1 **Behavioural stress feedback loops in benthic invertebrates caused by pH drop-induced metabolites**

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Abstract

9

10   Studies on pH stress in marine animals typically focus on direct or species-specific aspects. We here test the

11   hypothesis that a drop to pH = 7.6 indirectly affects the intra- and interspecific interactions of benthic

12   invertebrates by means of chemical communication. We recorded fitness-relevant behaviours of small hermit

13   crabs *Diogenes pugilator*, green shore crabs *Carcinus maenas*, and harbour ragworms *Hediste diversicolor* in

14   response to short-term pH drop, and to putative stress metabolites released by conspecifics or gilt-head sea

15   bream *Sparus aurata* during 30 minutes of acute pH drop. Not only did acute pH drop itself impair time to find

16   a food cue in small hermit crabs and burrowing in harbour ragworms, but similar effects were observed under

17   exposure to pH drop-induced stress metabolites. Stress metabolites from *S. aurata,* but not its regular control

18   metabolites, also induced avoidance responses in all recipient species. Here, we confirm that a short-term

19   abrupt pH drop, an abiotic stressor, has the capacity to trigger the release of metabolites which induce

20   behavioural responses in conspecific and heterospecific individuals, which can be interpreted as a behavioural

21   cost. Our findings that stress responses can be indirectly propagated through means of chemical

22   communication warrant further research to confirm the effect size of the behavioural impairments caused by

23   stress metabolites and to characterise their chemical nature.

24

25   **Keywords:** behavioural cost, stress propagation, stress metabolites, chemical communication, ocean

26   acidification

Introduction

28 Compared to the open ocean, coastal areas and particularly intertidal zones are highly variable environments
29 characterised by abrupt changes in water parameters. This includes fluctuations in pH beyond 0.3 units, the
30 levels predicted for average change related to the process of ocean acidification towards the end of the century
31 (Caldeira and Wickett, 2003; Sabine et al., 2004; IPCC, 2019). While this may mean that intertidal species are
32 more resilient to climate change due to their acquired tolerances of pH fluctuations, it also forces them to live
33 more frequently near their physiological tolerance limits (Truchot and Duhamel-Jouve, 1980; Hofmann et al.,
34 2011; Sokolova, 2018; Wolfe et al., 2020). Superimposing ocean acidification on natural pH fluctuations may
35 further increase the variability of organisms' responses (Eriander et al., 2015). Therefore, organisms inhabiting
36 intertidal areas are interesting models to study the effects of short-term pH fluctuations within the IPCC
37 predicted range. A low environmental pH can alter animal behaviour through several direct or indirect
38 pathways, which include (i) deviation of energy budgets towards the stress response (Pörtner, 2008), (ii) fleeing
39 to avoid the sources of stress (Pörtner and Peck, 2010; Abreu et al., 2016), (iii) disrupted information detection
40 and processing leading to impaired decision-making (Briffa et al., 2012), and (iv) alteration of the chemical
41 signals themselves impacting the sensory environment and the transfer of information (Wyatt et al., 2014;
42 Roggatz et al., 2016, 2019). Behavioural effects triggered by lowered pH are known to occur in different
43 taxonomic groups such as crustaceans (de la Haye et al., 2011), marine ragworms (Bond, 2018), and fish
44 (Munday et al., 2009), although recent research debate on both their ubiquitousness and their effect size (Clark
45 et al., 2020a; Clements et al., 2020b, but see Clark et al., 2020b; Munday et al., 2020).

46 In aquatic environments, where visibility can be limited, infochemicals and chemosensory functions, such as
47 pheromones used for mating (Karlson and Lüscher, 1959) are crucial for communication (Hardege, 1999;
48 Jordão and Volpato, 2000; Hay, 2009; Ashur et al., 2017). However, they are also a mechanism to propagate
49 stress – but to date mostly researched within the context of biotic stressors. For example, alarm cues released
50 following mechanical damage from fish epidermal club cells can trigger panic reactions in conspecifics and
51 heterospecifics (Toa et al., 2004; Júnior et al., 2010). Such mechanisms of chemical communication also occur
52 in invertebrates, as evidenced by the reduced out-of-burrow activity of the marine polychaete *Alitta virens*
53 exposed to damaged conspecifics (Watson et al., 2005; Ende et al., 2017). Disturbance cues refer to chemicals
54 that may be stored in gill epithelium or urine, and are released voluntarily or involuntarily by disturbed or
55 stressed prey, to induce early antipredator responses in recipients to anticipate potential threats (Bairos-Novak
56 et al., 2017; Goldman et al., 2020a). The central role of chemical communication in the aquatic environment
57 and the recent evidence of its impairment by ocean acidification pinpoints the need for a deeper understanding
58 of such potential environmental modulation of chemical signalling (Chivers et al., 2013).

59 Overall, though, it is not well understood yet whether abiotic stressors such as pH drop can also induce the
60 release of chemical cues and whether these can propagate the stress response to other individuals. For such
61 cues, we here introduce the term '*stress metabolites*' as defining such compounds released, voluntarily or not,
62 by an organism in response to abiotic stressors. These can be detected and processed by conspecifics and/or
63 heterospecifics, leading to the induction of a behavioural stress response (Hazlett, 1985). Such a mechanism

64   of indirect stress propagation might constitute positive feedback loops, defined as the propagation of stress

65   responses within or between species by means of stress metabolites. Detecting stress metabolites may allow

66   other individuals to modify their behaviour to avoid a change in the abiotic environment, and communities to

67   coordinate or potentiate their response to ensure survival (Mothersill et al., 2007; Giacomini et al., 2015; Abreu

68   et al., 2016). However, to investigate the function of any pH drop-induced chemical cues, the original stressor

69   (pH drop) must first be removed from the experimental design.

70   In this study, we investigate the indirect effects of pH drop through stress metabolites induced by it on fitness-

71   relevant behaviours within and among species inhabiting the intertidal zone. Using a full factorial design, we

72   test the hypothesis that an acute pH drop to end-of-century level (7.6) will both directly and indirectly affect

73   behaviour through the induction of stress metabolites. We observed three marine invertebrates (small hermit

74   crab *Diogenes pugilator*, green shore crab *Carcinus maenas*, and harbour ragworm *Hediste diversicolor*)

75   exposed to control pH and pH drop in combination with conditioned water from both conspecifics and their

76   potential vertebrate predator, the gilt-head sea bream *S. aurata*.

