1    **Ocean acidification affects marine chemical communication by changing**

2    **structure and function of peptide signalling molecules**

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4    **Running head: Ocean acidification affects signalling cues**

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16   Paper type: Primary research

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18  **Abstract**

19  Ocean acidification is a global challenge that faces marine organisms in the near future with a

20  predicted rapid drop in pH of up to 0.4 units by the end of this century. Effects of the change

21  in ocean carbon chemistry and pH on the development, growth and fitness of marine animals

22  are well documented. Recent evidence also suggests that a range of chemically mediated

23  behaviours and interactions in marine fish and invertebrates will be affected. Marine animals

24  use chemical cues, for example, to detect predators, for settlement, homing and reproduction.

25  But while effects of high $CO_2$ conditions on these behaviours are described across many

26  species, little is known about the underlying mechanisms, particularly in invertebrates. Here

27  we investigate the direct influence of future oceanic pH conditions on the structure and

28  function of three peptide signalling molecules with an interdisciplinary combination of

29  methods. NMR spectroscopy and quantum chemical calculations were used to assess the

30  direct molecular influence of pH on the peptide cues and we tested the functionality of the

31  cues in different pH conditions using behavioural bioassays with shore crabs (*Carcinus*

32  *maenas*) as a model system. We found that peptide signalling cues are susceptible to

33  protonation in future pH conditions, which will alter their overall charge. We also show that

34  structure and electrostatic properties important for receptor-binding differ significantly

35  between the peptide forms present today and the protonated signalling peptides likely to be

36  dominating in future oceans. The bioassays suggest an impaired functionality of the signalling

37  peptides at low pH. Physiological changes due to high $CO_2$ conditions were found to play a

38  less significant role in influencing the investigated behaviour. From our results we conclude

39  that the change of charge, structure and consequently function of signalling molecules

40  presents one possible mechanism to explain altered behaviour under future oceanic pH

41  conditions.

42

43    Abbreviations: $CO_2$, carbon dioxide; GGR, Gly-Gly-Arg, glycyl-glycyl-L-arginine; GHK,

44    Gly-His-Lys, glycyl-L-histidyl-L-lysine; LR, Leu-Arg, L-leucyl-L-arginine; NMR, nuclear

45    magnetic resonance.

## Introduction

The absorption of atmospheric carbon dioxide ($CO_2$) by the oceans leads to a shift of the dynamic carbonate equilibrium resulting in an increase in bicarbonate ion and proton concentrations. Through this mechanism, global average ocean pH has already decreased by more than 0.1 units since pre-industrial times to pH 8.1 (IPCC, 2013) and is predicted to drop further to pH 7.7 by the year 2100 (Bopp *et al.*, 2013; IPCC, 2013). This 'ocean acidification' represents a major challenge that faces marine organisms in the near future. The extent of ocean acidification is tightly linked to anthropogenic $CO_2$ emissions, which are certain to continue for the foreseeable future (Bopp *et al.*, 2013). High concentrations of $CO_2$ in the ocean are also referred to as ocean hypercapnia (McNeil & Sasse, 2016).

So far research has focused on the impact of ocean acidification on the biology of organisms, in particular calcification and physiological processes. These studies have shown clear effects of a decreased environmental pH on aerobic performance, growth and overall fitness of marine animals (Fabry *et al.*, 2008; Wittmann & Pörtner, 2013). In recent years, it has also been demonstrated that high $CO_2$ conditions affect marine animal behaviour (reviewed by Briffa *et al.*, 2012 and Clements & Hunt, 2015). This includes a range of chemically mediated behaviours, for example in marine fish, where homing, predator detection in larvae, feeding and habitat choice have been found to be altered through olfactory disruption in reduced pH conditions (Munday *et al.*, 2009; Leduc *et al.*, 2013). There are also indications that ocean acidification influences interactions of organisms and communities (Munday *et al.*, 2009; Leduc *et al.*, 2013; Dodd *et al.*, 2015). In fact, chemical cues are omnipresent in marine systems and regulate critical aspects of the behaviour of marine organisms across the phylogenetic tree (Hay, 2009). These molecules are often produced unintentionally, which defines them as cues (Steiger *et al.*, 2011). However, they mostly evoke highly specific and stereotyped responses (Wyatt, 2014a) and so possess a signalling function. We therefore refer

71 to them in the following as signalling cues or signalling molecules. These signalling cues are

72 as diverse as their biological functions, and can be based on every form of biological

73 molecule from amino acids to nucleic acids and carbohydrates (Hay, 2009; Wyatt, 2014a).

74 However, their exact structures and in particular their active conformations are mostly

75 unknown. Only a very limited number of signalling cue structures and their respective

76 biological function have been identified so far (Hay, 2009).

77 Cues derived from amino acids constitute one of the most important classes of signalling

78 molecules (Decho *et al.*, 1998; Wyatt, 2014b) with a vast range of ecological functions

79 ranging from foraging (Hayden *et al.*, 2007) to reproduction (Hardege *et al.*, 2004), larval

80 release, settlement and homing (Rittschof & Cohen, 2004). Peptide and protein cues are

81 mostly water soluble due to their zwitterion form (one positively and one negatively charged

82 terminus) under natural conditions in solution. They are a natural choice for signalling

83 molecules as the building blocks (amino acids), the machinery (enzymes) and the templates

84 (DNA/RNA) are already available in cells (Decho *et al.*, 1998; Zimmer & Butman, 2000).

85 Furthermore, the 20 proteinogenic amino acids allow a huge variety, and therefore specificity,

86 of signalling molecules when polymerised into a peptide (Rittschof, 1990). Peptide-mediated

87 behaviours in marine organisms have not yet been investigated in depth with regard to

88 changing ocean conditions. However, their potential vulnerability to pH has already been

89 hypothesised (Hardege *et al.*, 2011; Wyatt *et al.*, 2014) and first indications were shown for

90 crustaceans (de la Haye *et al.*, 2012; Kim *et al.*, 2015). Hermit crabs were found to be less

91 able to locate food in reduced-pH conditions, which is often a peptide-mediated behaviour (de

92 la Haye *et al.*, 2012; Kim *et al.*, 2015).

93 The pH-dependent alteration of behaviour has several plausible explanations. First, it could be

94 a consequence of systemic physiological changes that reduce the energy available to the

95 organism or alter its metabolic processes (Pörtner *et al.*, 2004). Second, the change in pH

96　could affect the neural mechanisms required for processing information (Nilsson *et al.*, 2012).

97　Thirdly, the reduced behavioural response may be due to disruption of the signal reception,

98　which itself can have a number of reasons. For example, the organism's ability to detect

99　chemical cues, also referred to as chemoreception, could be impaired by physical damage to

100　the receptive organs (Briffa *et al.*, 2012), alteration of the receptors (Tierney & Atema, 1988)

101　or changes to the signalling molecules in low pH environments (Brown *et al.*, 2002). All of

102　these effects lead to a reduced recognition between signalling cue and receptor. While

103　physical damage to the receptive organs has already been investigated as potential factor (de

104　la Haye *et al.*, 2012), the alteration of receptors and changes to signalling molecules have only

105　been suggested based on behavioural bioassays in different conditions. Molecular evidence

106　for these pH effects is scarce and only reported for one freshwater system with irreversible

107　change to the molecules at very low pH conditions (Brown *et al.*, 2002). The effects of pH on

108　signalling molecules in marine systems and in the context of ocean acidification have not yet

109　been investigated on a molecular level.

110　Peptide-mediated behaviours are particularly suitable to investigate the pH-induced change of

111　signalling molecules as a potential reason for altered behaviour in high $CO_2$ environments.

