularly  
179 recorded at 5-sec intervals. After each test, the flume chamber was emptied and washed with  
180 the CS. The seawater treatments were then switched from one channel to the other, providing a  
181 control for potential channel preferences that were not associated with the water source. After  
182 seawater switching, the entire assay was repeated with a new specimen, with each individual  
183 being used in a single assay. In this way, the time (in seconds) spent by the crustacean individual  
184 in each of the three compartments of the choice flume (*i.e.* the downstream and the two  
185 channels) was recorded.

186 Using the protocol described above, three experiments were conducted with a minimum  
187 of 40 individuals per experiment and species, with the exception of *P. elegans*, for which only  
188 25 individuals were tested per experiment. Moreover, these experiments were conducted both  
189 in the morning (half of the total individuals; 7 h – 11 h UTC) and in the afternoon (half of the

190 total individuals; 14 h – 18 h UTC) in order to assess the daily variability of the crustaceans’  
191 response to the seawater treatments. For each experiment, +CS and the two types of conditioned  
192 seawater (SCS and SCE) were tested against CS. Because palaemonids were not available for  
193 the first experiment (CS vs. +CS), only the two mysids were used. All four crustaceans were  
194 used for the second (CS vs. SCS) and the third (CS vs. SCE) experiments.

195 The SCS was expected to contain the sponge community exometabolome (i.e. the  
196 metabolites naturally released into the seawater by the four selected sponge species). The SCS  
197 was obtained by maintaining living individuals of the four sponge species, measuring about  
198 25 cm<sup>3</sup> each, in a 10 L aerated-tank for 16 hours. The SCE was expected to contain the global  
199 metabolome of the same sponge community, that is to say, all metabolites contained in the  
200 tissues of the four selected sponges. This SCE was obtained from the sponges used to obtain  
201 the SCS, which were weighed, frozen, ground in a blender, filtered through a 500 µm sieve and  
202 then mixed with seawater in order to obtain a homogenous solution. The sponge community  
203 used to prepare the SCS and SCE represented a total weight of 108 ± 10 g.

204 To ensure that the modifications of the seawater used to prepare the SCS and SCE was  
205 due to the introduction of sponge exometabolomes or extracts only and not by a microbial  
206 community present in the pumped open flow seawater, a further CS was prepared for the  
207 metabolomic analyses by maintaining this open flow seawater for 16 hours in the same  
208 condition as the SCS.

209

### 210 *Metabolomic analyses*

211 An untargeted metabolomic approach was used to compare the metabolite composition  
212 of the different seawater treatments: the control seawater (CS), the cave seawater (+CS) and the  
213 two conditioned seawater treatments (SCS and SCE). .

214 Briefly, a volume of 500 mL of seawater samples was collected in the tanks during the  
215 two-flume choice experiments and passed successively through 0.45  $\mu\text{m}$  pore-sized nylon  
216 filters and octadecyl-bonded silica extraction discs (Empore<sup>TM</sup> C<sub>18</sub> SPE discs) to obtain  
217 particulate and dissolved phases of each seawater. The filters were extracted for particulate  
218 phase (or eluted for dissolved phase) with methanol. The extracts were then concentrated to  
219 dryness, treated to remove salts, dissolved in methanol and filtered at 0.2  $\mu\text{m}$  before  
220 metabolomic analysis. On-line Ultra High-Performance Liquid Chromatography coupled to a  
221 High-Resolution Mass Spectrometer (UHPLCHRMS) analyses were performed using a Dionex  
222 Ultimate 3000 system equipped with an autosampler and connected to a quadrupole Time of  
223 Flight (qToF) mass spectrometer equipped with an electrospray ionization interface (Bruker  
224 Impact II) to record mass spectra in the positive and negative modes (see Supplementary  
225 Information for detailed methods).

226 Following the UHPLC-HRMS data acquisition, base peak chromatograms (BPC) were  
227 exported as line spectra and converted into the netCDF file format to process unit mass  
228 resolution data in centroid mode with XCMS (Smith et al. 2006) on R software (3.4.2). The  
229 XCMS approach involved peak picking and integration, peak grouping for identification  
230 between samples, chromatogram alignment to avoid retention time deviation and, finally, peak  
231 filling to avoid missing values in chromatograms. XCMS generated a matrix  
232 (ions/retention time  $\times$  sample) that was exported on Microsoft Excel. Home-made scripts were  
233 used to clean the matrix, removing 1) ions detected in solvent blanks (ratio pools/blanks < 10),  
234 2) ions presenting an intensity variation above 30% for pool samples, and 3) ions with correlated  
235 intensities for pool samples (threshold = 0.8). Extracted Ion Chromatograms of variable  
236 importance in projection (VIP) chemomarkers were drawn to measure their concentrations in  
237 the different seawater treatments, taking into account the most important value of ion intensity  
238 at each VIP retention time. The heatmap based on data acquired in positive mode was finally

239 selected to tentatively annotate chemomarkers of seawater samples using the molecular  
240 formulae provided by Bruker Data-analysis 4.2.

241

### 242 *Statistical analyses*

243 Paired samples *t* tests (or Wilcoxon tests when normality and homoscedasticity  
244 conditions were not met) were performed to compare the time spent in each of the two upstream  
245 channels containing the different seawater treatments (CS vs. +CS; CS vs. SCS; CS vs. SCE),  
246 in order to determine the channel chosen by each crustacean species. Two-sample *t* tests (or  
247 Mann Whitney tests when normality and homoscedasticity conditions were not met) were  
248 performed to compare the time spent in the channels filled with CS, +CS, SCS or SCE between  
249 morning and afternoon, in order to determine if crustacean choice is affected by the time of day.  
250 Principal Component Analyses (PCA) were performed to explore and identify similarities  
251 between the chemical fingerprints of the different seawater samples. A permutation test (999  
252 permutations, PPLS-DA models, based on a double cross-validation, outer loop = 4 fold cross-  
253 validation, and inner loop = 3 fold cross-validation) was performed for the differentiation of the  
254 chemical fingerprints of the different seawater samples followed by post hoc permutational  
255 pairwise test (Hervé, 2018). The classification error rate (CER, 0 – 1) was calculated to estimate  
256 the strength of the generated models. Kruskal Wallis tests, followed by a post-hoc multiple  
257 range test (Fisher's Least Significant Difference), were performed to test differences in  
258 chemomarker concentrations between seawater treatments.