Methods

Experimental design

80   Small hermit crabs *Diogenes pugilator* and green shore crabs *Carcinus maenas* were collected in autumn at

81   low tide in the Ria Formosa lagoon (Portugal). Harbour ragworms (*Hediste diversicolor*) were sourced via a

82   local supplier (Valbaits) from the Setubal lagoon (Portugal). Animals were acclimated for one week at pH 8.2

83   in large communal tanks mimicking natural habitats at the Ramalhete Marine Station (CCMAR, Faro,

84   Portugal) in a direct CO_2-controlled system with pCO_2 constantly measured and adjusted as described in Sordo

85   et al. (2016). Seawater parameters were measured daily (mean temperature: $18.90°C \pm 0.97°C$, mean salinity:

86   35.58 ± 0.19, mean dissolved oxygen: $91.60\% \pm 2.15\%$). A total pool of approx. 100-120 *D. pugilator*, 150-

87   200 *C. maenas*, and over 150 *H. diversicolor* was used during the experimental period, yielding a total of 921

88   observations. Because no individual marking could be achieved, we aimed to space out the reuse of animals in

89   successive behavioural assays by circulating animals through recovery tanks afterwards, before returning them

90   to their stock population tanks once a day. Animal reuse was randomized across treatments and treatments

91   randomized per day to prevent any confounding effects on the measured behaviours. All experiments were

92   conducted in October 2019, except one independent set of observations (n = 40) in *C. maenas* conducted in

93   September 2018, which was analysed together with the 2019 data with the year treated as a covariate.

94   A three-way factorial design of pH drop x stress metabolites x donor species was generated to study the effects

95   of acute pH drop, stress metabolites induced by it, and their combination versus a control containing regular

96   metabolites (control metabolites) (Figure 1). Metabolite donor species were *D. pugilator*, *C. maenas*, *H.*

97   *diversicolor* (called conspecific donors) and the potential predator *Sparus aurata* (called heterospecific donor).

98   Metabolite donors were conditioned for 30 minutes in seawater at regular pH (pH 8.2, 400 µatm CO_2,

99   putatively inducing control metabolite release), or pH drop (pH 7.6, 700 µatm CO_2, putatively inducing stress

100   metabolite release). Recipient species were *D. pugilator, C. maenas,* and *H. diversicolor* which received water

101 containing either conspecific or heterospecific metabolites). To achieve the full factorial design, recipient

102 species received either control or stress metabolites and were tested in either control pH (8.2) or pH drop (pH

103 = 7.6), by re-adjusting the pH of the conditioned water before each bioassay. Overall, four different

104 experimental treatments were obtained: (i) putative control metabolites at pH 8.2 (CM), (ii) putative control

105 metabolites at pH 7.6 (pH drop), (iii) putative stress metabolites at pH 8.2 (SM), (iv) and putative stress

106 metabolites at pH 7.6 (pH drop+SM). Testing conspecific and heterospecific metabolites with three recipient

107 species amounted to a total of twelve treatments. Prior to use in recipient species, conditioned seawater from

108 *H. diversicolor* and *S. aurata* (0.0067 fish/L) was tenfold diluted in fresh system seawater at the desired pH.

109 To explore the possibility that stress metabolites are equivalent to highly concentrated control metabolites, we

110 additionally tested the effects of undiluted *S. aurata* control metabolites at pH 8.2 in *H. diversicolor* and *D.*

111 *pugilator*. See supplementary information for further details on treatments (Table S1) and water conditioning

112 (supplementary methods).

113

114 *Behavioural bioassays and respirometry*

115 For each of the four conditions, we measured the time to find a feeding cue (1/10 diluted mussel juice) *vs.* a

116 mock (seawater) cue in *D. pugilator* and *C. maenas*, or to bury the head entirely in the sand in *H. diversicolor*,

117 and dubbed this variable 'time-to-success'. Behavioural assays were terminated once the animal successfully

118 grabbed the ballasted sponge containing the feeding cue with their pincers (*D. pugilator* and *C. maenas*) or

119 buried its entire head (*H. diversicolor*), or at a maximum time of 300 seconds. Both feeding and burrowing

120 behaviours are tested response variables in crabs (de la Haye et al., 2012; Wang et al., 2018) and ragworm

121 (Bhuiyan et al., 2021) exposed to pH drop. Additionally, avoidance behaviour was binary coded. In *D.*

122 *pugilator* and *C. maenas*, these avoidance behaviours included freezing (suddenly retreating into the shell or

123 attempting to burrow, sudden and lasting arrest in locomotion), and escaping (walking along the walls or

124 retreating into a corner of the tank). Such freeze and escape behaviours indicate danger in crustaceans (Katz

125 and Rittschof, 1993; Perrot-Minnot et al., 2017; Tomsic et al., 2017). In *H. diversicolor*, avoidance behaviours

126 consisted of freezing, which might be accompanied by spread of jaws, sideways-undulating behaviour, and

127 formation of a noticeable slime cap whilst outside the sediment. These behaviours indicate extreme stress in

128 marine polychaetes (Mouneyrac et al., 2003; Burlinson and Lawrence, 2007; McBriarty et al., 2018). The

129 feeding cues were randomly assigned left or right to the crabs. Additionally, respiration rates of *C. maenas*

130 were monitored immediately after their use in feeding behaviour assays in a two-way factorial design of pH

131 drop x stress metabolites using only conspecific donor metabolites. See supplementary methods for further

132 details on behavioural assays and respirometry.

133

134 *Statistical analysis*

135 Avoidance behaviours were analysed using generalised linear models for logit regression with binomial

136 distribution using the *stats* R package (R Core Team, 2020). Time-to-success was modelled for the time to

137 reach a feeding cue in *D. pugilator* and *C. maenas* or to burrow the head in *H. diversicolor* depending on the

138   three predictors: pH drop, stress metabolites, and donor. Time-to-success data was analysed with a time-to-
139   event analysis (also called survival analysis) using Cox proportional hazard models from the *survival* R
140   package (Therneau and Grambsch, 2000; Therneau, 2021). The event being the success of food cue location
141   (in crabs) or burrowing the head in sediment (in worms), the analysis was hereafter referred to as 'time-to-
142   success analysis'. The exponentiated estimates (hazard ratios) from the Cox proportional hazard models were
143   expressed as 'success ratio'. Success ratios were visualised using the *plot_model* function from the *sjPlot* R
144   package (Lüdecke, 2021). The 'success probability' (the probability of the feeding or burrowing event
145   occurring at any time over 300s) over time was represented by Kaplan-Meier curves drawn using the *survminer*
146   R package (Kassambara et al., 2020). Animals not reaching the feeding cue or not burying their head were
147   censored and assigned the maximum time of '300+'. The oxygen measurements were transformed using the
148   additive-inverse slope coefficients standardised to the carapace width and rescaled to the mean additive-inverse
149   slope coefficient of the control CM as shown in equation (1) to yield respiration rates.

150   $respiration\ rate = (-a/size)/(mean - a\ of\ control)$ Equation (1)

151   where a is the slope of the oxygen levels (%) as a function of time (s) and the size is proxied by the carapace
152   width.