112　Amino acids and therefore peptides possess a number of chemical functional groups that can

113　be protonated (addition of a $H^+$) depending on the pH in the surrounding medium (see Fig. 1).

114　This includes a carboxylic group at the C-terminus, an amino group at the N-terminus and

115　other groups at the side chains if present. The pH conditions at which these groups will be

116　protonated is group specific and expressed using p$K_a$ values: negative logarithmic acid

117　dissociation constants expressing the pH value at which 50% of the molecules in solution are

118　deprotonated and 50% are protonated at the corresponding group.

119    We suggest that the change of pH in future oceans could lead to profound changes in

120    protonation states of peptide signalling molecules containing groups with p$K_a$ values close to

121    8, which in turn may lead to significant alterations in their structure and function.

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123    To test this hypothesis and understand the real impact of pH to the signalling cues and the

124    associated behaviour requires a molecular approach using interdisciplinary tools and methods.

125    Therefore in this study we combine for the first time NMR spectroscopy with quantum

126    chemical calculations and bioassays to obtain a more complete picture of the direct molecular

127    impacts of ocean acidification. As a model system we focus on three peptides that mimic cues

128    for egg ventilation in crustaceans: two tripeptides glycyl-L-histidyl-L-lysine (GHK) and

129    glycyl-glycyl-L-arginine (GGR) as well as the dipeptide L-leucyl-L-arginine (LR).

130    First, we assess the peptides' susceptibility to protonation with increasing ocean acidification

131    through NMR spectroscopic determination of the p$K_a$ values for each ionisable group. These

132    values are also used to calculate the abundance of the different protonation states over the pH

133    range. Secondly, we explore the differences in conformation and charge distribution of the

134    relevant protonation states using quantum chemical calculations. Thirdly, we test the effects

135    of pH on the peptides' functionality in behavioural bioassays. These experiments also aim to

136    establish whether signal reception or physiological and neurological changes play a more

137    significant role in causing behavioural changes with pH. We discuss how changes in

138    signalling molecules with pH could be linked to change in peptide-mediated behaviour

139    through impaired chemoreception. Finally, we evaluate the transferability of our model

140    system and the ecological significance of our results before giving an overview of possible

141    consequences and perspectives.
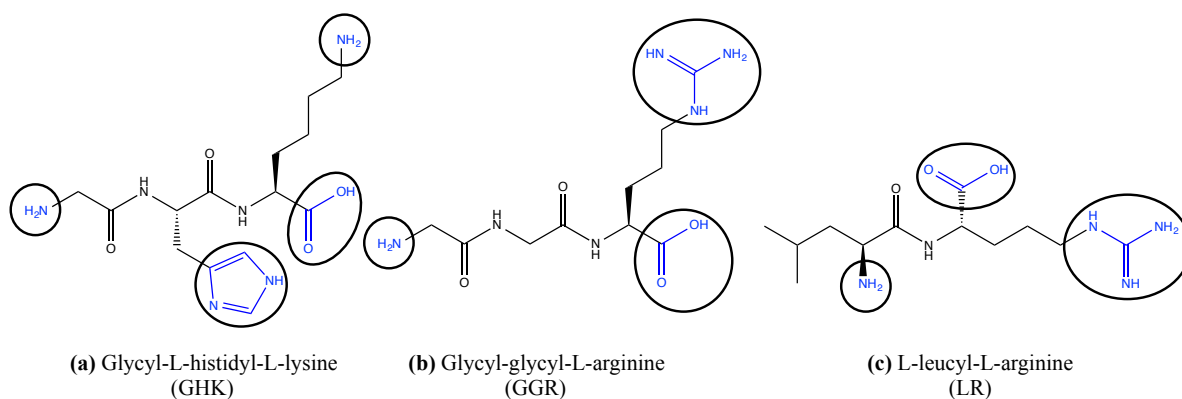
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## Materials and methods

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*Choice of signalling molecules & model system*

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For our study of the direct influence of pH on structure and function of peptide signalling

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molecules we chose glycyl-L-histidyl-L-lysine (GHK), glycyl-glycyl-L-arginine (GGR) and

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L-leucyl-L-arginine (LR). These three peptide cues are synthetic mimics of the yet-

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unidentified natural signalling molecules known to mediate egg ventilation (Forward Jr *et al.*,

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1987). The three mimics are good model systems as they have a chemically diverse amino

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acid sequence and side chains, but display the same documented biological function (egg

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ventilation). The use of a well-defined system with a known chemical signalling cue and a

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stereotyped behaviour allowed us to link molecular changes to the signalling cue function.

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Egg ventilation is a naturally occurring stereotyped behaviour of female decapods carrying an

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egg clutch (Reinsel *et al.*, 2014). Regular probing and movement of the eggs, which are

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attached to the female's abdomen, ensures oxygen supply, waste removal and larval

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development (Crothers, 1967; Reinsel *et al.*, 2014). This behaviour is mediated by peptides

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released from the eggs, allowing chemical communication between the female and her brood

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(Reinsel *et al.*, 2014). The ventilation frequency depends on the developmental stage of the

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embryos (De Vries & Forward Jr, 1991) and peaks during larval release, allowing

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synchronised hatching (Forward Jr *et al.*, 1987; Reinsel *et al.*, 2014).

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**(a)** Glycyl-L-histidyl-L-lysine
(GHK)

**(b)** Glycyl-glycyl-L-arginine
(GGR)

**(c)** L-leucyl-L-arginine
(LR)

**Fig. 1** Chemical structures of the signalling peptides glycyl-L-histidyl-L-lysine (a), glycyl-glycyl-L-arginine (b) and L-leucyl-L-arginine (c). Functional groups with potential for de-/protonation are highlighted with circles.

*Assessment of peptides' susceptibility to protonation*

$pK_a$ values are useful measures to assess the protonation state of ionisable functional groups at a given pH. However, to date they remain unknown for most signalling molecules, including peptides. We determined the $pK_a$ of all ionisable groups of glycyl-L-histidyl-L-lysine (GHK), glycyl-glycyl-L-arginine (GGR) and L-leucyl-L-arginine (LR) with NMR spectroscopy based on the pH dependent change of ^{1}H chemical shifts. Samples were prepared with a concentration of 2.5 mM (GHK) or 10 mM (GGR, LR) in sodium phosphate buffer (10 mM, pH adjusted) with 10% $D_2O$ and TMS as internal standard. The sample pH was adjusted (Mettler Toledo Five Easy FE20 pH meter with InLab Flex-Micro electrode) with minimal quantities of HCl or NaOH to obtain a sequence of 0.3 to 0.5 pH unit steps. The preparation of peptide samples in buffer and the adjustment of the pH with hydrochloric acid instead of $CO_2$ allowed for chemically stable samples over the course of the NMR measurements. [1]H spectra were measured with a Bruker Avance II Ultrashield 500 MHz spectrometer at 298 K. Proton chemical shifts were determined with WATERGATE 3-9-19 water suppression (Piotto *et al.*, 1992; Sklenář *et al.*, 1993) and 32 scans. For peak assignment 2D correlation and total correlation spectroscopy measurements of at least two samples of

185    different pH were performed per peptide (see Supporting information (SI) for peak

186    assignment and more details on sample preparation). All spectra were processed using the

187    Topspin software (Version 1.3, Bruker Instruments, Karlsruhe, Germany). $^1$H chemical shifts

188    ($\delta$) of each nucleus that could be obtained over the pH range were plotted against the sample

189    pH. The p$K_a$ was determined by the inflection point of a fitted sigmoid or double sigmoid

190    curve to the data using the IGOR pro software (Version 6.02, WaveMetrics, Inc. 1988-2007).