259 Multivariate analyses (PCA) and heatmaps were performed with MetaboAnalyst 3.5  
260 (Xia and Wishart 2016). Univariate analyses were performed with R software (version 3.4.2.)  
261 and Statgraphics centurion (version 15.2.11.0).

262

## 263 **RESULTS**

264 ***Control experiment***

265           When both upstream channels were filled with CS, the four crustaceans showed no  
266 significant preference between upstream channels (Supplementary Fig. S4) and remained  
267 mainly in the downstream compartment (Supplementary Fig. S5). These results confirmed the  
268 equivalence of the two channels for the assay. Moreover, both mysid species exhibited higher  
269 locomotor activity than the two palaemonid species, staying twice as long in the upstream  
270 channels (Supplementary Fig. S4).

271

272 ***Comparative crustacean responses to the seawater treatments***

273           During the 5-min tests of +CS vs. CS, most crustaceans exhibited stationary swimming  
274 behaviour at the intersection of the seawater currents from the two upstream channels before  
275 exhibiting their preference. However, occasionally after making their first choice, individuals  
276 would switch and explore the opposite channel, showing constant activity while investigating  
277 the seawater from both upstream channels. *Hemimysis margalefi* showed a clear preference for  
278 the +CS, staying 16 times longer in this channel (Fig. 1) than in the CS. In contrast, *Leptomysis*  
279 sp. demonstrated a significant preference for the CS channel, staying 4 times longer in this  
280 channel (Fig. 1) than in the +CS. The behaviour of the two mysids varied with the time of day,  
281 with *H. margalefi* having a higher preference for the +CS in the morning (Fig. 2). Interestingly,  
282 *Leptomysis* sp. had a higher preference for the CS in the afternoon (Fig. 2).

283           In the SCS vs. CS experiment, *Hemimysis margalefi* exhibited a significant preference  
284 for the SCS, staying 4 times longer in this channel (Fig. 1) than in the CS. This behaviour varied  
285 with the time of day, with a higher preference for the SCS in the morning, when it spent 7 times  
286 longer than in the CS channel, whereas this difference was only 2.5 times longer in the afternoon  
287 (Fig. 3). *Palaemon serratus* also exhibited a preference for the SCS compared to the CS channel  
288 (Fig. 1), but this attraction was less pronounced than for *H. margalefi* and was not affected by

289 the time of day (Fig. 3). Comparatively, *Leptomysis* sp. confirmed its clear preference for the  
290 CS channel (Fig. 1) and, in this experiment too, their behaviour varied with the time of day,  
291 with no clear preference in the morning, but with a 5-fold longer time spent in the CS channel  
292 than in the SCS in the afternoon (Fig. 3). Finally, as was the case for *Leptomysis* sp., *P. elegans*  
293 showed no preference in the morning but stayed 4 times longer in the CS than in the SCS  
294 channel in the afternoon (Fig. 3).

295

296 In the SCE vs. CS experiment, both *H. margalefi* and *P. serratus* clearly avoided the  
297 SCE channel and spent about 15 times longer in the CS channel compared to the SCE channel  
298 (Fig. 1). This trend was less marked for *Leptomysis* sp. and *P. elegans*, as these crustaceans  
299 stayed in the CS channel only 2 to 3 times longer than they did in the SCE channel (Fig. 1). The  
300 two cave-dwelling crustaceans (i.e. *H. margalefi* and *P. serratus*) presented the same  
301 behavioural response regardless of the time of day, whereas *Leptomysis* sp. and *P. elegans*  
302 exhibited a significant avoidance of SCE in the afternoon only (Fig. 4). Moreover, the  
303 swimming behaviour appeared quite different than in the previous experiments. *Hemimysis*  
304 *margalefi* and *P. serratus* did not exhibit a stationary phase at the intersection of the seawater  
305 currents, but they swam more quickly and presented a faster choice, mostly in favour of the CS  
306 channel. In a distinct manner, *Leptomysis* sp. showed a reduced swimming activity and spent  
307 most of its time on the bottom of the downstream compartment (Supplementary Fig. S5).

308

### 309 ***Metabolomic analyses***

310 The comparative untargeted analyses of the CS, +CS, SCS and SCE chemical  
311 fingerprints were performed by UHPLC-HRMS in both negative and positive modes and for  
312 both dissolved and particulate phases. After XCMS processing and filtering, 156 ions in the  
313 negative mode and 144 ions were detected in the positive mode, and these were used to compare

314 the chemical fingerprints of the various seawater treatments (Fig. 5, Fig. S9). As a preliminary  
315 result, no change could be detected when comparing the chemical fingerprints of the two CS,  
316 i.e. the CS freshly pumped to perform the behavioural experiment or the CS kept for 16 hours  
317 as was the conditioned seawater SCS.

318 The chemical fingerprints of the dissolved phases obtained in both negative and positive  
319 modes resulted in the separation of +CS on one side and CS, SCS and SCE on the other side  
320 along the first axis of the PCA (Fig. 5, Table S1). Along the second axis, the chemical  
321 fingerprints recorded in the negative mode resulted in the grouping of SCS and SCE that  
322 overlapped opposite to CS (Fig. 5), whereas in the positive mode, SCS grouped with CS  
323 opposite to SCE (Fig. 5), but SCS and CS appeared different according to the post hoc  
324 permutational pairwise test (Table S1). Chemical fingerprints of particulate phases obtained in  
325 both negative and positive modes showed similar patterns with the grouping of CS with SCS  
326 separated from both SCE and +CS (Fig. 5).