153   All statistical models always included the main effects and interaction terms of the binary predictors pH drop
154   (pH = 8.2 vs. pH = 7.6), stress metabolites (stress metabolites vs. control metabolites), and donor (conspecific
155   vs. heterospecific). Covariates (year, number of water uses, crab size, where relevant) were included in models
156   when deemed significant compared to the null model by an ANOVA Chi-square test from the *stats* R package
157   (R Core Team, 2020). Overall model fit p-values were retrieved from the ANOVA Chi-squared test or the
158   Likelihood ratio test for generalised linear models and Cox-proportional models, respectively. Pairwise
159   comparisons between treatments involved in the interaction term between the three predictors were obtained
160   from statistical models using the *emmeans* R package (Lenth, 2021), wherein false discovery rate p-value
161   adjustments were applied for post-hoc term-wide multiple testing.

162   To investigate the possibility that stress metabolites are equivalent to highly concentrated control metabolites
163   (which were present in CM and pH drop treatments but in tenfold dilution), additional tests compared the
164   behavioural response of *D. pugilator* and *H. diversicolor* to tenfold diluted stress metabolites and control
165   metabolites to that of undiluted control metabolites from *S. aurata*.

166   Effect sizes of the respiration rates were measured as Cohen's d using the effsize R package (Torchiano, 2020)
167   for estimates between two groups, or according to Lenhard and Lenhard (2016) for estimates of the interaction
168   term between pH drop and stress metabolites predictors. Effect sizes were classified following the
169   classification given in Sawilowsky (2009). All statistical analyses were conducted in RStudio (RStudio Team,
170   2020) with a significance threshold of P ≤ 0.05. See R script, datasets, and supplementary information for
171   further details.

172   **Results**

173   *Response of small hermit crab* **Diogenes pugilator** *to pH, stress metabolite, and donor predictors*

174   The time-to-success analysis was not conclusive for the stress metabolite predictor in *D. pugilator* (Figure 2A,

175   Table 1, $Z = -0.66$, $P = 0.5073$). On the other hand, the Cox proportional hazard model (overall Likelihood

176   ratio test model fit: $P = 0.06$) found that both pH drop (feeding success ratio = 0.47, $Z = -2.14$, $P = 0.0325$) and

177   metabolites from *S. aurata* (feeding success ratio = 0.35, $Z = -2.76$, $P = 0.0058$) had significant negative effects

178   on the feeding success ratio of *D. pugilator*. In addition, there was a significant interaction between pH drop

179   and metabolite donor terms on the feeding success of *D. pugilator* ($Z = 2.19$, $P = 0.0289$). Overall, pH drop

180   induced a significantly lower success (risk) score in the conspecific treatment (Figure 2B). Post-hoc analyses

181   evidenced that all treatments (CM, pH drop, SM, and pH drop+SM) induced similar feeding time responses in

182   both the conspecific and heterospecific donor groups (Table S2).

183   Avoidance responses of *D. pugilator* did not depend on pH drop ($Z = 0.25$, $P = 0.8049$) nor stress metabolites

184   ($Z = -0.25$, $P = 0.7995$, overall Chi-square test model fit: $P < 0.0001$, Table 2), but were significantly more

185   pronounced when metabolites originated from *S. aurata* (69%) instead of conspecifics (26%, $Z = 2.46$, $P =$

186   0.0139, Figure 3, Table 2). After splitting the display of avoidance behaviour by donor, pairwise comparisons

187   failed to find differences between the four treatments (CM, pH drop, SM, and pH drop+SM), but confirmed

188   that the donor effect existed across all four treatments (Table S3, Figure 3). However, partitioning the

189   avoidance behaviour of *D. pugilator* into freezing and escaping responses evidenced that escaping significantly

190   increased with *S. aurata* control metabolites only when tested in pH drop (Figures S1-S2, Tables S4-S7, see

191   supplementary results).

192

193   *Response of green shore crab* **Carcinus maenas** *to pH, stress metabolite, and donor predictors*

194   The Cox proportional hazard model (overall Likelihood ratio test model fit: $P = 0.009$) showed that the feeding

195   success ratio of *C. maenas* did not significantly vary with pH ($Z = -0.80$ $P = 0.4247$), metabolites ($Z = 0.68$, P

196     $= 0.4989$), nor donor ($Z = 1.09$, $P = 0.2773$) terms (Figure 2, Table 1). Nevertheless, all interaction terms were

197   significant. Post-hoc tests revealed that time-to-success responses were similar across treatments CM, pH drop,

198   SM, and pH drop+SM when *C. maenas* received conspecific metabolites (Table S8). This pattern changed

199   when *S. aurata* was the metabolite donor as evidenced by significantly lower feeding success scores in *C.*

200   *maenas* exposed to pH drop ($Z = 2.97$, $P = 0.0179$), SM ($Z = 2.48$, $P = 0.0394$), and the trend for pH drop+SM

201   ($Z = 2.20$, $P = 0.0563$), compared to the control CM.

202   In *C. maenas*, the predictors pH drop ($Z = -1.23$, $P = 0.2195$), stress metabolites ($Z = -1.23$, $P = 0.2195$), and

203   donor ($Z = -1.23$, $P = 0.5785$) did not alter avoidance responses but their interaction terms were significant

204   (overall Chi-square test model fit: $P < 0.0001$, Table 2, Figure 3). Pairwise comparisons showed no differences

205   between the four treatments (CM, pH drop, SM, pH drop+SM) in avoidance patterns of *C. maenas* receiving

206   conspecific metabolites. Conversely, *C. maenas* facing *S. aurata* metabolites while experiencing treatments of

207   pH drop (94%, $Z = -3.60$, $P = 0.0020$), SM (75%, $Z = -3.28$, $P = 0.0031$), and pH drop+SM (56%, $Z = -2.33$,

208     $P = 0.04$) significantly increased their avoidance display compared to control CM (18%). Moreover, pH drop

209 combined with stress metabolites (pH drop+SM treatment) instead of control metabolites (pH drop treatment)

210 significantly lowered avoidance behaviours (Z = 2.21 P = 0.0405, Table S9, Figure 3). Partitioning the

211 avoidance behaviour of *C. maenas* into freezing and escaping responses evidenced that escaping explained the

212 avoidance response whereas no clear patterns were observed in the freezing behaviour (Figures S1-S2, Tables

213 S10-S13, see supplementary results). Lastly, neither pH drop nor stress metabolites altered the respiration rates

214 of *C. maenas* although the interaction was near to significant (P = 0.0531, Figure S3, Table S14).

215

216 ***Response of harbour ragworm* Hediste diversicolor *to pH, stress metabolite, and donor predictors***

217 Burrowing success responses of *H. diversicolor* did not depend on the donor species (P = 0.1093, Figure 2,

218 Table 1). However, the Cox proportional hazard model (overall Likelihood ratio test model fit: P < 0.0001)

219 evidenced that both pH drop (burrowing success ratio = 0.30, Z = -3.91, P < 0.0001) and stress metabolites

220 (burrowing success ratio = 0.35, Z = -3.42, P = 0.0006) terms significantly altered burrowing time responses

221 of *H. diversicolor*. Significant interactions between the main predictors required to decipher the involvement

222 of within-donor treatment effects (Table S15). Post-hoc tests revealed that pH drop (Z = 3.91, P = 0.0006), SM

223 (Z = 3.43, P = 0.0012), and pH drop+SM (Z = 3.47, P = 0.0012) treatments significantly lowered burrowing

224 success scores compared to the control CM in *H. diversicolor* exposed to conspecific metabolites. Moreover,

225 *H. diversicolor* facing *S. aurata* metabolites had lower burrowing success scores in the pH drop+SM treatment

226 compared to the control CM (Z = 3.35, P = 0.0049).