191    For each ionisable group the p$K_a$ value obtained from the closest suitable $^1$H nucleus was

192    used.

193    Based on the p$K_a$, the concentration and therefore proportion of each protonation state over

194    the pH range could be calculated using the Henderson—Hasselbalch equation

195
$$\text{pH} = \text{p}K_a + \log\frac{[\text{A}^-]}{[\text{HA}]}$$

196    that relates the pH to the p$K_a$ and the concentrations of the acid (HA) and its corresponding

197    base (A$^-$) (for details see Po & Senozan, 2001 and references therein).

198

199    *Exploring differences between protonation states*

200    A change in protonation states of the chemical cues could be accompanied by structural

201    changes to the cues in the lowered pH of future oceans. To investigate this we used quantum

202    chemical calculations to obtain the energetically most favourable conformers for each

203    possible protonation state. These model conformers were then used to assess conformational

204    differences between the protonation states, as well as differences in their molecular

205    electrostatic potential (MEP), which describes the charge distribution around the molecule.

206    An optimal initial conformer of each protonation state for each peptide (GHK, GGR and LR)

207    was generated using the openbabel program (version 2.2.3) (O'Boyle *et al.*, 2011). Our

208 approach generates 5000 random starting conformers for each state/peptide and then performs

209 up to 2000 optimisation steps towards the minimum for each conformer using the mmff94

210 force field (Halgren, 1996). The resulting optimised conformers are then ranked and the

211 lowest energy conformer is used as starting conformation for another cycle of random

212 conformer generation and subsequent optimisation. The final conformer obtained after three

213 such cycles was further optimised using the PBE0 exchange correlation functional (Adamo &

214 Barone, 1999) with a pc-2 basis set (Jensen, 2001, 2002a, 2002b) and water as implicit

215 solvent using COSMO (Klamt, 1995) implemented in the ORCA suite of programs (Version

216 3.0.0) (Neese, 2012). We used the RIJ-COSX approximation (Neese *et al.*, 2009) with a def2-

217 TZVPP/J auxiliary basis set (Weigend & Ahlrichs, 2005) and included D3 dispersion

218 corrections following Grimme (Grimme *et al.*, 2010, 2011). The VeryTightSCF and TightOpt

219 criteria implemented in ORCA were used to stop the SCF gradient and the optimisation at a

220 total energy change of $< 10^{-8}$ $E_h$ respectively. The calculation of the molecular electrostatic

221 potential (MEP) was performed with the GAMESS program (vJan122009R1) using the

222 Perdew-Burke-Ernzerhof exchange functional (PBE) (Perdew *et al.*, 1996) in conjunction

223 with a STO-3G basis set (Hehre, 1969). A three-dimensional electron density isosurface was

224 visualised with 100 grid points, a medium grid size and a contour value of 0.03 $e \cdot a_0^{-3}$ using

225 the wxMacMolPlot program (v7.5141) (Bode & Gordon, 1998). The density isosurface was

226 coloured according to the MEP with a maximum value of 0.25 $E_h \cdot e^{-1}$ and the RGB colour

227 scheme with red representing positive, green neutral and blue negative charge.

228 To validate our approach and the obtained conformations, we compared the experimental

229 chemical shifts measured during the $pK_a$ determination with calculated ^{1}H NMR chemical

230 shifts of GHK II and GHK III. The shielding values of ^{1}H nuclei were calculated at the

231 PBE0/aug-pc-2 level of theory, using the RIJ-COSX approximation with a def2-TZVPP/J

232 auxiliary basis set and the individual gauge for localised orbitals method (IGLO) (Kutzelnigg

233    *et al.*, 1991) in ORCA (Version 3.0.0). The VeryTightSCF criteria implemented in ORCA

234    were used to stop the SCF gradient at a total energy change of $< 10^{-8}$ $E_h$. Chemical shift

235    values were obtained by calculating the difference between the proton shielding values of the

236    protonation state conformer and those of the standard tetramethylsylane (TMS), which was

237    optimised and its shielding constants calculated as stated earlier. For comparison, the

238    experimental $^1$H chemical shift values from samples with the pH closest to the maximum

239    proportion of each protonation state were used. The conformer validation by comparison was

240    performed for GHK II and GHK III, as an example (see Table S1).

241

242    *Determination of cue functionality in bioassays*

243    In our behavioural assays, we observed the number of abdominal egg ventilation strokes of

244    shore crabs (*Carcinus maenas*) before and after addition of a given concentration of one of

245    two peptide cues (GHK: glycyl-L-histidyl-L-lysine or GGR: glycyl-glycyl-L-arginine) or

246    seawater (control). The shore crabs respond to the signalling cues by increasing the rate at

247    which they ventilate their eggs. This stereotyped behavioural response to these specific

248    peptides has been reported previously for mud crabs (*Rhithropanopeus harrisii*) (Forward Jr

249    *et al.*, 1987), but is tested here for *C. maenas* for the first time.

250    The egg ventilation frequency of ovigerous crabs was determined with a bowl assay (Forward

251    Jr *et al.*, 1987) before and after the addition of signalling cue (GHK or GGR) or seawater as

252    control. Only two cues were tested due to seasonal time-constrains. During this type of assay,

253    the crabs are placed individually in non-reflecting plastic containers with 1L of seawater and

254    observed for a given time. The duration of the assay was kept as short as possible in order to

255    minimise effects of the crabs themselves on the seawater pH in this closed system. The

256    bioassay procedure therefore contained a habituation phase (1 min), an interval of counting

257    the abdominal pumps (5 min), slow addition of the peptide cue close to the crab's abdomen

258    (100 µL) and a further 5 min counting interval. Tests were performed with 10 replicates in

259    natural (pH $8.1 \pm 0.1$) and future (pH $7.7 \pm 0.1$) oceanic pH conditions for four concentrations

260    per peptide. The concentration range and steps were chosen based on the threshold values

261    published for mud crabs ($10^{-9}$ M, mixed uniformly in bowl) (Forward Jr *et al.*, 1987) and

262    adapted to the average volume around a shore crab (50 mL) due to the application as a signal

263    trail next to the crabs abdomen. This yielded a concentration range of $10^{-10}$ M to $10^{-7}$ M

264    around the crab with cue solutions ranging from $5 \times 10^{-8}$ M to $5 \times 10^{-5}$ M (see SI for details on

265    cue solution preparation).

266    In order to estimate the extent of physiological and neuronal impairment of the ventilation

267    behaviour relative to the chemoreceptive ability, the complete set of bioassays for both cues

268    was performed twice: with crabs kept in natural pH conditions (pH 8.1) for at least 4 days

269    prior to experiments and crabs pre-acclimated to pH 7.7 for one week. All crabs were tested in

270    both pH conditions, however they were tested first in the conditions they were kept in. For

271    example, crabs acclimated to pH 7.7 were tested first in pH 7.7 before being tested in pH 8.1

272    and vice versa.

273    The natural egg ventilation frequency varies greatly amongst individuals with 3.2 ($\pm$ 3.1)

274    strokes per 5 min in pH 8.1 and 4.7 ($\pm$ 2.6) strokes per 5 min in pH 7.7. Hence the

275    experimental set up described above was chosen to allow for direct immediate comparison. A

276    higher ventilation frequency after addition of the cue was counted as positive response. The

277    ratio of positive to no responses was compared pairwise between the seawater control and the

278    treatment with a given concentration of one of the cues using a one-sided Fisher's exact test

279    of independence (F-test) of the "R" statistical package (version 3.1.2, R Development Core

280    Team 2014). This test allows testing for differences between two proportions of nominal

281    variables with a small sample size (McDonald, 2014). Significant differences to the control

282    were established for significance level of $p < 0.05$ (*) and $p < 0.01$ (**). Figures show

283      proportion of significantly responding crabs out of the total number of crabs tested in

284      percentage, therefore no standard error is given.