327 To highlight some chemical markers potentially responsible for the crustaceans'  
328 behaviour, heatmaps of the most important features that discriminate groups in both modes and  
329 phases were constructed (Fig. 6, and Supplementary Figs. S6, S7 and S8). Many features  
330 present in SCE waters in high quantities may help to explain the crustaceans' repulsion. Among  
331 them, the brominated derivatives known from *A. cavernicola* were easily identifiable on the  
332 basis of their isotopic pattern, exact mass and in comparison with standards (Fig. S10). For  
333 example, aeroplysinin-1 (m/z 335.8871, rt 400 s, M338T401 on Fig. S7) was identified mostly  
334 in the dissolved phase and arothionin (m/z 812.8406, rt 470 s, M819T471 and M820T472 on  
335 Fig. S8, and possible stereoisomer with the same isotopic pattern at rt 500: M817T501 and  
336 M819T502, Figs. S8 and S10) was identified mostly in the particulate phase. On the contrary,  
337 only a few chemical features were found to possibly explain the attraction of *H. margalefi* by

338 both +CS and SCS. The chemical fingerprints obtained in the negative mode from the dissolved  
339 phases were the only ones separating CS from +CS and SCS/SCE (Fig. 5).

340 In the positive mode of the dissolved phase, two sets of features were tentatively  
341 annotated. The 1<sup>st</sup> set of features grouped ions present in both +CS and SCE (Supplementary  
342 Table S2, Figs. 6 and 7). Four metabolites, A (C<sub>24</sub>H<sub>44</sub>N<sub>4</sub>O<sub>4</sub>), B (C<sub>30</sub>H<sub>55</sub>N<sub>5</sub>O<sub>5</sub>), C (C<sub>36</sub>H<sub>66</sub>N<sub>6</sub>O<sub>6</sub>)  
343 and D (C<sub>42</sub>H<sub>77</sub>N<sub>7</sub>O<sub>7</sub>), were detected in this group and are likely small peptides made of leucine  
344 or iso-leucine units. Their concentrations were significantly higher in +CS, whereas the few  
345 visible differences between SCS and CS were never statistically significant (Fig. 7). The 2<sup>nd</sup> set  
346 of features grouped ions which were more concentrated in SCS and SCE, present in +CS and  
347 overall poorly represented in CS (Supplementary Table S2, Figs. 6 and 7). They were  
348 consistently significantly more concentrated in SCS than in other waters. However, although  
349 some of them were twice as concentrated in +CS than in CS, these differences were generally  
350 not supported statistically except for the metabolite L (C<sub>17</sub>H<sub>34</sub>N<sub>2</sub>O<sub>3</sub>) detected in higher  
351 concentration in SCS and +CS compared to CS (Fig. 7). Molecular formulae of the marker  
352 metabolites E (C<sub>18</sub>H<sub>36</sub>O<sub>6</sub>), F (C<sub>18</sub>H<sub>30</sub>O<sub>3</sub>), G (C<sub>18</sub>H<sub>34</sub>O<sub>5</sub>) and H (C<sub>14</sub>H<sub>26</sub>O<sub>3</sub>) were confidently  
353 assigned thanks to the high accuracy of the mass and the consistency of the molecular formulae  
354 provided by data analysis. These compounds likely belong to the oxylipin family, some  
355 derivatives of a family previously found in the marine environment (Qiao et al. 2011; Lamari  
356 et al. 2013; Lauritano et al. 2016). Halogenated metabolites harbouring the same number of  
357 carbons were also detected, I (C<sub>18</sub>H<sub>31</sub>BrN<sub>2</sub>O<sub>5</sub>), J (C<sub>18</sub>H<sub>34</sub>Cl<sub>2</sub>O<sub>4</sub>) and K (C<sub>18</sub>H<sub>33</sub>ClO<sub>4</sub>).

358

## 359 DISCUSSION

360 Distance interactions due to the putative role of chemical mediation in habitat detection  
361 by fishes and crustaceans have been previously studied among benthic ecosystems dominated  
362 by macroalgae (Lecchini et al. 2007, 2010, 2013; Lecchini et al. 2011) or seagrasses

363 (Nagelkerken et al. 2002; Arvedlund and Takemura 2006; Huijbers et al. 2008; Igulu et al.  
364 2013), in coral reefs (Lecchini et al. 2005; Lecchini et al. 2007; Huijbers et al. 2008; Ben-Tzvi  
365 et al. 2010; Lecchini et al. 2010; McCormick et al. 2010; Lecchini et al. 2011; Devine et al.  
366 2012; Coppock et al. 2013; Igulu et al. 2013; Lecchini et al. 2013; Lecchini et al. 2014) and in  
367 mangroves (Huijbers et al. 2008; Igulu et al. 2013). For some model interactions, such as clown  
368 fishes and sea anemones, their description demonstrated how these processes are crucial for  
369 ecosystem functioning, and how this mediation can be affected by global change (Arvedlund  
370 and Nielsen 1996; Arvedlund et al. 1999; Dixson et al. 2008; Munday et al. 2009; Dixson 2012;  
371 Dixson et al. 2014; Lecchini et al. 2014).

372         Here, we report for the first time clear behaviours of cave-dwelling crustaceans attracted  
373 by cave seawater (+CS) and sponge conditioned seawater (SCS), and the concomitant presence  
374 of chemomarkers in these waters. Sponges being dominant in benthic communities such as  
375 underwater caves, we hypothesized that sponges and/or their associated microbiota may be  
376 responsible for the production of chemical cues that significantly contribute to the network of  
377 chemical interactions shaping the ecosystem. Conversely to previous studies in this field, we  
378 anticipated that only part of the chemical diversity produced by these sessile invertebrates  
379 would contribute to an overall chemical sea-scape conditioning the network of interactions.  
380 Indeed, most previous studies on sponge chemical ecology used tissue homogenates or crude  
381 extracts (*i.e.* the global metabolome) to highlight their deterrent properties against predators,  
382 competitors for space or microorganisms (Bakus and Green 1974; Thompson et al. 1985;  
383 Thacker et al. 1998; Kubanek et al. 2002; Pawlik et al. 2002; Pawlik 2011). Our experiment  
384 exposing crustaceans to SCE confirmed the strong deterrent effect of seawater containing the  
385 entire sponge metabolome. Most likely, deterrent or toxic properties of specialized metabolites  
386 present in the sponges are responsible for these behavioural responses. In line with this  
387 hypothesis, aerothionin, which is one of the discriminating compounds of the SCE, and other