227 *H. diversicolor* displayed more avoidance in response to *S. aurata* metabolites (47%) compared to conspecific

228 metabolites (20%, Z = 1.09, P = 0.0006, overall Chi-square test model fit: P < 0.0001, Table 2, Figures 3). *H.*

229 *diversicolor* exposed to *S. aurata* metabolites were not affected by pH drop (Z = -0.01, P = 0.9906) whereas

230 stress metabolites induced significantly more avoidance than control metabolites (Z = -5.30, P < 0.0001, overall

231 Chi-square test model fit: P < 0.0001, Table 2). There were no differences across pH drop, SM, and pH

232 drop+SM treatments in *H. diversicolor* receiving conspecific metabolites (Table S16). Pairwise comparisons

233 revealed that the SM treatment (93%) triggered significantly more avoidance than CM (33%, Z = -4.31, P =

234 0.0001) and pH drop+SM treatments (51%, Z = 3.92, P = 0.0003). Specimens of *H. diversicolor* exposed to *S.*

235 *aurata* metabolites displayed different avoidance responses in the control treatment CM (video 1) compared

236 to pH drop (slowed-down burrowing response, video 2), SM (sideways-undulating behaviour with body flips,

237 video 3), and pH drop+SM (raised head and spread mouth parts with freezing behaviour in video 4 and slowed-

238 down movement with sideways undulating behaviour in video 5).

239

240 **Discussion**

241 ***Direct effects of pH drop and donor type on assay success and avoidance behaviours***

242 Changes in fitness-relevant behaviours to acute pH change can be expected to show high variance and small

243 effect sizes (Clark et al., 2020a), and may therefore require large observational datasets. In this study, we

244 performed over 900 behavioural observations, and our results reflect this high variance. *Sparus aurata*-

245 conditioned water, whether it contained control metabolites or pH stress-induced metabolites, elicited

246 avoidance in all recipient species which can be attributed to antipredator behaviours. Despite the donor effect

247 however, much of the behavioural variance was explained by the effects of pH drop and stress metabolites,

248 their interaction, or their interaction with the donor term (Figure 2).

249 We found that pH drop lowered success ratios in feeding and burrowing in *Diogenes pugilator* and *Hediste*

250 *diversicolor*, respectively. Such low pH-induced impairments of fitness-relevant behaviours were previously

251 found in hermit crabs *Pagurus bernhardus* and *P. tanneri* and may indicate impaired decision-making (de la

252 Haye et al., 2011, 2012; Kim et al., 2015). Past literature also reported delayed burrowing responses in the

253 harbour ragworm *H. diversicolor* exposed for 28 days to pH drop (Bhuiyan et al., 2021), and an inverse

254 relationship between burrowing and pH in the king ragworm *Alitta virens* (Batten and Bamber, 1996). These

255 behavioural alterations may reflect the cost of acid-base regulation to counteract hypercapnia (Pörtner, 2008),

256 as marine ragworms overexpress the acid-base regulator *Carbonic Anhydrase* (*CA*) at the expense of energy

257 reserves in response to acidification (Freitas et al., 2016; Wage et al., 2016). In the longer term, behavioural

258 costs of an altered burrowing activity may extend to crucial ecological functions as shown by the diminished

259 bioturbation activity of *H. diversicolor* in similar conditions (Bond, 2018). In contrast, *Carcinus maenas* did

260 not delay its feeding response to pH drop in presence of conspecific metabolites, which supports recent

261 observations of ocean acidification having weaker effects than previously expected on marine animal

262 behaviour (Clark et al., 2020a; Clements et al., 2020a, 2020b, 2021) including feeding responses in aquatic

263 arthropods (Clements and Darrow, 2018). The resilience to pH stress in *C. maenas* may be due to their

264 extracellular pH regulatory capacities allowing them to maintain feeding during short-term assays (Appelhans

265 et al., 2012). Therefore, short-term hypercapnia is not a strong stressor in *C. maenas*, due to its pH-variable

266 intertidal habitat (Fehsenfeld et al., 2011), which is supported by its ability to maintain respiration rates in our

267 study. Other than reduced success ratios, pH drop itself did not induce any avoidance behaviours indicative of

268 stress.

269 Besides the direct effect of pH drop on behaviour, previous studies on ocean acidification also found

270 contrasting effects of pH drop on predator-prey interactions (Draper and Weissburg, 2019). Here, we observed

271 an increased escaping tendency of *C. maenas* in response to *S. aurata* control metabolites in pH drop but not

272 in control pH. Although this was not the main focus of our study, it could mean that pH drop renders predator

273 odour more potent for its prey, for example through pH-dependent changes in odour molecular structure,

274 receptor binding, or information processing (Munday et al., 2009; Roggatz et al., 2016, 2019; Schirrmacher et

275 al., 2020).

276

Indirect effects of pH drop on recipients' behaviour through stress chemical communication

278 Despite the above-mentioned species-specific direct effects of pH drop on behaviour success ratios, we could

279 show here that pH drop also has indirect effects on behaviour by altering chemical communication. We found

280 that stress metabolites released by animals exposed to pH drop altered feeding and burrowing, and increased

281 avoidance behaviours, but that the response was donor and recipient species-specific. Hermit crabs *D.*

282 *pugilator* did not react to any stress metabolites. Significant responses in the other two recipient species,

283 however, were directionally equivalent to those induced by pH drop itself since it took longer to find a food
284 cue or to burrow the head in the presence of stress metabolites. Although behavioural stress response
285 propagation through chemical communication is well documented for alarm substances and disturbance cues
286 upon predation stress (Giacomini et al., 2015; Abreu et al., 2016; Mathuru, 2016), our results show here that
287 chemical communication induced by abiotic stressors such as pH drop can trigger the release of waterborne
288 chemicals that in turn trigger a behavioural stress response in its recipients. Stress metabolites conditioned by
289 the potential predator *S. aurata* induced more avoidance behaviours and delayed feeding in *C. maenas* and
290 burrowing in *H. diversicolor,* compared to *S. aurata* control metabolites. Similarly, the presence of conspecific
291 stress metabolites significantly lowered *H. diversicolor* burrowing success ratio compared to conspecific
292 control metabolites.