285 **Results**

286 *Peptide-cue susceptibility to protonation with pH change*

287 The p$K_a$ values of all ionisable groups of glycyl-L-histidyl-L-lysine (GHK), glycyl-glycyl-L-

288 arginine (GGR) and L-leucyl-L-arginine (LR) respectively are summarised in Table 1. For all

289 three peptides, the p$K_a$ values of the L-lysine and L-arginine at the peptide C-termini were

290 found to lie outside the physiological pH range. The p$K_a$ for the Arg side chain could not be

291 obtained accurately by curve fitting due to an insufficient number of data points. Only two

292 samples $\geq$ pH 12 were measured in order to minimise potential errors often associated with

293 NMR measurements in high pH conditions (see SI for details on this). However, the

294 determined p$K_a \approx 15$ and literature values for isolated L-arginine of 12.1 (Lide, 2004) clearly

295 lie outside the pH range affected by ocean acidification and can therefore be neglected in this

296 context. In contrast, the N-terminal glycine and L-leucine residues possessed p$K_a$ values

297 within an ocean pH range likely to be experienced before the end of this century. The p$K_a$ of

298 the L-histidine side chain of GHK was found to lie slightly below this pH range. Therefore all

299 three peptide cues are susceptible to pH changes and will most likely change their protonation

300 state with on-going ocean acidification. It is important to note that this susceptibility would

301 not be apparent based purely on p$K_a$ values of isolated glycine or L-leucine, which are 1.58 to

302 1.65 units higher than the values observed in the peptides. Indeed, the p$K_a$ values of the

303 individual amino acids would suggest they are not significantly affected by a pH change from

304 8.1 to 7.7, but the effect of neighbouring amino acids in peptides plays a significant role on

305 the protonation of an ionisable group. This has been previously shown by Wishart *et al.*

306 (Wishart *et al.*, 1995) and stresses the importance of compound-specific p$K_a$ determination.

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308

309     **Table 1** pK$_a$ values (± SD) of the ionisable groups of the signalling peptides glycyl-L-
310     histidyl-L-lysine (GHK), glycyl-glycyl-L-arginine (GGR) and L-leucyl-L-arginine (LR).

| Peptide | Ionisable group | pK$_a$ | | |
|---------|-----------------|--------|---|------|
| GHK | Gly NH$_2$ | 7.98 | ± | 0.04 |
| | His side chain | 6.45 | ± | 0.05 |
| | Lys COOH | 2.8 | ± | 0.4 |
| | Lys side chain | 11.44 | ± | 0.06 |
| GGR | Gly NH$_2$ | 8.00 | ± | 0.05 |
| | Arg COOH | 2.89 | ± | 0.08 |
| | Arg side chain | 15 | ± | 9[a] |
| LR | Leu NH$_2$ | 7.93 | ± | 0.03 |
| | Arg COOH | 2.71 | ± | 0.08 |
| | Arg side chain | 15 | ± | 9[a] |

311     [a] No accurate pK$_a$ for the Arg side chain could be obtained due to an insufficient number of
312     data points for curve fitting ≥ pH 12. However, a literature value of 12.1 (Lide, 2004) strongly
313     suggests that the Arg side chain will not be affected by ocean acidification.
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327  Based on the determined group-specific p$K_a$ values and the Henderson–Hasselbalch equation,

328  the abundance of the different protonation states over the pH range can be calculated and is

329  shown in Fig. 2. We found that upon acidification there will be a 23% decrease of the

330  currently present GHK and LR protonation states and a 22% decrease of the current GGR

331  form. In turn there will be a corresponding increase of peptide forms protonated at the N-

332  terminus. In the case of GHK a second protonation state, which is additionally protonated at

333  the L-histidine side chain, becomes more prominent at low pH. These protonated forms are

334  positively charged while the currently present forms are overall neutral (zwitterionic).

335  Our results suggest that peptide cues are highly susceptible to pH alteration and that a change

336  in abundance from neutral to positively charged protonation states will occur with on-going

337  acidification.
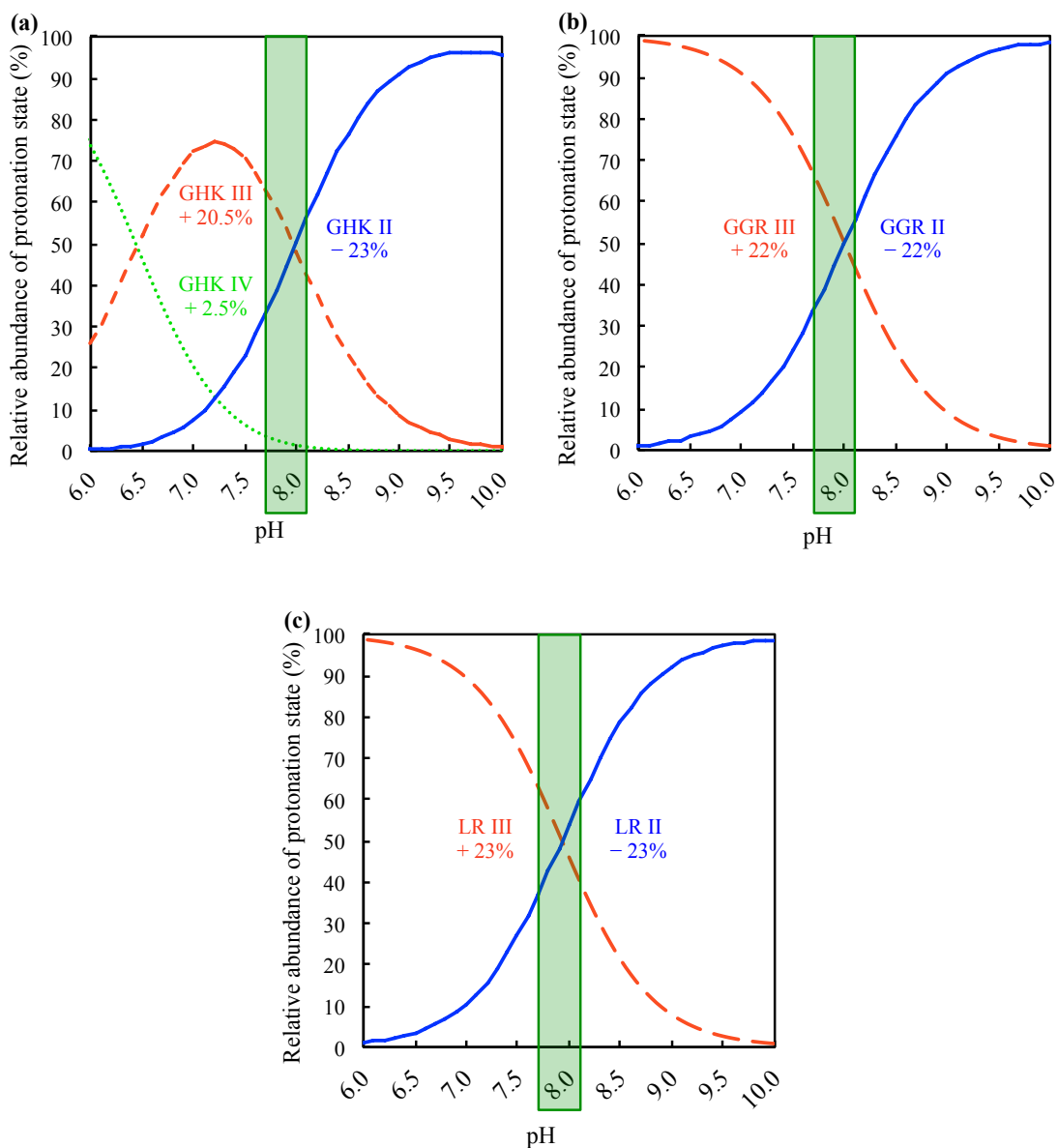
**Fig. 2** Relative abundance of individual protonation states of glycyl-L-histidyl-L-lysine (a), glycyl-glycyl-L-arginine (b) and L-leucyl-L-arginine (c) and their percentage change with ocean acidification. Proportions are shown for protonation states present between pH 6 and 10. The green shaded area indicates the pH range of ocean acidification from today's pH 8.1 to the projected pH 7.7 for 2100. (a) GHK II (blue, continuous line): L-lysine side chain protonated; GHK III (red, dashed): glycine N-terminus and L-lysine side chain protonated; GHK IV (green, dotted): L-histidine side chain, glycine N-terminus and L-lysine side chain protonated: (b) GGR II (blue, continuous line): L-arginine side chain protonated; GGR III (red, dashed): glycine N-terminus and L-arginine side chain protonated; (c) LR II (blue, continuous line): L-arginine side chain protonated; LR III (red, dashed): L-leucine N-terminus and L-arginine side chain protonated.

