Ocean acidification affects marine chemical communication by changing structure and function of peptide signalling molecules

Running head: Ocean acidification affects signalling cues

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Abstract

Ocean acidification is a global challenge that faces marine organisms in the near future with a predicted rapid drop in pH of up to 0.4 units by the end of this century. Effects of the change in ocean carbon chemistry and pH on the development, growth and fitness of marine animals are well documented. Recent evidence also suggests that a range of chemically mediated behaviours and interactions in marine fish and invertebrates will be affected. Marine animals use chemical cues, for example, to detect predators, for settlement, homing and reproduction. But while effects of high CO_2 conditions on these behaviours are described across many species, little is known about the underlying mechanisms, particularly in invertebrates. Here we investigate the direct influence of future oceanic pH conditions on the structure and function of three peptide signalling molecules with an interdisciplinary combination of methods. NMR spectroscopy and quantum chemical calculations were used to assess the direct molecular influence of pH on the peptide cues and we tested the functionality of the cues in different pH conditions using behavioural bioassays with shore crabs (*Carcinus maenas*) as a model system. We found that peptide signalling cues are susceptible to protonation in future pH conditions, which will alter their overall charge. We also show that structure and electrostatic properties important for receptor-binding differ significantly between the peptide forms present today and the protonated signalling peptides likely to be dominating in future oceans. The bioassays suggest an impaired functionality of the signalling peptides at low pH. Physiological changes due to high CO_2 conditions were found to play a less significant role in influencing the investigated behaviour. From our results we conclude that the change of charge, structure and consequently function of signalling molecules presents one possible mechanism to explain altered behaviour under future oceanic pH conditions.

Abbreviations: CO_2, carbon dioxide; GGR, Gly-Gly-Arg, glycyl-glycyl-L-arginine; GHK, Gly-His-Lys, glycyl-L-histidyl-L-lysine; LR, Leu-Arg, L-leucyl-L-arginine; NMR, nuclear 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

could affect the neural mechanisms required for processing information (Nilsson *et al.*, 2012). Thirdly, the reduced behavioural response may be due to disruption of the signal reception, which itself can have a number of reasons. For example, the organism's ability to detect chemical cues, also referred to as chemoreception, could be impaired by physical damage to the receptive organs (Briffa *et al.*, 2012), alteration of the receptors (Tierney & Atema, 1988) or changes to the signalling molecules in low pH environments (Brown *et al.*, 2002). All of these effects lead to a reduced recognition between signalling cue and receptor. While physical damage to the receptive organs has already been investigated as potential factor (de la Haye *et al.*, 2012), the alteration of receptors and changes to signalling molecules have only been suggested based on behavioural bioassays in different conditions. Molecular evidence for these pH effects is scarce and only reported for one freshwater system with irreversible change to the molecules at very low pH conditions (Brown *et al.*, 2002). The effects of pH on signalling molecules in marine systems and in the context of ocean acidification have not yet been investigated on a molecular level.

Peptide-mediated behaviours are particularly suitable to investigate the pH-induced change of signalling molecules as a potential reason for altered behaviour in high CO_2 environments. Amino acids and therefore peptides possess a number of chemical functional groups that can be protonated (addition of a H^+) depending on the pH in the surrounding medium (see Fig. 1). This includes a carboxylic group at the C-terminus, an amino group at the N-terminus and other groups at the side chains if present. The pH conditions at which these groups will be protonated is group specific and expressed using pK_a values: negative logarithmic acid dissociation constants expressing the pH value at which 50% of the molecules in solution are deprotonated and 50% are protonated at the corresponding group.

We suggest that the change of pH in future oceans could lead to profound changes in protonation states of peptide signalling molecules containing groups with pK_a values close to 8, which in turn may lead to significant alterations in their structure and function.