293 On the other hand, *C. maenas* did not react to conspecific stress metabolites. The different behavioural effect
294 of conspecific stress metabolites between crabs and *H. diversicolor* has several possible explanations ranging
295 from different defence strategies related to stress risk perception and evaluation (Hazlett, 1985; Bairos-Novak
296 et al., 2017, 2018; Goldman et al., 2020b) to different types, concentrations, or ratios of the released chemicals
297 (Júnior et al., 2010; Morishita and Barreto, 2011). As we have shown here a pH drop to 7.6 is not a strong
298 stressor in crabs (Fehsenfeld et al., 2011) which may explain that we failed to observe responses to water from
299 pH drop-conditioned conspecific green shore crabs. However, the presence of stress metabolites conditioned
300 by *S. aurata* caused a marked drop in the success ratio of *C. maenas* at low (but not control) pH, evidenced by
301 significant interaction terms.

302 Whilst we did not investigate their chemical structure, our experiment allows us to characterize novel
303 properties of stress metabolites. Stress metabolites, like disturbance cues, potentially consist of regularly
304 excreted metabolites such as urea and ammonia (Bairos-Novak et al., 2017; Shrivastava et al., 2019). The fact
305 that behavioural responses of *H. diversicolor* and *D. pugilator* remained unchanged even when we used
306 undiluted control metabolites (Tables S17-S18), might mean that stress metabolites are not just up-
307 concentrated control metabolites. We also observed that avoidance behaviours by both *C. maenas* and *H.*
308 *diversicolor* became less pronounced with subsequent water uses, showing that the cues are either volatile or
309 have a very short half-life (less than half a day) in seawater. Alternatively, recipients may be able to
310 discriminate degraded versus fresh metabolites and react accordingly to the degree of threat they may indicate
311 (Fuselier et al., 2009; Bairos-Novak et al., 2018). This shows that stress metabolites induced by pH drop are
312 either not identical with metabolites released in normal pH, and/or are at least more than tenfold higher
313 concentrated than control metabolites.

314 The avoidance behaviours we recorded in this study are known indicators of stress. *S. aurata* stress metabolites
315 elicited atypical freezing, eversion of the proboscis, mucus secretion, flipping and sideways-undulating
316 behaviours in *H. diversicolor* which were previously described as indicators for a physiological stress response
317 following exposure to copper sulphate (Burlinson and Lawrence, 2007), antiparasitic drugs (McBriarty et al.,
318 2018), and trace metals (Mouneyrac et al., 2003). We also observed increased freeze and escape responses in

319   crabs, which indicate a stress response in crustaceans (Katz and Rittschof, 1993; Perrot-Minnot et al., 2017;

320   Tomsic et al., 2017).

321   The finding that *S. aurata* pH-induced stress metabolites induced such responses in potential prey species

322   suggests that responses to disturbance cues are not depending on the 'audience', similar as what has been

323   observed in tadpoles (Bairos-Novak et al., 2020). In our study, stress metabolites from *H. diversicolor* altered

324   the burrowing success of conspecifics whereas Watson and colleagues (2005) found that whole-body extracts

325   of *H. diversicolor* did not alter out-of-burrow activities of the king ragworm *Alitta virens* (of the same

326   subfamily Nereidinae), indicating that stress metabolites may also differ from alarm cues.

327   A directionally similar response to pH drop and to the stress metabolites it induces shows that pH drop can

328   have indirect effects, reminiscent of a positive feedback loop, by which stressed animals negatively influence

329   fitness-relevant behaviours of community members. This was first observed by Hazlett (1985) after freshwater

330   crayfish exposed to water conditioned with heat-stressed conspecifics displayed increased alertness, after

331   returning the conditioned water to normal temperature. Similar indirect effects through chemical signalling

332   from stressed donors to unstressed recipients were also shown in response to different types of biotic stressors

333   such as mock predator chase (Toa et al., 2004; Giacomini et al., 2015), handling (Barcellos et al., 2011), and

334   acute fasting (Abreu et al., 2016), and to physical injury including irradiation (Mothersill et al., 2007) and

335   predation (Frisch, 1938; Mathuru et al., 2012; Oliveira et al., 2013).

336

337   ***Conclusions and Perspectives***

338   In this study, we could show that short-term pH drop of a similar magnitude of that experienced within the

339   intertidal zone, but also aligned to end-of-century predicted average values (Chavez et al., 2017; Landschützer

340   et al., 2018), had negative consequences on fitness-relevant behaviours in harbour ragworm *H. diversicolor*

341   and small hermit crab *D. pugilator*. Additionally, we confirm that pH drop events also impede the same

342   behaviours in the same way indirectly via chemical communication, albeit these effects depended on donor

343   and recipient species. Sea bream *S. aurata* and harbour ragworm *H. diversicolor* stressed by pH drop released

344   stress metabolites which likely differ from control metabolites and negatively affect fitness-relevant

345   behaviours in metabolite recipients. Stress metabolites induced similar avoidance behaviours as those exhibited

346   under physiological stress, meaning that a stress response was propagated from donor to recipient. These

347   negative indirect effects, or positive feedback loops, warrant further study, especially as our results were

348   inconclusive with regards to the combined treatments of low pH and stress metabolites. Short-term pH drops

349   thus involve behavioural additionally to metabolic trade-offs, which is also of interest for better predicting the

350   response of natural and aquaculture systems under ocean acidification combined with tidal pH fluctuations.

351   We are hopeful that our more than 900 observations and balanced experimental design could overcome most

352   potential limitations, in relation to pseudoreplication due to reusing some animals on different days, and any

353   overestimation of effect sizes related to small sample size (Clements et al., 2020b). Given the potential high

354   ecological significance of indirect negative effects of pH fluctuations on population fitness, studies aiming to

355   replicate our results should aim to further optimise our experimental design with better technical equipment as

356   that available to us (Baker, 2016).

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Competing Interests

The authors declare no conflicts of interest.

Ethics

All experiments were approved by the University of Hull Ethics Committee under the approvals U020 and FEC_2019_81.

Authors' contributions

KWV designed the study. Experiments were performed by LF, SS, LA, JF, RN, KR, and KWV. LF analysed the data and wrote the first manuscript draft with KWV, JDH and HBH. All authors contributed to the final manuscript.