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*Structural difference between protonation states*

To assess structural changes to the cues in the lowered pH of future oceans, which could have implications for how the cues may dock with their receptors, we compared model conformers of different protonation states. Our results are shown in Fig. 3a and reveal that the conformations of the protonation states of each peptide differ considerably. The conformations of the neutral forms (GHK II, GGR II and LR II) are more compact in comparison to the protonated forms (GHK III and GHK IV, GGR III and LR III). The protonated forms are more open and planar. This is particularly apparent for GHK, where the position of the L-histidine side chain changes from close proximity to the L-arginine side chain (GHK II) to a stretched out conformation upon protonation. Furthermore, we found that the MEP differs significantly for the different protonation states when represented on their electron density isosurfaces (Fig. 3b). The neutral forms display distinct patches of positive or negative charge and large neutral areas. In contrast, the protonated forms show large positively charged areas with only some neutral or slightly negative patches. Based on the p$K_a$ values, the neutral peptide forms could be identified as the protonation states dominating in today's ocean. The protonated forms will be increasingly present in future oceanic pH conditions.
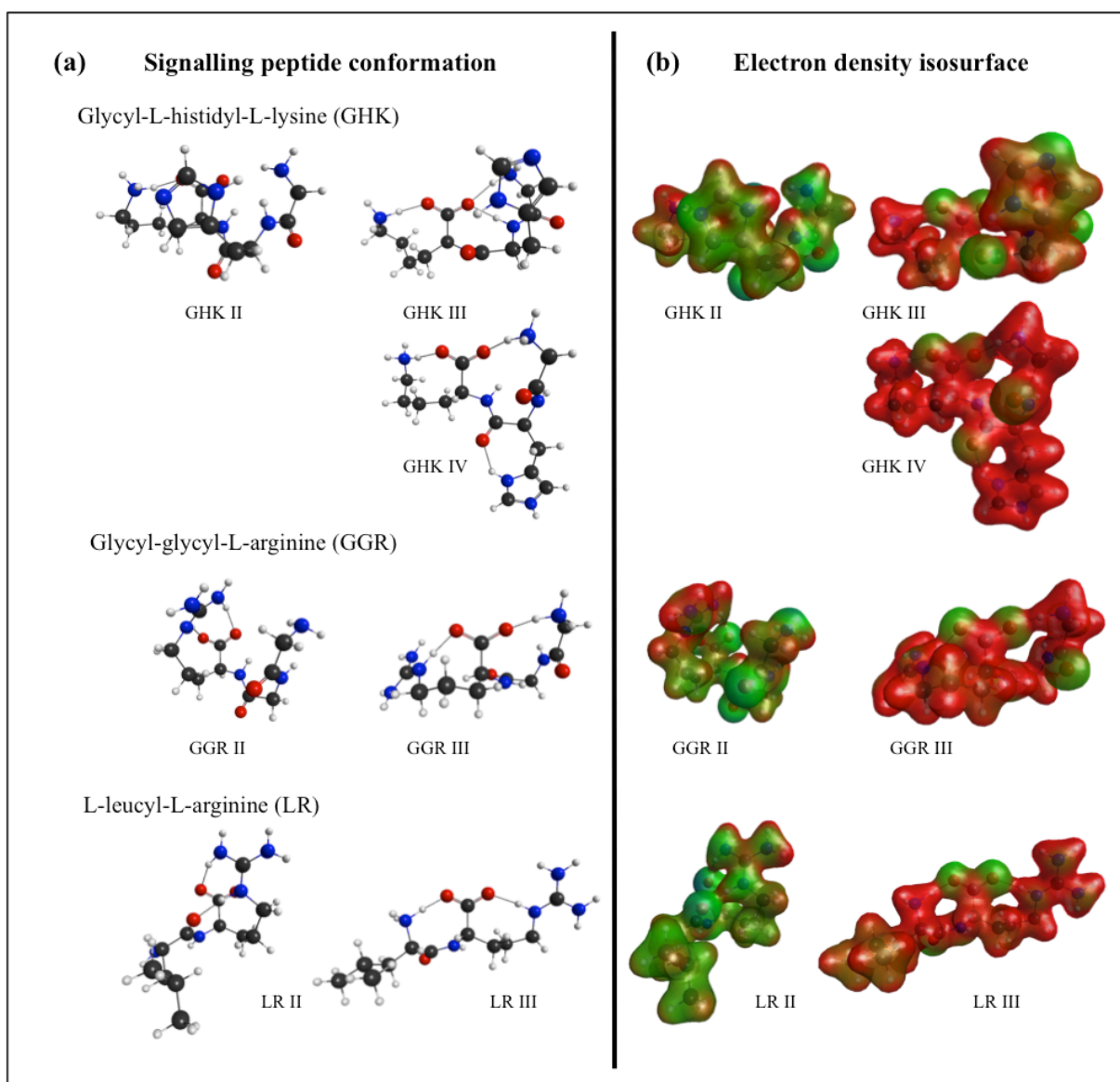
**Fig. 3** Conformations and charge distribution of the protonation states of GHK, GGR and LR. (a) Conformations of the different peptide protonation states with carbon atoms in black, hydrogen in white, nitrogen in blue and oxygen in red. (b) Electron density isosurfaces (contour value 0.03 $e \cdot a_0^{-3}$) are colour coded according to the molecular electrostatic potential of each conformer with a maximum value to map of 0.25 $E_h \cdot e^{-1}$. Blue indicates negative, green neutral and red positive charge.

380    We also compared experimentally obtained and quantum chemically calculated ^{1}H chemical

381    shift values of GHK II and GHK III. Very similar approaches have been previously used for

382    structure determination and validation of compounds in solution (see for example Lodewyk *et*

383    *al.*, 2012). The chemical shift of a nucleus is influenced by the position of all neighbouring

384    nuclei, which can cause either deshielding or shielding effects from the applied magnetic field

385    during the NMR experiment. [1]H shifts have been shown to be sensitive to chemical structure

386    and even small conformational changes can result in significant variations of the

387    corresponding proton shifts (Hunter *et al.*, 2005). Therefore a comparison between the

388    measured and calculated proton chemical shifts enables us to assess if the calculated

389    conformations are in agreement with the protonation state conformations present in solution.