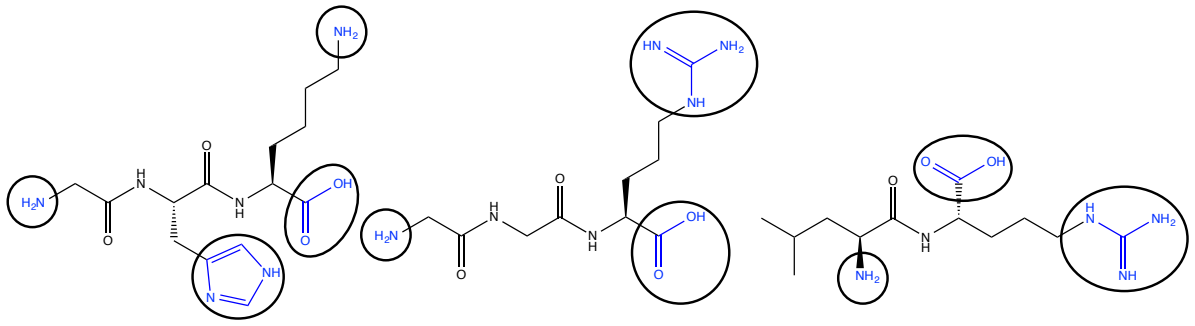
To test this hypothesis and understand the real impact of pH to the signalling cues and the associated behaviour requires a molecular approach using interdisciplinary tools and methods. Therefore in this study we combine for the first time NMR spectroscopy with quantum chemical calculations and bioassays to obtain a more complete picture of the direct molecular impacts of ocean acidification. As a model system we focus on three peptides that mimic cues for egg ventilation in crustaceans: two tripeptides glycyl-L-histidyl-L-lysine (GHK) and glycyl-glycyl-L-arginine (GGR) as well as the dipeptide L-leucyl-L-arginine (LR).

First, we assess the peptides' susceptibility to protonation with increasing ocean acidification through NMR spectroscopic determination of the pK_a values for each ionisable group. These values are also used to calculate the abundance of the different protonation states over the pH range. Secondly, we explore the differences in conformation and charge distribution of the relevant protonation states using quantum chemical calculations. Thirdly, we test the effects of pH on the peptides' functionality in behavioural bioassays. These experiments also aim to establish whether signal reception or physiological and neurological changes play a more significant role in causing behavioural changes with pH. We discuss how changes in signalling molecules with pH could be linked to change in peptide-mediated behaviour through impaired chemoreception. Finally, we evaluate the transferability of our model system and the ecological significance of our results before giving an overview of possible consequences and perspectives.

Materials and methods

Choice of signalling molecules & model system

For our study of the direct influence of pH on structure and function of peptide signalling molecules we chose glycyl-L-histidyl-L-lysine (GHK), glycyl-glycyl-L-arginine (GGR) and L-leucyl-L-arginine (LR). These three peptide cues are synthetic mimics of the yet-unidentified natural signalling molecules known to mediate egg ventilation (Forward Jr *et al.*, 1987). The three mimics are good model systems as they have a chemically diverse amino acid sequence and side chains, but display the same documented biological function (egg ventilation). The use of a well-defined system with a known chemical signalling cue and a stereotyped behaviour allowed us to link molecular changes to the signalling cue function. Egg ventilation is a naturally occurring stereotyped behaviour of female decapods carrying an egg clutch (Reinsel *et al.*, 2014). Regular probing and movement of the eggs, which are attached to the female's abdomen, ensures oxygen supply, waste removal and larval development (Crothers, 1967; Reinsel *et al.*, 2014). This behaviour is mediated by peptides released from the eggs, allowing chemical communication between the female and her brood (Reinsel *et al.*, 2014). The ventilation frequency depends on the developmental stage of the embryos (De Vries & Forward Jr, 1991) and peaks during larval release, allowing synchronised hatching (Forward Jr *et al.*, 1987; Reinsel *et al.*, 2014).



(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 (δ) of each nucleus that could be obtained over the pH range were plotted against the sample
189 pH. The pK_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 pK_a value obtained from the closest suitable ^{1}H nucleus was
192 used.

193 Based on the pK_a, the concentration and therefore proportion of each protonation state over
194 the pH range could be calculated using the Henderson—Hasselbalch equation

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

196 that relates the pH to the pK_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