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595   **Tables**
596
597   **Table 1**. Results of Cox proportional hazard model for the main effects of predictors (pH drop, stress
598   metabolites, donor) on the time-to-success analysis in small hermit crab (*Diogenes pugilator*, n = 320
599   observations with 120 events of finding a food cue), green shore crab (*Carcinus maenas*, n = 189 observations
600   with 122 events of finding a food cue), and harbour ragworm (*Hediste diversicolor*, n = 325 observations with
601   234 events of burrowing head in sediment). Unsuccessful observations are censored. Significance (P ≤ 0.05)
602   is shown by p-values in bold. Overall significance of the models using Likelihood ratio tests were: *D. pugilator*:
603   P = 0.06; *C. maenas*: P = 0.009; *H. diversicolor*: P < 0.0001). Covariates were dropped from models after
604   analyses of deviance showed that they passed the Chi-squared test (*D. pugilator*: number of water uses: P =
605   0.1163, crab size: P = 0.6318; *C. maenas*: number of water uses: P = 0.6104, crab size: P = 0.9995, year: P =
606   0.3348; *H. diversicolor*: number of water uses: P = 0.2649). Success ratio (aka hazard ratio) is the
607   exponentiated estimate. SE is the standard error of estimate.
608

Predictors	Success (hazard) ratio	Estimate	SE	Z	P
D. pugilator					
pH drop	**0.4702**	-0.7546	0.3530	-2.1378	**0.0325**
stress metabolites	0.8106	-0.2100	0.3167	-0.6631	0.5073
donor	**0.3460**	-1.0613	0.3843	-2.7613	**0.0058**
pH drop:stress metabolites	1.7878	0.5810	0.4858	1.1959	0.2317
pH drop:donor	**3.2842**	1.1891	0.5441	2.1853	**0.0289**
stress metabolites:donor	1.4970	0.4034	0.5326	0.7575	0.4487
pH drop:stress metabolites:donor	0.4168	-0.8752	0.7498	-1.1672	0.2431
C. maenas					
pH drop	0.7490	-0.2890	0.3620	-0.7982	0.4247
stress metabolites	1.2156	0.1953	0.2887	0.6762	0.4989
donor	1.4445	0.3678	0.3385	1.0865	0.2773
pH drop:stress metabolite	0.8674	-0.1422	0.4630	-0.3072	0.7587
pH drop:donor	**0.2870**	-1.2484	0.6312	-1.9777	**0.0480**
stress metabolites:donor	**0.2981**	-1.2102	0.5008	-2.4165	**0.0157**
pH drop:stress metabolites:donor	**6.0273**	1.7963	0.8261	2.1743	**0.0297**
H. diversicolor					
pH drop	**0.3043**	-1.1899	0.3045	-3.9075	**< 0.0001**
stress metabolites	**0.3520**	-1.0442	0.3049	-3.4251	**0.0006**
donor	0.6965	-0.3616	0.2258	-1.6013	0.1093
pH drop:stress metabolites	**3.9914**	1.3841	0.4411	3.1376	**0.0017**
pH drop:donor	**2.4669**	0.9030	0.3816	2.3663	**0.0180**
stress metabolites:donor	1.7318	0.5492	0.3816	1.4390	0.1501
pH drop:stress metabolites:donor	**0.2291**	-1.4736	0.5633	-2.6158	**0.0089**

609

610 **Table 2**. Results of the binomial generalised linear model for the main effects of predictors (pH drop, stress
611 metabolites, donor) on the avoidance behaviour in small hermit crab (*Diogenes pugilator*, n = 320 observations
612 of finding a food cue), green shore crab (*Carcinus maenas*, n = 151 observations of finding a food cue), and
613 harbour ragworm (*Hediste diversicolor*, n = 325 observations of burrowing head in sediment). Due to missing
614 observations in conspecific control metabolites at control pH in Hediste diversicolor, data was analysed with
615 two models. Model 1: effect of donor across all treatments (sample size: 253 observations). Model 2: effects
616 of pH and metabolites in subset receiving *S. aurata* metabolites (sample size: 154 observations). Overall
617 significance of models from Chi-squared analyses of deviance when including only predictors were: *D.*
618 *pugilator*: P < 0.0001; *C. maenas*: P < 0.0001; *H. diversicolor*: P < 0.0001 (model 1) and P < 0.0001 (model
619 2). Covariates were dropped from models after analyses of deviance showed that they passed the Chi-squared
620 test (*D. pugilator*: number of water uses: P = 0.5963, crab size: P = 0.9158; *C. maenas*: crab size: P = 0.2710).
621 Significance (P ≤ 0.05) is shown by p-values in bold. SE is the standard error of estimate.

622

| Predictors	Estimate	SE	Z	P
D. pugilator				
(Intercept)	-0.9694	0.3541	-2.7380	0.0062
pH drop	0.1221	0.4944	0.2470	0.8049
stress metabolites	-0.1292	0.5086	-0.2540	0.7995
donor	1.1701	0.4758	**2.4590**	**0.0139**
pH drop:stress metabolites	-0.2603	0.7219	-0.3610	0.7185
pH drop:donor	0.7758	0.6919	1.1210	0.2622
stress metabolites:donor	1.0272	0.7022	1.4630	0.1435
pH drop:stress metabolites:donor	-0.8890	1.0039	-0.8860	0.3759
C. maenas				
(Intercept)	-0.2224	0.6725	-0.3307	0.7409
pH drop	-1.1263	0.9173	-1.2279	0.2195
stress metabolites	-1.1263	0.9173	-1.2279	0.2195
donor	-0.4626	0.8326	-0.5556	0.5785
number of water uses	-0.3077	0.1556	**-1.9772**	**0.0480**
pH drop:stress metabolites	2.2527	1.2978	1.7357	0.0826
pH drop:donor	5.5551	1.5392	**3.6090**	**0.0003**
stress metabolites:donor	3.8799	1.2465	**3.1127**	**0.0019**
pH drop:stress metabolites:donor	-7.5415	1.9329	**-3.9017**	**0.0001**
H. diversicolor				
Model 1 (both donors)				
(Intercept)	0.2801	0.3790	0.7391	0.4598
donor	1.0944	0.3169	**3.4534**	**0.0006**
number of water uses	-0.7745	0.1462	**-5.2980**	**< 0.0001**
Model 2 (*S. aurata* donor)				
(Intercept)	-0.6931	0.5000	-1.3863	0.1657
pH drop	-18.8729	1603.1137	-0.0118	0.9906
stress metabolites	3.3557	0.7788	**4.3086**	**< 0.0001**
pH drop:stress metabolites	16.2548	1603.1138	0.0101	0.9919