388 compounds of the same family had already been identified as feeding deterrents in other  
389 bioassays and may be responsible for the observed repulsive effects (Thoms et al. 2004;  
390 Gochfeld et al. 2012). Interestingly, none of the main specialized metabolites previous known  
391 in the four sponges tested were detected in the SCS or the +CS, so they do not appear to belong  
392 to what can be called the sponge exometabolome and, thus, do not contribute to the chemical  
393 seascape. So far, the exudation of major compounds has been demonstrated in the single case  
394 of the sponge *Crambe crambe* (Ternon et al. 2016), but there is crucial need for new  
395 experiments to better distinguish between the global metabolome, which is usually extracted  
396 from organisms using solvent, and the exometabolome which is the part naturally released by  
397 an organism into the surrounding water.

398       Following previous hypotheses by Bingham and Young (1991), our study demonstrates  
399 for the first time that seawater conditioned by a sponge assemblage (SCS), potentially  
400 containing components of the exometabolome only, exerts an attractant effect on some mobile  
401 invertebrate species, leading to a specific behavioural response related to their ecology.  
402 Demonstrating such a “positive” effect of sponge compounds would have been impossible with  
403 only the rather traditional use of crude extracts or pure major compounds in our experimental  
404 plan. This past strategy explains the important knowledge gap that exists concerning processes  
405 of chemical mediation within marine benthic ecosystems, in particular where ecosystems are  
406 dominated by sponges. Few studies have attempted to demonstrate the putative release of  
407 metabolites into the surrounding medium (Walker et al. 1985; Porter and Targett 1988; Duque  
408 et al. 2001; Ternon et al. 2016). After characterizing the exudation of chemical cues by the  
409 sponge *Crambe crambe*, and assessing the toxic activity of these compounds on invertebrate  
410 larvae, Ternon et al. (2016) hypothesized that this process might explain the ecological success  
411 of this encrusting sponge. Here, we provide the first demonstration that some sponge  
412 metabolites may also be involved in distance interactions, acting as kairomones to shape the

413 behaviour of mobile organisms and, thus, structuring biodiversity. We thus confirm the  
414 hypothesis of [Bingham and Young \(1991\)](#) that chemicals released by sponges have the potential  
415 to attract organisms. Moreover, we have demonstrated a species-specific response to  
416 conditioned seawater, since *H. margalefi* and *P. serratus* only showed a significant attraction  
417 for the seawater containing the exometabolome of the sponge community. The opposite  
418 response of *Leptomysis* sp. can be explained by the fact that the CS was pumped from heart to  
419 the *Leptomysis* sp. Habitat, which is dominated by macrophytes and macroalgae and where  
420 sponges are poorly distributed.

421         The responses of our cave-dwelling species support our hypothesis that chemical cues  
422 released by the sponges located at the entrance of underwater caves might be a key factor in the  
423 homing behaviour of *H. margalefi* and a refuge signal for *P. serratus*. The difference recorded  
424 between their behavioural responses is likely related to their life habits. *Hemimysis margalefi*  
425 is a cave-dweller living in swarms and is thought to migrate out of the cave for night feeding  
426 ([Rastorgueff et al. 2011](#); [Rastorgueff et al. 2015](#)). This circadian movement is presumed to be  
427 linked to the decrease in light intensity ([Passelaigue and Bourdillon 1986](#)), at least to explain  
428 how these mysids get out of the cave at the end of the day. However, our goal was to provide  
429 clues to support the idea that the return to the cave in the night or early morning may be related  
430 to the chemical seascape of the cave, through chemical cues potentially produced by sponges  
431 guiding these mysids to the entrance of their habitat. We acknowledge that we only conducted  
432 our experiments in the morning, and future experiments could be performed in darkness to  
433 mimic if the same trend is observed during the night. Interestingly, *H. margalefi* exhibited the  
434 same positive attraction when faced with the choice of the natural cave seawater and the sponge  
435 conditioned seawater containing exometabolomes. A better understanding of these migrations  
436 of cave-dwelling organisms is crucial as they represent important vectors of organic matter  
437 transfer from the outside euphotic littoral zone to various locations inside caves, where this

438 matter becomes available to other cave dwellers such as molluscs, crustaceans and teleost fishes  
439 ([Rastorgueff et al. 2011](#)). There is likely a strong link between this type of migration pattern,  
440 and how it mitigates cave oligotrophy, and the diversity of sessile organisms at the origin of the  
441 chemical seascape.

442 *Palaemon serratus* is a crustacean which may occasionally inhabit underwater caves. In  
443 this case too, we hypothesize that the sponge seascape might influence the swimming behaviour  
444 of this species and contribute to locate its habitat refuge. Interestingly, *P. serratus* spent most  
445 of its time in the downstream compartment and exhibited a much less pronounced attraction for  
446 the sponge conditioned seawater, compared to *H. margalefi*. In the case of palaemonids, the  
447 process of chemical mediation in habitat detection might not be related to gradients of  
448 waterborne molecules only. Whereas mysids are swimming crustaceans, palaemonids swim  
449 close to and walk on the bottom using their well-developed legs. They continuously explore the  
450 bottom, sensing more hydrophobic molecules with leg chemoreceptors. They also use pincers  
451 to bring fragments of substrate into contact with chemosensory mouthparts in order to follow  
452 hydrophobic cues adherent to the substrate ([Mollo et al. 2014](#)).