approach generates 5000 random starting conformers for each state/peptide and then performs up to 2000 optimisation steps towards the minimum for each conformer using the mmff94 force field (Halgren, 1996). The resulting optimised conformers are then ranked and the lowest energy conformer is used as starting conformation for another cycle of random conformer generation and subsequent optimisation. The final conformer obtained after three such cycles was further optimised using the PBE0 exchange correlation functional (Adamo & Barone, 1999) with a pc-2 basis set (Jensen, 2001, 2002a, 2002b) and water as implicit solvent using COSMO (Klamt, 1995) implemented in the ORCA suite of programs (Version 3.0.0) (Neese, 2012). We used the RIJ-COSX approximation (Neese *et al.*, 2009) with a def2-TZVPP/J auxiliary basis set (Weigend & Ahlrichs, 2005) and included D3 dispersion corrections following Grimme (Grimme *et al.*, 2010, 2011). The VeryTightSCF and TightOpt criteria implemented in ORCA were used to stop the SCF gradient and the optimisation at a total energy change of $< 10^{-8}$ E_h respectively. The calculation of the molecular electrostatic potential (MEP) was performed with the GAMESS program (vJan122009R1) using the Perdew-Burke-Ernzerhof exchange functional (PBE) (Perdew *et al.*, 1996) in conjunction with a STO-3G basis set (Hehre, 1969). A three-dimensional electron density isosurface was visualised with 100 grid points, a medium grid size and a contour value of 0.03 $e{\cdot}a_0^{-3}$ using the wxMacMolPlot program (v7.5141) (Bode & Gordon, 1998). The density isosurface was coloured according to the MEP with a maximum value of 0.25 $E_h{\cdot}e^{-1}$ and the RGB colour scheme with red representing positive, green neutral and blue negative charge.

To validate our approach and the obtained conformations, we compared the experimental chemical shifts measured during the pK_a determination with calculated ^{1}H NMR chemical shifts of GHK II and GHK III. The shielding values of ^{1}H nuclei were calculated at the PBE0/aug-pc-2 level of theory, using the RIJ-COSX approximation with a def2-TZVPP/J 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

(100 µL) and a further 5 min counting interval. Tests were performed with 10 replicates in natural (pH 8.1 ± 0.1) and future (pH 7.7 ± 0.1) oceanic pH conditions for four concentrations per peptide. The concentration range and steps were chosen based on the threshold values published for mud crabs (10^{-9} M, mixed uniformly in bowl) (Forward Jr *et al.*, 1987) and adapted to the average volume around a shore crab (50 mL) due to the application as a signal trail next to the crabs abdomen. This yielded a concentration range of 10^{-10} M to 10^{-7} M around the crab with cue solutions ranging from 5 x 10^{-8} M to 5 x 10^{-5} M (see SI for details on cue solution preparation).

In order to estimate the extent of physiological and neuronal impairment of the ventilation behaviour relative to the chemoreceptive ability, the complete set of bioassays for both cues was performed twice: with crabs kept in natural pH conditions (pH 8.1) for at least 4 days prior to experiments and crabs pre-acclimated to pH 7.7 for one week. All crabs were tested in both pH conditions, however they were tested first in the conditions they were kept in. For example, crabs acclimated to pH 7.7 were tested first in pH 7.7 before being tested in pH 8.1 and vice versa.

The natural egg ventilation frequency varies greatly amongst individuals with 3.2 (± 3.1) strokes per 5 min in pH 8.1 and 4.7 (± 2.6) strokes per 5 min in pH 7.7. Hence the experimental set up described above was chosen to allow for direct immediate comparison. A higher ventilation frequency after addition of the cue was counted as positive response. The ratio of positive to no responses was compared pairwise between the seawater control and the treatment with a given concentration of one of the cues using a one-sided Fisher's exact test of independence (F-test) of the "R" statistical package (version 3.1.2, R Development Core Team 2014). This test allows testing for differences between two proportions of nominal variables with a small sample size (McDonald, 2014). Significant differences to the control 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 pK_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 pK_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 pK_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 ≥ 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 $pK_a \approx 15$ 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 pK_a values
297 within an ocean pH range likely to be experienced before the end of this century. The pK_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 pK_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 pK_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 pK_a determination.
307
308

Table 1 pK_a values (± SD) of the ionisable groups of the signalling peptides glycyl-L-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]

[a] No accurate pK_a for the Arg side chain could be obtained due to an insufficient number of data points for curve fitting ≥ pH 12. However, a literature value of 12.1 (Lide, 2004) strongly suggests that the Arg side chain will not be affected by ocean acidification.

Based on the determined group-specific pK_a values and the Henderson–Hasselbalch equation, the abundance of the different protonation states over the pH range can be calculated and is shown in Fig. 2. We found that upon acidification there will be a 23% decrease of the currently present GHK and LR protonation states and a 22% decrease of the current GGR form. In turn there will be a corresponding increase of peptide forms protonated at the N-terminus. In the case of GHK a second protonation state, which is additionally protonated at the L-histidine side chain, becomes more prominent at low pH. These protonated forms are positively charged while the currently present forms are overall neutral (zwitterionic).

Our results suggest that peptide cues are highly susceptible to pH alteration and that a change in abundance from neutral to positively charged protonation states will occur with on-going acidification.