390    For most protons of GHK II (RMSD: 0.43 ppm) and GHK III (RMSD: 0.68 ppm) the

391    experimental and calculated values agreed within an error margin of 0.7 ppm. Only two

392    protons differed by 0.9 ppm and 1.6 ppm for GHK II and GHK III respectively (see Table

393    S1). This shows that there is still some need for refinement and we suggest that including

394    solvent effects could help to explain the few observed deviations. However, the agreement

395    between experimental and calculated proton chemical shift values suggests that the

396    conformers obtained by our quantum chemical calculations are reasonably accurate models of

397    the observed peptides and validates the chosen approach.

398    Note that the same approach is used for all protonation states, which also allows direct

399    comparison between them. All three peptides consistently show similar trends and display

400    less compact conformation and more uniformly distributed (positive) charge upon

401    protonation. This stresses that there are considerable differences in conformation and MEP

402    between protonation states present in today's oceans and those that will be present in future

403    oceans.

404

*Effects of pH on peptide-mediated behaviour*

When tested in pH 8.1, a significant number of shore crabs increased the egg ventilation frequency compared to the control in response to $5 \times 10^{-8}$ M of cue. This corresponds to a concentration of $10^{-10}$ M around the crab (50 mL) or $5 \times 10^{-12}$ M in the bowl (1L). Future oceanic pH conditions negatively affected the behavioural response to both cues. In pH 7.7, an at least tenfold higher concentration ($5 \times 10^{-7}$ M, $10^{-9}$ M around the crab, $5 \times 10^{-11}$ M in the bowl) than at pH 8.1 was required for a significant number of crabs to respond to GHK and GGR (Fig. 4). The natural concentration has not been reported in the literature as the natural cue is unknown to date. However, studies with synthetic cue mimics triggering this behaviour found similar or slightly higher threshold values for several shrimp species and mud crabs (Forward Jr et al., 1987; Reinsel et al., 2014).
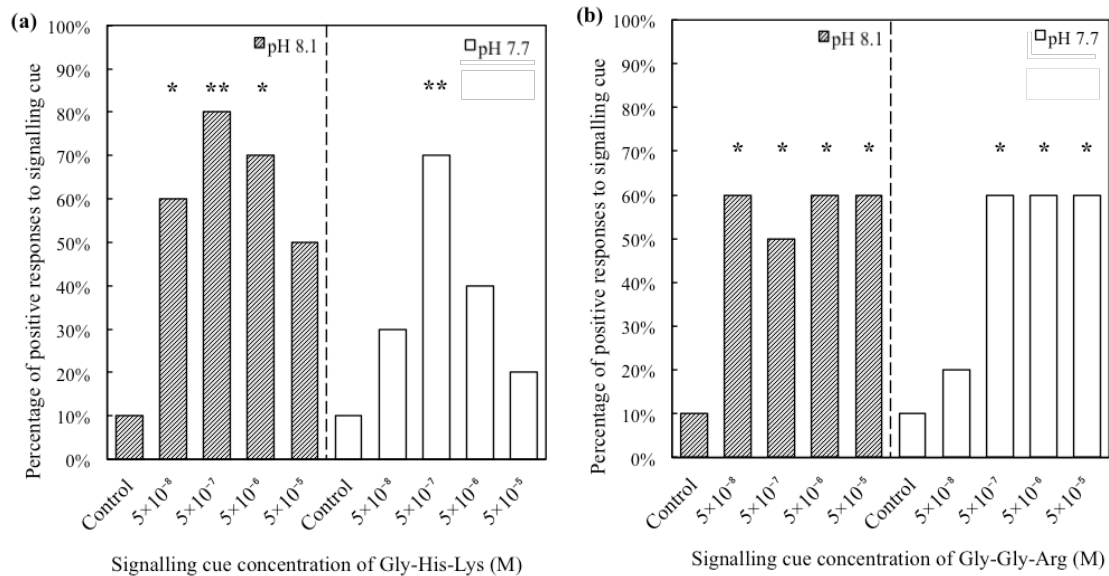
**Fig. 4** Effects of pH on peptide-mediated egg-ventilation behaviour of shore crabs. Percentage of positive egg-ventilation responses of female *Carcinus maenas* to increasing concentrations of the signalling cues glycyl-L-histidyl-L-lysine (GHK, a) and glycyl-glycyl-L-arginine (GGR, b) in two different pH test conditions. Results for pH 8.1 are shown in grey (left) and for pH 7.7 in white (right). Significant differences between the proportion of positive answers at each concentration and the proportion of positive answers in controls with seawater are indicated by asterisks with * for a significance level of $p < 0.05$ and ** for $p < 0.01$ (F-test, n=10).

In order to further investigate the factors causing the observed change in threshold concentration, bioassays were also performed after animals were left to acclimate in a pH 7.7 environment for one week. It was assumed that their metabolism and physiological processes such as acid-base regulation would be clearly affected by then (Pörtner *et al.*, 2004; de la Haye *et al.*, 2012; Henry *et al.*, 2012) and potentially cause inhibition of the behavioural response if these were the main influencing factors. Crabs acclimated to low pH failed to respond to the cue at any tested concentration in pH 7.7 test conditions. This highlights the important role of physiology and metabolism in the inhibition of peptide-mediated behaviour. However, even after a seven-day acclimation to pH 7.7, when returned to pH 8.1 a significant

number of shore crabs responded immediately to the signalling cues (Fig. 5). There was not enough time for the crabs to re-acclimate. This reversible effect on the ability of the shore crabs to detect the signalling cues indicates that although changes to metabolism and physiology play an important role, the negative effect of lower pH on immediate behavioural response to a signalling cue is mainly caused by impaired signal reception.
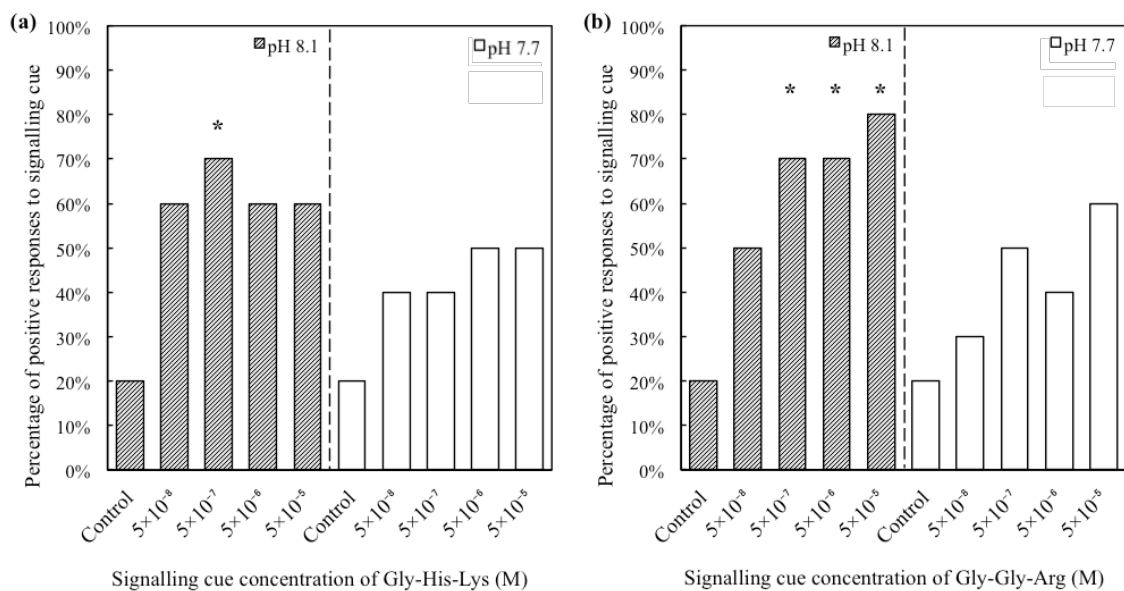