453 Variations of the behavioural responses with time of day differed among the species  
454 tested. The cave-dwelling mysid seemed to be much more attracted by sponge cues in the  
455 mornings, whereas the palaemonid showed no variation, which tends to confirm that their  
456 response does not rely on the same ecological process. Daily rhythm of chemosensitivity has  
457 already been reported for several arthropods, such as the Tsetse fly *Glossina morsitans*  
458 *morsitans* which presents a higher chemosensitivity in early morning and late afternoon when  
459 its feeding activity is the greatest ([Van der Goes van Naters et al. 1998](#)). The recent application  
460 of underwater electroantennography ([Machon et al. 2016](#)) offers new perspectives of  
461 chemosensitivity surveys of our model crustaceans that face various chemical cues at different  
462 period of the day. Moreover, the behavioural responses might also be correlated to the

463 expression level of some chemo-receptor such as IR25a ([Zbinden et al. 2017](#)) in order for the  
464 crustacean antennules to perceive chemical cues. For technical reason, most of our morning  
465 behavioural experiments could only be conducted during daylight hours and we believe that the  
466 combination of additional experiments performed in the darkness combined to chemosensitivity  
467 surveys might further support our hypothesis on the role of the chemical seascape on diurnal  
468 migrations of cave-dwelling crustaceans.

469         Very few studies in chemistry have attempted to identify the structure of the  
470 hypothesized marine chemical cues involved in habitat selection ([Lecchini et al. 2005](#); [Derby  
471 and Sorensen 2008](#); [Lecchini et al. 2010](#); [Lecchini et al. 2014](#)). Even though we expected to  
472 detect some of the previously known compounds of the four sponge models in the SCS, our  
473 metabolomics approach shows that none of the specialized metabolites reported so far from the  
474 global metabolome of our sponge models could be found in the SCS. Based on their molecular  
475 formulae as obtained unambiguously by HRMS, the chemomarkers present in the sponge  
476 conditioned seawater, and at low concentration in the cave seawater, correspond to C18-based  
477 oxylipins. Further confirmation of these structures is needed, however, using available  
478 standards for example. This hypothesis seems realistic as diatom oxylipins are known for their  
479 multiple functions, such as sexual pheromones or food-finding cues detectable by grazers ([Fink  
480 et al. 2006](#); [Kâ et al. 2014](#)), but also as chemical-alarm cues when facing grazing pressure  
481 ([Ianora et al. 2004](#); [Pohnert 2005](#)). Thus, the ecological function of oxylipins may be very  
482 broad. The putative oxylipins may be produced by any of the sponge holobionts ([McClintock  
483 et al. 2005](#)) and they may be responsible for the homing behaviour of crustaceans. The four  
484 peptides naturally found in high concentrations in the cave seawater are also likely produced  
485 by sponge holobionts, although in this case their very low concentrations in the sponge  
486 conditioned seawater do not provide information on their putative function in the cave chemical  
487 seascape.

488 In addition to the organic compounds in the seawater which are detectable by our  
489 metabolomics approach, we assume that inorganic compounds may also influence the  
490 biological responses of our model organisms. A significant difference in nutrient concentrations  
491 between seawater treatments would only result from the fluxes generated by the sponge  
492 holobiont catabolism (for a review see [Maldonado et al. 2012](#)), releasing ammonia, nitrate or  
493 phosphate for instance during the water conditioning. However, as the sponge volume used in  
494 our experiments represented only 1% of the aquarium volume, we can reasonably consider that  
495 there was a very low probability of a significant change in nutrient concentration during the  
496 preparation of our seawater treatments.

497 Finally, elucidating the mechanisms and strategies used in habitat detection is crucial to  
498 predict how individual species or assemblages may respond to the degradation of their  
499 environment ([Swihart et al. 2003](#); [Jones et al. 2004](#)). Changes affecting chemical mediation  
500 processes are likely to impact subsequent distribution of marine organisms. Sponges are known  
501 to be sensitive to global changes, and most of our study species have already suffered mass  
502 mortalities related to extreme climate events ([Lejeusne et al. 2010](#)). In a context of increasing  
503 frequency of such events ([Giorgi and Lionello 2008](#)), we can expect further alteration of sponge  
504 communities to impact distance interactions mediated by sponge chemical cues. Underwater  
505 caves are fragmented habitats and their biodiversity patterns are greatly shaped by their  
506 connectivity. Therefore, any alteration of the chemical seascape within and surrounding marine  
507 caves, directly or indirectly caused by human activities, could have severe consequences on  
508 these ecosystems. As has been evidenced in previous studies by [Dixon et al. \(2008, 2011\)](#),  
509 connectivity between distinct marine habitats may partly rely on chemical mediations, and not  
510 only on currents.

511

512 **AUTHOR CONTRIBUTION STATEMENT**

513 MS and TP designed the experiments. MS and MLC performed the experiments. MS,  
514 SG, MLC and OPT analysed the data. MS, SG, MLC, OPT and TP wrote the manuscript.

515

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527

## 528 **CONFLICT OF INTEREST**

529 The authors declare that they have no conflict of interest.

530

## 531 **ETHICAL APPROVAL**

532 All applicable international, national, and/or institutional guidelines for the care and use  
533 of animals were followed.

534

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760

761 **Figure legends**

762

763

764 **Fig. 1** Response of the four crustaceans to: (a) underwater cave seawater (+CS) vs. control  
765 seawater (CS), (b) sponge conditioned seawater (SCS) vs. CS, and (c) sponge chemical extracts  
766 dissolved in seawater (SCE) vs. CS. Results are mean time ( $\pm$  SE) in seconds spent by each  
767 species in the channel filled with water containing chemical cues (grey bar) and the CS channel  
768 (white bar). Paired samples *t* tests (or Wilcoxon tests) were performed to compare the time  
769 spent in each of the two upstream channels. Significant differences are indicated with \* for  $P <$   
770 0.05, \*\* for  $P <$  0.01, and \*\*\* for  $P <$  0.001. HM = *Hemimysis margalefi*, LS = *Leptomysis* sp.,  
771 PE = *Palaemon elegans*, PS = *Palaemon serratus*.