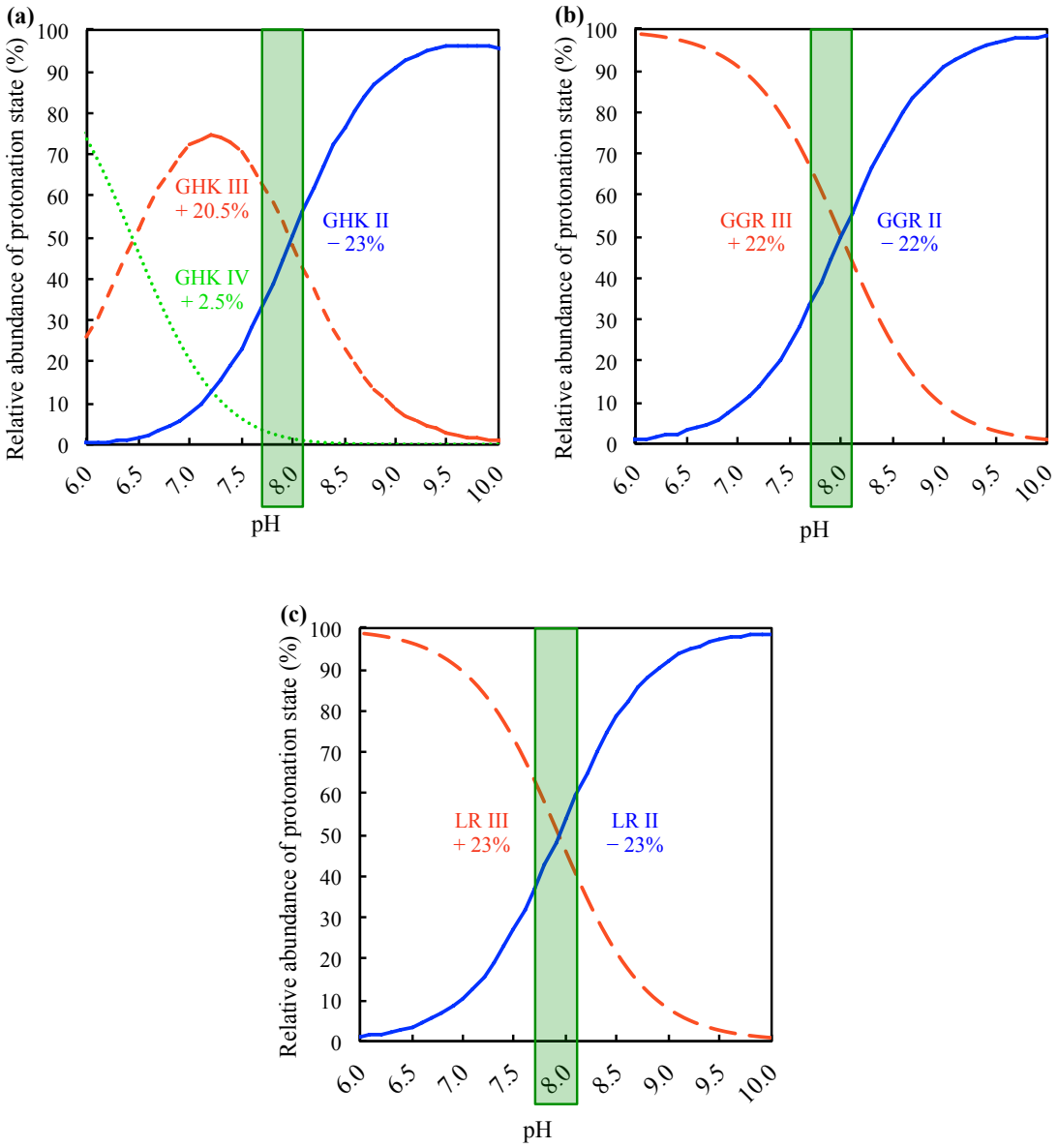


Fig. 2 Relative abundance of individual protonation states of glycyl-L-histidyl-L-lysine (a), glycyl-glycyl-L-arginine (b) and L-leucyl-L-arginine (c) and their percentage change with ocean acidification. Proportions are shown for protonation states present between pH 6 and 10. The green shaded area 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.

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 pK_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.