**Fig. 5** Effects of pH on peptide-mediated behaviour of shore crabs after acclimation to pH 7.7. Percentage of positive egg-ventilation responses of female *Carcinus maenas* to increasing concentrations of the signalling cues glycyl-L-histidyl-L-lysine (GHK, a) and glycyl-glycyl-L-arginine (GGR, b) in two different pH test conditions after one week of acclimation in pH 7.7. Results for pH 8.1 are shown in grey (left) and for pH 7.7 in white (right). Significant differences between the proportion of positive answers at each concentration and the proportion of positive answers in controls with seawater are indicated by asterisks with * for a significance level of $p < 0.05$ (F-test, n=10).

460　**Discussion**

461　Chemical communication amongst organisms involves a sender, a receiver and signalling

462　molecules that carry the information from one to the other. Successful signal reception by the

463　receiver depends on the interaction of the signalling molecule and a receptor, which triggers a

464　cellular response. Changes caused by pH to the components involved in signal reception,

465　including the signalling cues as well as the receptors, could therefore significantly impair

466　chemoreception and so alter the associated behaviour.

467

468　*Potential influence of pH on receptor-ligand interaction crucial for signal reception*

469　Our results show that the investigated peptide signalling molecules are likely to change

470　reversibly with pH. They are not only susceptible to protonation with progressing ocean

471　acidification but are also likely to change conformation and charge distribution. The exact

472　receptor and binding site involved in mediating egg-ventilation behaviour in crustaceans are

473　both currently unknown. However, according to Rittschof *et al.* (1990), the binding site likely

474　resembles the catalytic site of a trypsin-like serine protease. Pettis *et al.* (1993) suggested that

475　the binding site contains a hydrophobic component and a positively charged group a few

476　amino acids away. The hydrophobic binding site component could interact with hydrophobic

477　parts of the peptide molecules, especially at their N-terminal and central amino acids. The

478　positively charged binding site group is likely to interact with the peptide carboxyl group. The

479　authors also found that the length of the peptide's hydrophobic side chains and the partial

480　charge of the L-arginine guanidinium side chain significantly affect binding affinity (Pettis *et*

481　*al.*, 1993). The large neutral areas and distinct negatively charged patch at the carboxyl group

482　found in the protonation states of GHK, GGR and LR at today's oceanic pH conditions

483　provide a good match to this proposed receptor model. However, the protonated forms of the

484　peptide cues found at pH 7.7 do differ significantly from those present at pH 8.1 in terms of

485  their conformation and their electrostatic properties. The stimulation of a receptor by a

486  signalling molecule depends on the signalling molecule's functional groups, charge, shape,

487  hydrophobicity and flexibility (Wyatt, 2014a). As some of these characteristics, especially

488  charge, shape and hydrophobicity are likely to be significantly altered by pH for all three

489  peptides in this study, it can be assumed that a successful interaction of the protonated peptide

490  cues and the proposed receptor would be less likely.

491  This correlates with our observation that shore crabs tested in low pH conditions required a

492  higher signalling cue concentration before showing a behavioural response compared to

493  normal pH conditions. An increased threshold concentration can be linked to a lower binding

494  affinity between the signalling molecule and the receiving receptor proposed by Rittschof *et*

495  *al.* (1989). Therefore our results suggest a pH-dependent reduction of binding affinity. This

496  could be linked to the observed significant changes of the signalling molecules and the

497  potential mismatch of signalling cues and receptors in future oceanic conditions. Many

498  receptors and ligands involved in chemical signalling processes are known to be highly

499  specific to avoid eavesdropping and enable species specificity (Wyatt, 2014a). Even small

500  changes to either the molecules or receptors can have significant effects on the binding

501  affinity (Reisert & Restrepo, 2009).

502

503  *Could pH effects on signal reception explain altered behaviour?*

504  The extent to which ocean acidification may impair signal reception is difficult to estimate.

505  On the one hand, our p$K_a$ results show that there will be changes in the abundance of the

506  different peptide protonation states. Not all signalling molecules are protonated within the pH

507  range associated with ocean acidification. However, there will be approximately 23% less of

508  the current bioactive peptide forms available in future pH conditions. This translates into a 1.3

509 times higher concentration of the molecule required in solution to elicit a behavioural

510 response.

511 On the other hand, the bioassay experiments showed that the impaired shore crab behaviour at

512 pH 7.7 could only be compensated by a much higher ($\geq$ tenfold increased) signalling cue

513 concentration. This overcompensates the loss of bioactive molecules in low pH conditions

514 calculated above and could be seen as discrepancy between the scale of change in signalling

515 molecule properties and the extent of impact on the behaviour. However, it has to be

516 considered that behaviour is influenced by a multitude of factors including animal physiology

517 and metabolism as well signal reception and decision-making (see Table 1 in Briffa *et al.*,

518 2012). Signal reception itself could not only be affected by pH-induced changes of the

519 signalling molecules but also the corresponding receptor sites. Possible vulnerability of the

520 receiving receptors and in particular their active binding sites to pH has been already

521 hypothesised by Tierney and Atema (1988). Changes to receptors through protonation would

522 potentially change the number, type and alignment of intermolecular forces (e.g. hydrogen

523 bonding, electrostatic forces and hydrophobic regions) required for the successful interaction

524 between ligand and receptor (Hardege *et al.*, 2011; Wyatt, 2014a). This would exacerbate the

525 effect of pH on signal reception and concurrently the chemically mediated behaviour and

526 explain the much higher concentration of the cues required at low pH.

527 The importance of signal reception in the context of behaviour affected by ocean acidification

528 is illustrated by the results of our second set of bioassays. The shore crabs were able to

529 immediately restore their chemically mediated behaviour when returned to normal pH

530 conditions despite being acclimated to low pH conditions for a week (Fig. 5). Lower overall

531 response levels of crabs acclimated to pH 7.7 compared to pH 8.1 (Fig. 5 vs. 4) suggest an

532 impact of the low-pH incubation on crab physiology. However, it was not sufficiently high to

533 generally and fully impair the crab's behavioural response to the signalling molecules in

534   normal pH. This could suggest that signal reception is not significantly hindered by

535   physiological and metabolic acclimation to future pH conditions but by pH affecting the

536   signal reception mechanism. For our system, physiological and metabolic effects associated

537   with low pH conditions, such as the impact of acid-base regulation on signal transduction

538   (Nilsson *et al.*, 2012) or changes to the organism's fitness level (Pörtner *et al.*, 2004), were

539   therefore found to possess less significant influence.

540

541   *The different mechanisms behind altered behaviour in high $CO_2$ conditions*

542   Our results clearly identify the pH-induced change to peptide signalling molecules and the

543   associated impairment of signal reception as important mechanistic components to explain the

544   observed changes of chemically mediated behaviour in high $CO_2$ conditions.