772

773 **Fig. 2** Response of *Hemimysis margalefi* and *Leptomysis* sp. to the underwater cave seawater  
774 (+CS) vs. control seawater (CS) during the morning and the afternoon. Results are mean time  
775 ( $\pm$  SE) in seconds spent by each species in the +CS (grey bar) and the CS (white bar) channels.  
776 Paired samples *t* tests (or Wilcoxon tests) were performed to compare the time spent in each of  
777 the two upstream channels. Two-sample *t* tests (or Mann-Whitney tests) were performed to  
778 compare the time spent in the channels between morning and afternoon. Significant differences  
779 are indicated with \*\* for  $P <$  0.01, and \*\*\* for  $P <$  0.001.

780

781 **Fig. 3** Response of the four crustaceans to sponge conditioned seawater (SCS) vs. control  
782 seawater (CS) during the morning and the afternoon. Results are mean time ( $\pm$  SE) in seconds  
783 spent by each species in the SCS (grey bar) and the CS (white bar) channels. Paired samples *t*-  
784 tests (or Wilcoxon tests) were performed to compare the time spent in each of the two upstream  
785 channels. Two-sample *t* tests (or Mann Whitney tests) were performed to compare the time

786 spent in the channels between morning and afternoon. Significant differences are indicated with  
787 the respective symbols \* for  $P < 0.05$ , \*\* for  $P < 0.01$ , and \*\*\* for  $P < 0.001$ .

788

789 **Fig. 4** Response of the four crustaceans to sponge chemical extracts dissolved in seawater  
790 (SCE) vs. control seawater (CS) during the morning and the afternoon. Results are mean time  
791 ( $\pm$  SE) in seconds spent by each species in the SCE (grey bar) and the CS (white bar) channels.  
792 Paired samples  $t$  tests (or Wilcoxon tests) were performed to compare the time spent in each of  
793 the two upstream channels. Two-sample  $t$  tests (or Mann Whitney tests) were performed to  
794 compare the time spent in the channels between morning and afternoon. Significant differences  
795 are indicated with \* for  $P < 0.05$ , and \*\*\* for  $P < 0.001$ .

796

797 **Fig. 5** Principal Component Analysis (PCA) based on the chemical features of the different  
798 seawater samples analysed by UHPLC-MS in negative (a, c) and positive (b, d) modes, for the  
799 dissolved (a, b) and particulate (c, d) phases. A permanova test (PPLS-DA models, 999  
800 permutations with double-cross validation) was performed to test differentiation between  
801 chemical fingerprints (post hoc permutation pairwise test in Supplementary file S). The  
802 classification error rate (CER, 0 – 1) was calculated to estimate the strength of the generated  
803 models. CS: control seawater; +CS: cave seawater; SCS: sponge conditioned seawater; SCE:  
804 sponge chemical extract; p: particulate phase; d: dissolved phase.

805

806 **Fig. 6** Heatmap based on the variable importance in projection of the features ( $VIP > 1$ ) detected  
807 in the metabolic fingerprints of the dissolved phase of the seawater samples analysed by liquid  
808 chromatography coupled to mass spectrometry in positive mode. X-axis displays the different  
809 seawater treatments, Y-axis displays metabolite features coupling nominal mass (M as atomic  
810 mass unit) with chromatographic retention time (T in seconds). Connections of samples and

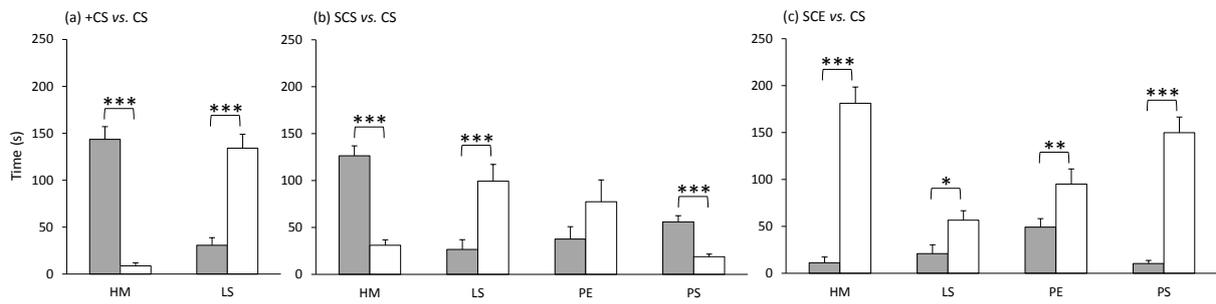
811 metabolic features are based on hierarchical clustering (Euclidian distances). The first group of  
812 features gathers ions present in +CS and SCE, the 2<sup>nd</sup> group of features gathers ions present in  
813 SCS and SCE. CS: control seawater; +CS: cave seawater; SCS: sponge conditioned seawater;  
814 SCE: sponge chemical extract.

815

816 **Fig. 7** Comparative ion intensities of the features corresponding to the chemical cues A–D (1st  
817 group of features that gathers ions present in +CS and SCE) and E–N (2nd group that gathers  
818 ions present in SCS and SCE) in the dissolved phase of the seawater samples analysed by liquid  
819 chromatography coupled to mass spectrometry in positive mode. The metabolites E, F, G and  
820 H likely belong to oxylipin family. Kruskal Wallis tests followed by a post hoc multiple range  
821 test (Fisher's Least Significant Difference),  $n=4$ : significant differences are indicated with  
822  $*P < 0.05$ , and  $**P < 0.01$ , and differences between treatments are shown by different letters.  
823 CS control seawater; +CS cave seawater; SCS sponge conditioned seawater; SCE sponge  
824 chemical extract