623

624   **Figures**
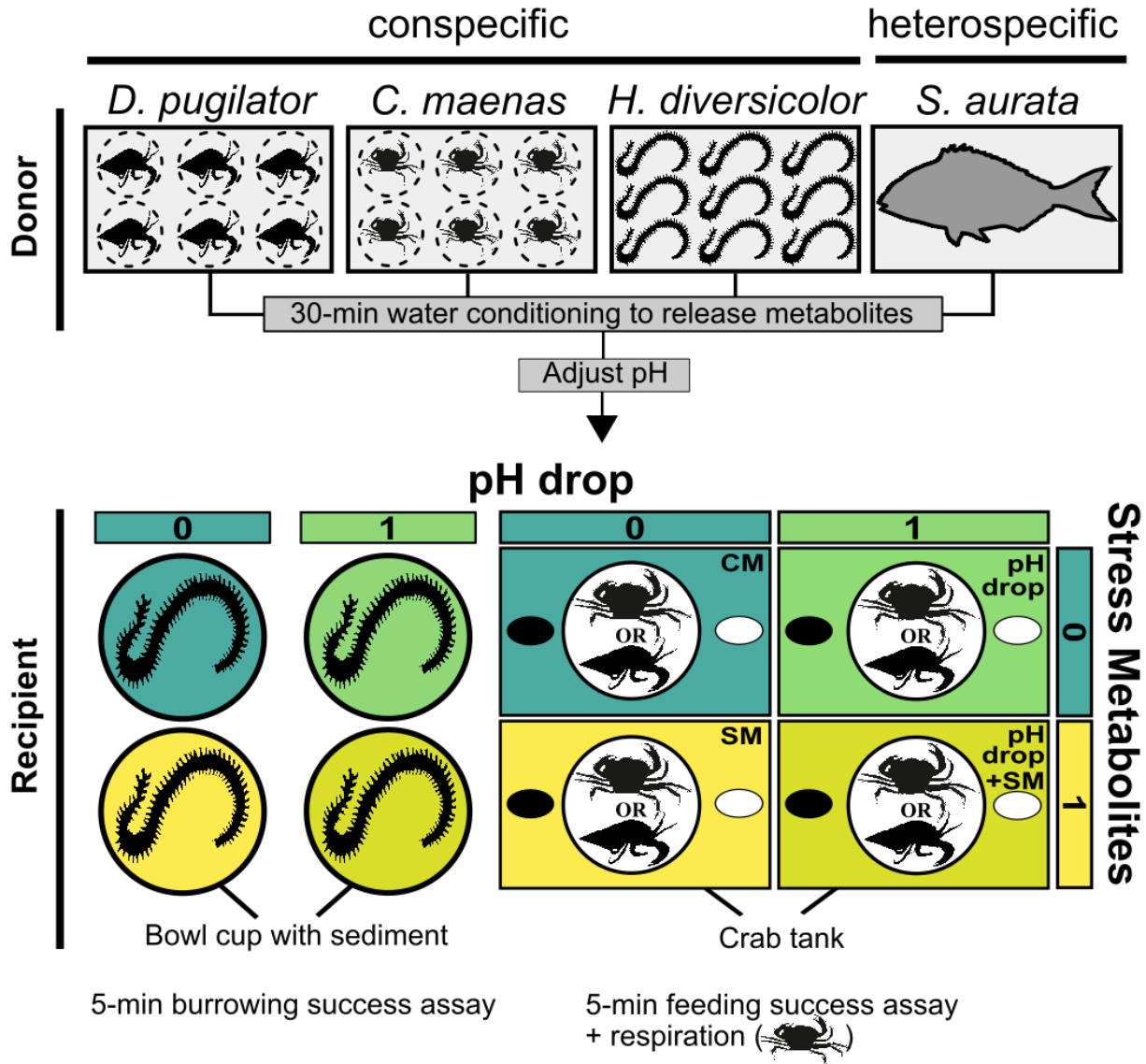
625

626   **Figure 1.** Schematic experimental design showing the presence vs. absence of predictors (pH drop, stress

627   metabolites, donor). Behavioural effects of short-term acute pH drop and stress metabolites it induced were

628   tested in three species: *Diogenes pugilator*, *Carcinus maenas*, and *Hediste diversicolor*. Metabolites were

629   obtained by conditioning donors in either control pH = 8.2 (releasing putative control metabolites), or pH drop

630   = 7.6 (releasing putative stress metabolites), followed by pH adjustments for factorial design yielding four

631   experimental conditions CM, pH drop, SM, and pH drop+SM. Predictors are binary coded as 0 (control

632   metabolites, control pH) and 1 (stress metabolites, pH drop). Metabolites originated from conspecifics or the

633   heterospecific *Sparus aurata*. Behaviour assays for crabs consisted in locating a feeding cue in 300 seconds.

634   Specimens of *H. diversicolor* were placed on top of sediment and burrowing behaviour was recorded for 300

635   seconds. A range of avoidance behaviours were also recorded. After completing the feeding assay, *C. maenas*

636   additionally underwent five minutes of respiration rate measurements. Experimental conditions were CM:

637   control metabolites in control pH, SM: control metabolites in control pH, pH drop: control metabolites in pH