545   This contrasts with the statements of Leduc *et al.* (2013) and Munday *et al.* (2009), who

546   excluded pH-induced effects for signalling molecules as likely reason of reduced behavioural

547   responses in marine organisms. However, as in most biological studies their experimental

548   design uses conditioned water with an unknown composition and concentration of signalling

549   cues. The use of compound mixtures poses the risk of synergistic or antagonistic effects and

550   does not allow assessing the impact of pH on the specific chemical(s) that trigger the

551   observed behaviour. It is important to note that many chemical cues in nature are actually

552   bouquets of chemicals (multicomponent) that are received in combination (Wyatt, 2014a).

553   However, peptide cues in particular are often single, unique cues due to their specific

554   sequence (Wyatt, 2014a). Conditioning water, e.g. by exposure to a predator for several hours

555   (Munday *et al.*, 2010), further inherits the risk of exceeding natural concentrations, which

556   could affect the specificity of the cues and the corresponding behaviour (Wyatt, 2014a). In

557   contrast, our choice of a test-system with known signalling molecules and concentrations

558   close to their threshold values (thus close to natural concentrations) allowed us to particularly

559  focus on the effects of pH on the individual signalling molecules and their biological

560  functionality.

561  Our results agree with findings of de la Haye *et al.* (2012), who observed significant effects of

562  pH the chemoreceptive ability of hermit crabs (*Pagurus bernhardus*) to food odours. Their

563  experimental set-up with cue preparation in different pH conditions further allowed testing for

564  irreversible changes to the chemical cues and potential effects. They found no indication of

565  covalent changes to the cues mediating the foraging of the hermit crabs (de la Haye *et al.*,

566  2012). However, reversible changes to the molecules were not investigated. They also found

567  no significant correlation between behavioural and physiological factors measured, for

568  example internal $Cl^-$ ion concentration, despite a five-day pH acclimation prior to the

569  experiments (de la Haye *et al.*, 2012). This contradicts the hypothesis of Nilsson *et al.*, who

570  suggested that the pH-induced physiological acid–base regulation interferes with the signal

571  transduction in marine species, particularly those using $HCO_3^-$ and $Cl^-$ to control their acid–

572  base balance (Nilsson *et al.*, 2012). Based on our study and that of de la Haye *et al.* (2012) it

573  seems that the mechanism by which pH affects chemically mediated behaviour in crustaceans

574  differs significantly from the mechanism proposed by Nilsson *et al.* (2012) for fish. Although

575  some processes of acid-base regulation in fish and crustaceans show similarities, e.g. the use

576  of cation and anion exchangers (Henry *et al.*, 2012), significant differences have also been

577  found. While in fish and molluscs the internal $Cl^-$ ion concentration decreases in acidified

578  waters, the haemolymph $[Cl^-]$ in crabs increases (Dodd *et al.*, 2015). Our chosen species (*C.*

579  *maenas*) was found to be significantly affected in its behaviour by pH, although it is known to

580  be highly adaptable to various environmental conditions (Compton *et al.*, 2010) and resilient

581  towards future ocean conditions (Hall-Spencer & Allen, 2015). Further indication of an

582  important mechanism other than acid-base balance affecting chemically mediated behaviour

583  is given in the comprehensive review of Clements & Hunt (2015) where they list diverse

584   effects of elevated $CO_2$ conditions on animal behaviour. This diversity of responses, even

585   amongst fish, cannot be explained by one mechanism alone.

586   In freshwater systems, acidified conditions have also been reported to significantly reduce the

587   response of fish and crayfish to food stimuli and alarm cues (Lemly & Smith, 1987; Leduc *et*

588   *al.*, 2013). In this context, Brown *et al.* (2002) showed that even weakly acidified conditions

589   could cause covalent, irreversible change to a signalling molecule and render it non-functional

590   as alarm cue for fathead minnows (*Pimephales promelas*). Their study presents the only

591   investigation of a specific signalling cue structure in the context of pH to date. Freshwater

592   systems are assumed to suffer from more acidic conditions and greater pH changes than the

593   well-buffered marine environment (Leduc *et al.*, 2013). The smaller pH fluctuations in the

594   ocean may reduce the likelihood of covalent changes to signalling molecules. However, this

595   does not preclude reversible changes of signalling molecules within the oceanic pH range as

596   we have shown here.

597

598   *Are the findings for our system transferable to other systems and cues?*

599   All three peptides investigated in our study were found to show similar changes in

600   conformations and electrostatic properties with pH despite their physical and chemical

601   differences. The abundances of their bioactive forms were also reduced in a similar manner in

602   future oceanic pH conditions. This could suggest that the results presented here could be

603   transferable to other similar peptides and could have mechanistic implications beyond the

604   system we investigated.

605   We used female shore crabs (*C. maenas*) with eggs as test system and their chemically

606   mediated egg ventilation behaviour had not been investigated before. However, we found that

607   the signalling cues GHK and GGR trigger the same stereotyped behavioural response in shore

608   crabs as reported for mud crabs (*R. harrisii*) (Forward Jr *et al.*, 1987). Structurally similar

609    peptide cues are also known to mediate egg-ventilation behaviour in blue crabs (*Callinectes*

610    *sapidus*) (Darnell & Rittschof, 2010) and different species of shrimp (Reinsel *et al.*, 2014).

611    Rittschof (1990) already suggested that peptide cues generated from protein degradation with

612    a trypsin-like serine protease could be a common theme. These peptide cues contain a number

613    of neutral residues like glycine or L-leucine in combination with a basic residue such as L-

614    arginine or L-lysine at the carboxyl terminus. They are not only known to mediate egg-

615    ventilation and larval release in brachyuran crabs (Rittschof & Cohen, 2004) but also play a

616    significant role in the location of a new shell by hermit crabs (Kratt & Rittschof, 1991) and

617    the settlement of barnacle and oyster larvae (Tegtmeyer & Rittschof, 1989; Zimmer-Faust &

618    Tamburri, 1994; Browne & Zimmer, 2001). We therefore consider our system and the results

619    obtained as representative for a number of different behaviours, which could be affected by

620    ocean acidification. In fact, peptides and amino acid derived cues mediate a vast number of

621    diverse behaviours that can affect species and communities and even have an impact at

622    ecosystem level (Hay, 2009; Wyatt, 2014b). Peptides similar to the mimics tested in our

623    study, for example, attract predatory snails to sites of barnacle settlement while

624    simultaneously functioning as settlement cues (Rittschof, 1990). These peptides are therefore

625    highly important in structuring communities. Based on our results, many of these interactions

626    could be highly influenced by pH and therefore potentially vulnerable to change with on-

627    going ocean acidification. However, there might be also systems where the organisms are

628    adapted to respond to the protonated peptide forms, for example in systems where pH is

629    naturally low, such as near $CO_2$ vents.

630

631    *Future perspective*

632    Our study presents, to the best of our knowledge, the first interdisciplinary investigation of

633    reversible molecular effects of pH on signalling cues and the associated peptide-mediated

634     behaviour in marine environments. We conclude from our results that the change of signalling

635     molecules by pH is an important mechanistic effect of ocean acidification, which could

636     explain some of the changes in chemically mediated behaviour not caused by physiological

637     influences. Future research needs to focus on the mechanism as well as the ecological

638     implications of the direct influence of pH on signal reception. In order to fully understand the

639     underlying processes, we currently determine the pH-dependent quantitative relationship

640     between signalling cue concentration and behavioural response as well as the actual signal

641     reception by electrophysiological methods. We are further attempting an estimation of

642     naturally occurring cue concentrations to better estimate the extent of ecological impact.

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826 Supporting information caption:

827     - SI_OA affects signalling cues_Method-details.pdf