825 **Fig. 1**

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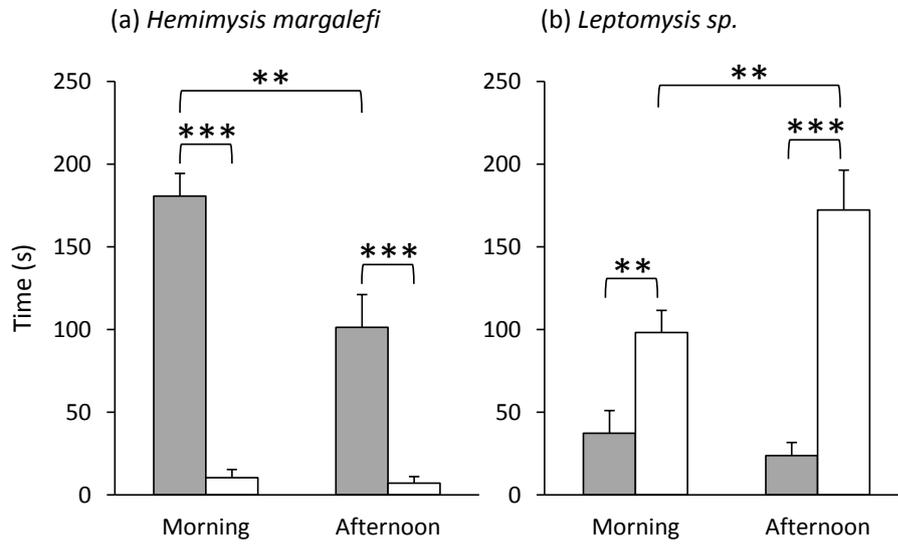


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829 **Fig. 2**

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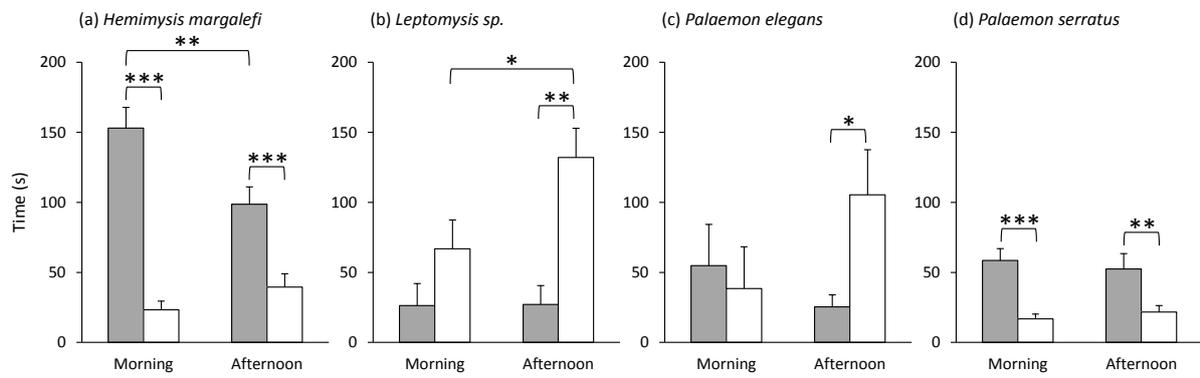


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833 **Fig. 3**

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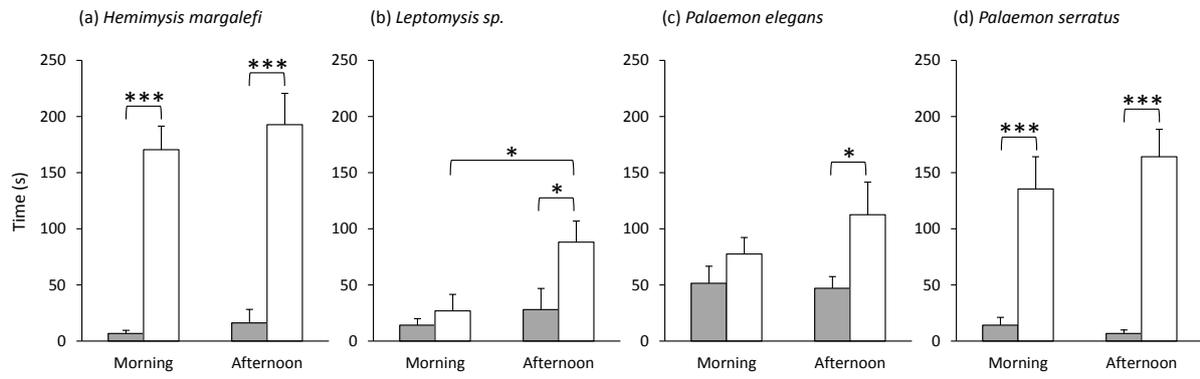