638   drop, pH drop+SM: stress metabolites in pH drop. Animal drawings by A. Murcia and KCWV.
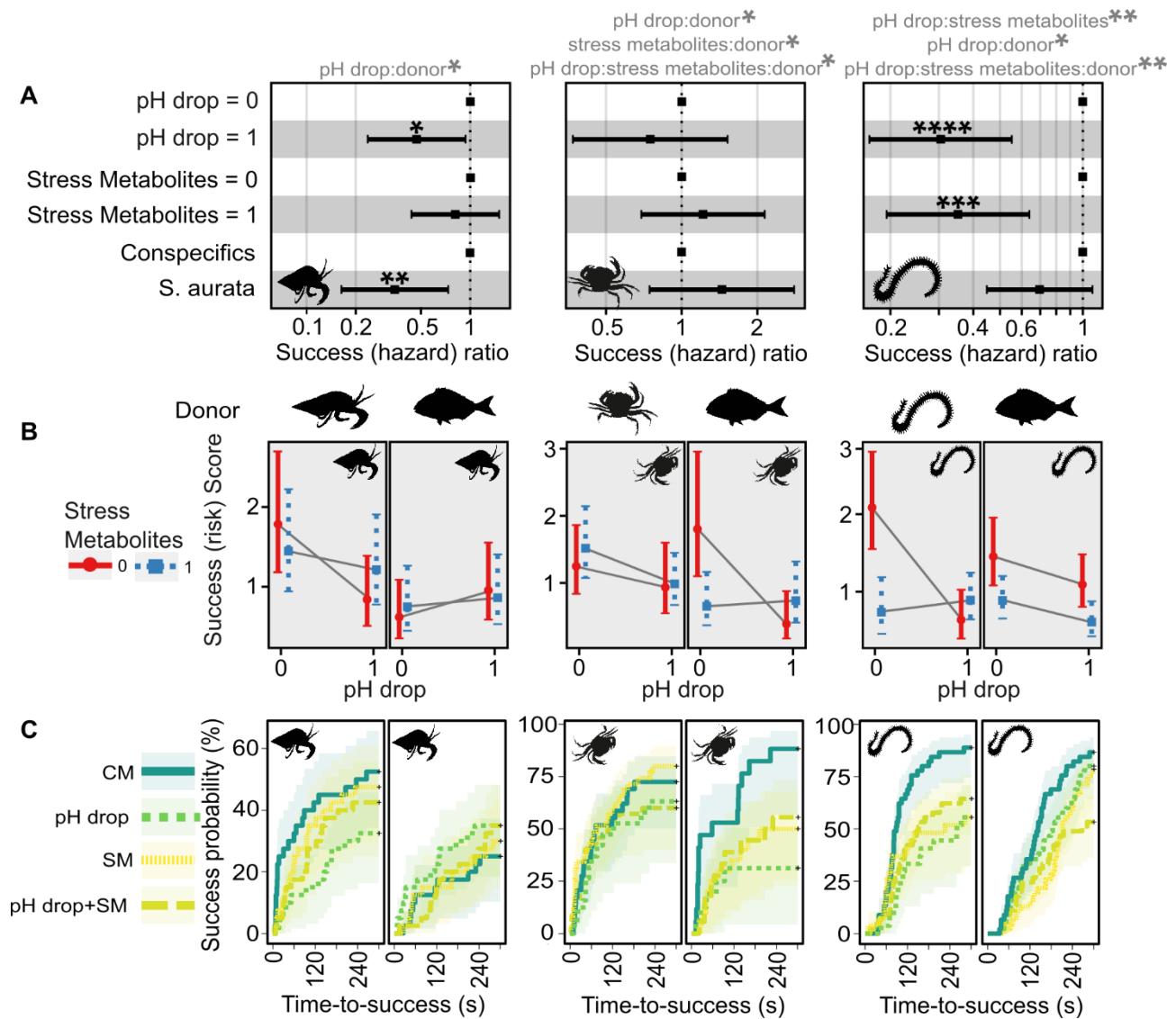
Figure 2. Effects of predictors (pH drop, stress metabolites, donor) on time-to-success in *Diogenes pugilator*, *Carcinus maenas*, and *Hediste diversicolor*. A) effects of predictors on success ratio (aka hazard ratio with success as event, arbitrary units). Likelihood ratio tests for overall model fit were: *D. pugilator*: P = 0.06; *C. maenas*: P = 0.009; *H. diversicolor*: P < 0.0001). Significant predictors from Cox proportional hazard models are shown with asterisks, and significant interaction terms are shown in grey above the plots. B) Interaction term plot of marginal effects showing predicted success score (aka risk score with success as event, arbitrary units ± confidence interval) split by donor and recipient species. Crossed solid grey lines represent an interacting effect of pH and metabolites. C) Kaplan-Meier curves to visualise success probability (cumulative event) for each experimental condition over time. *: P ≤ 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001. Experimental treatments were: CM: control metabolites in control pH, pH drop: control metabolites in pH drop, SM: stress metabolites in control pH, pH drop+SM: stress metabolites in pH drop.
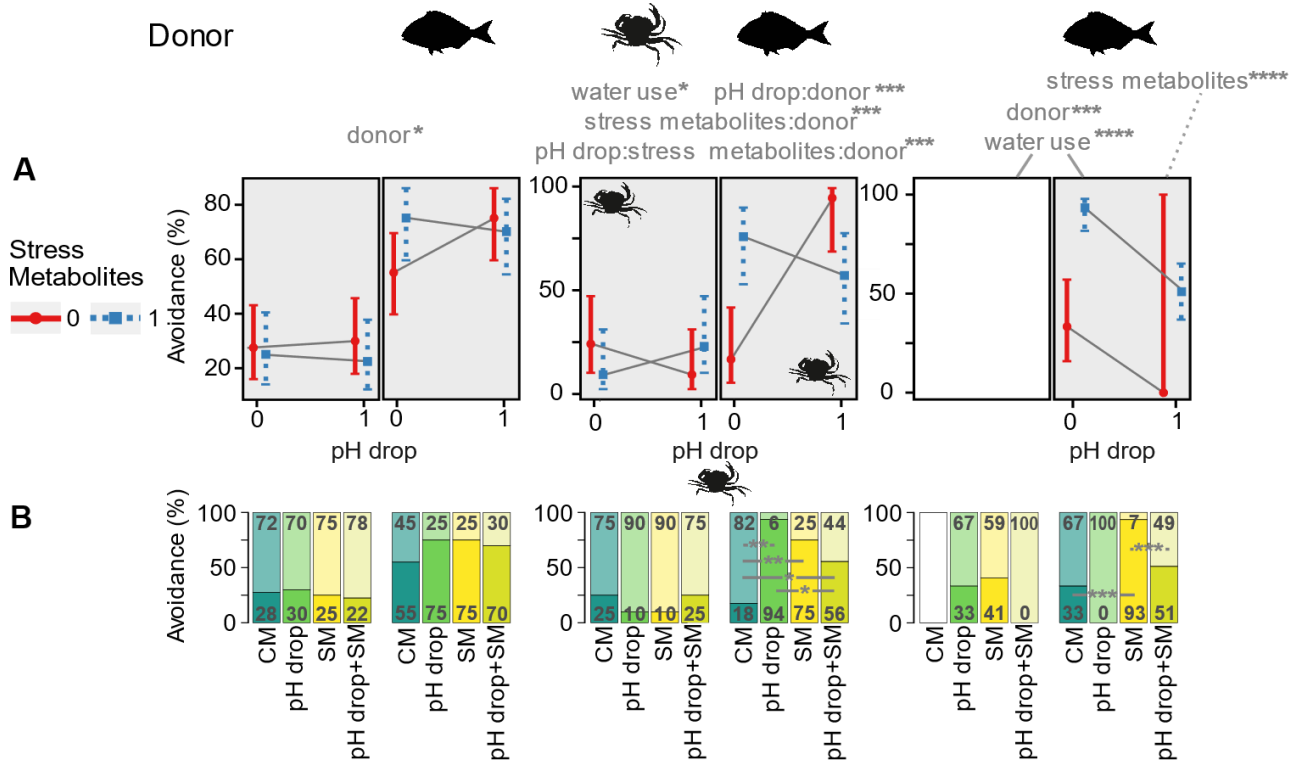
Figure 3. Effects of predictors (pH drop, stress metabolites, donor) on the percentage of avoidance behaviour in *Diogenes pugilator, Carcinus maenas, and Hediste diversicolor*. Avoidance behaviour included freezing and escaping (*D. pugilator* and *C. maenas*), or freezing, curling, flipping, and slime secretion (*H. diversicolor*). A) Split bars represent the presence (dark area) or absence (light area) of avoidance behaviours. Significant main predictors (and their interaction terms) and covariates are shown above plots. Significant pairwise comparisons between treatments in each donor/recipient are shown as horizontal grey lines. *: P ≤ 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001. Experimental treatments were: CM: control metabolites in control pH, pH drop: control metabolites in pH drop, SM: stress metabolites in control pH, pH drop+SM: stress metabolites in pH drop. (B) Interaction plot showing the marginal effects on the predicted avoidance percentages (± confidence interval) of stress metabolites and pH drop within the conspecific and heterospecific groups for each species. Crossing solid grey lines represent an interacting effect of pH and metabolites for each donor. The interactive effects of predictors for *H. diversicolor/H. diversicolor* are not represented due to the missing CM treatment. Overall effects of donor and water use across the response of *H. diversicolor* (for either donor) are shown with solid lines, whereas the effects of stress metabolites are for *S. aurata/H. diversicolor* only (grey dashed line).