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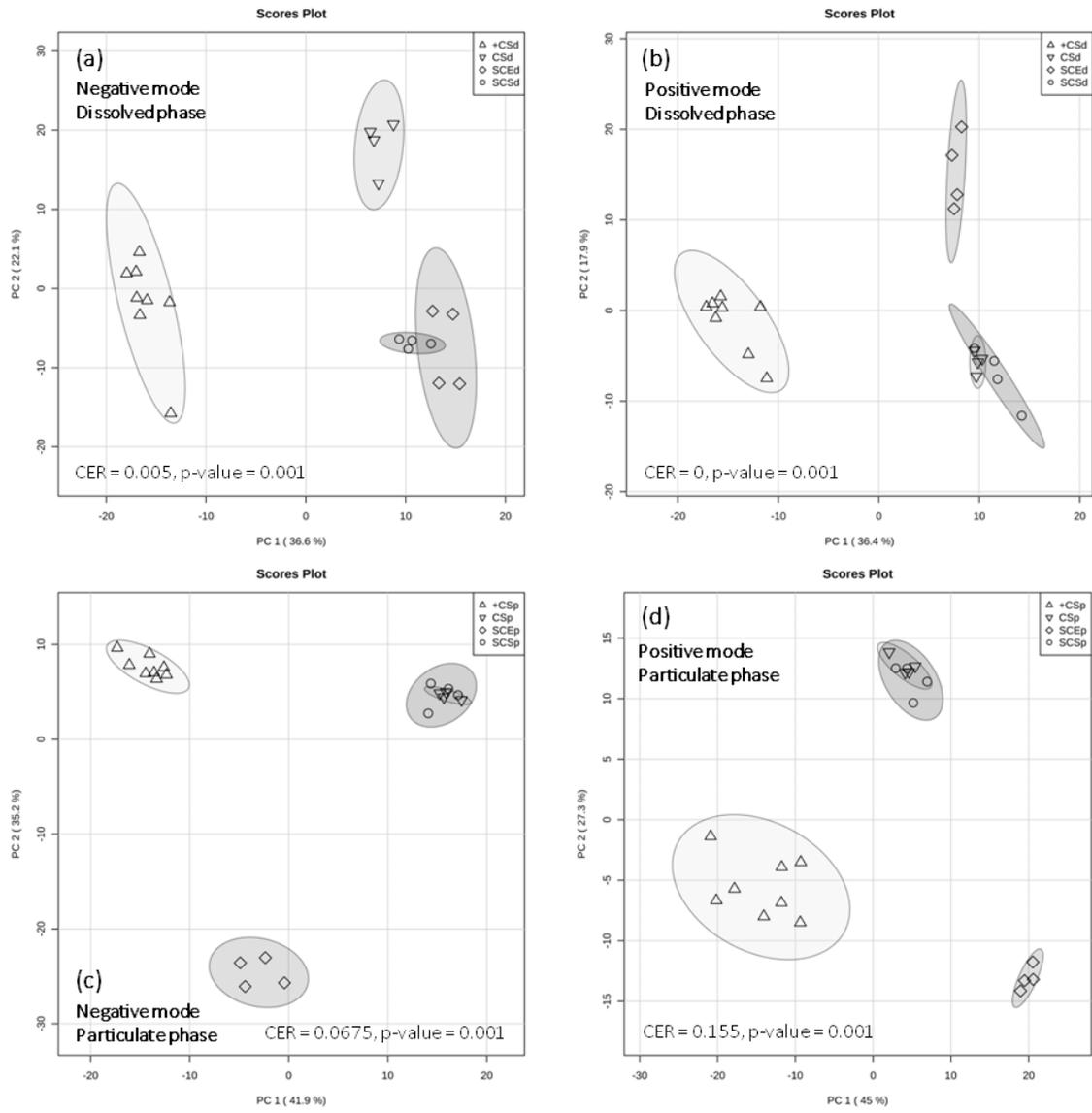
837 **Fig. 4**

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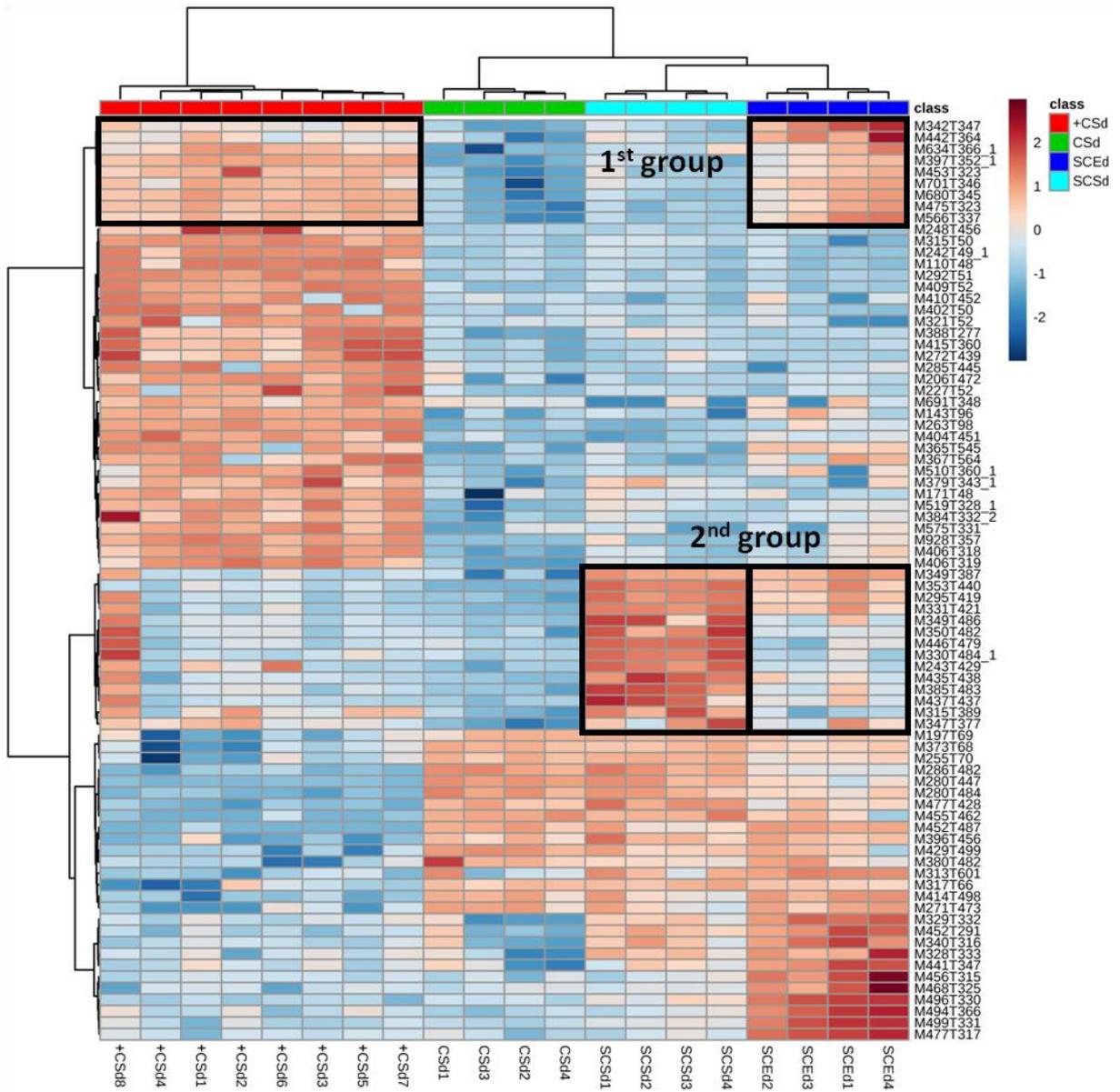
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