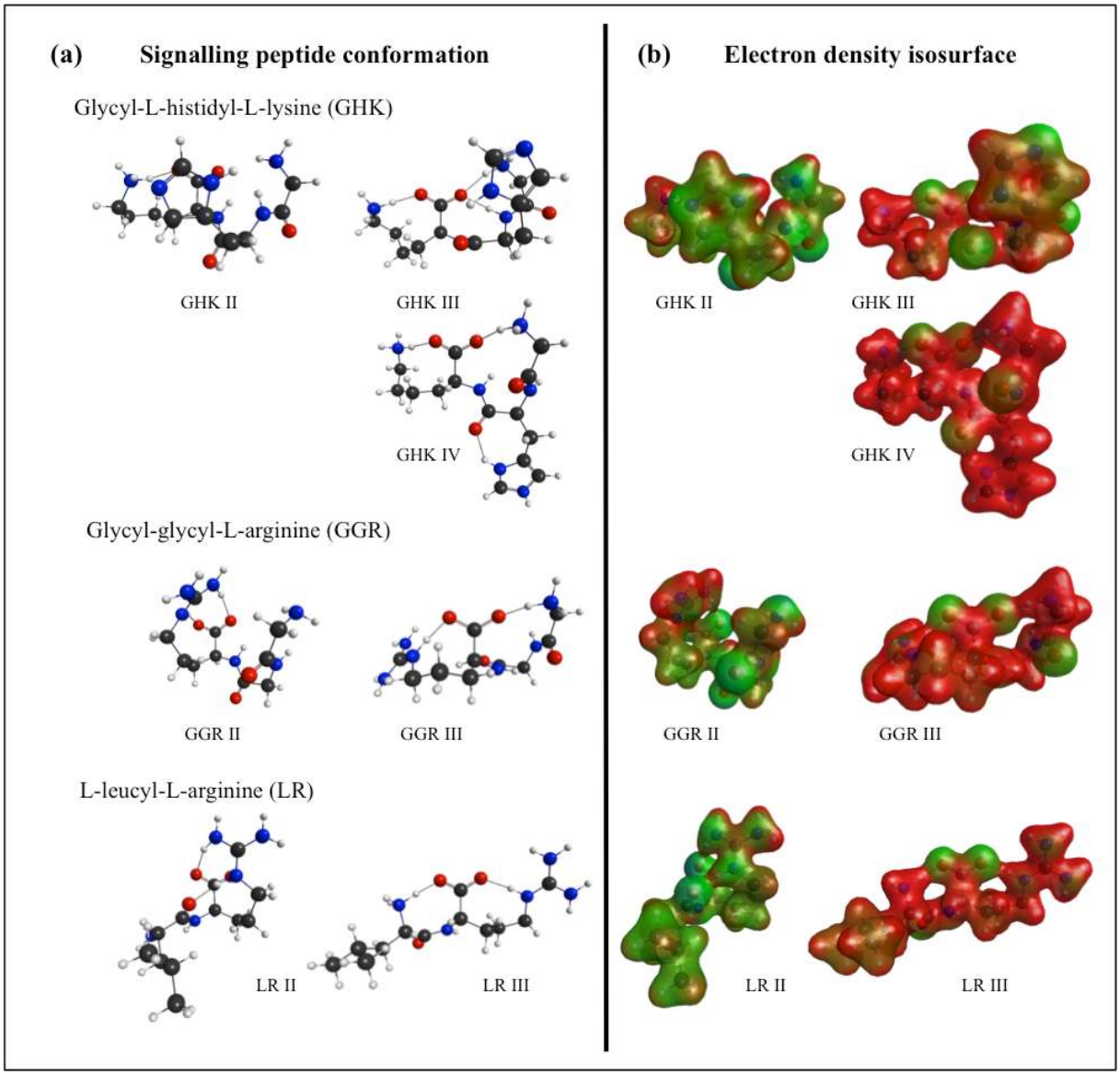


Fig. 3 Conformations and charge distribution of the protonation states of GHK, GGR and LR. (a) Conformations of the different peptide protonation states with carbon atoms in black, hydrogen in white, nitrogen in blue and oxygen in red. (b) Electron density isosurfaces (contour value 0.03 $e \cdot a_0^{-3}$) 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.

We also compared experimentally obtained and quantum chemically calculated ^{1}H chemical shift values of GHK II and GHK III. Very similar approaches have been previously used for structure determination and validation of compounds in solution (see for example Lodewyk *et al.*, 2012). The chemical shift of a nucleus is influenced by the position of all neighbouring nuclei, which can cause either deshielding or shielding effects from the applied magnetic field during the NMR experiment. ^{1}H shifts have been shown to be sensitive to chemical structure and even small conformational changes can result in significant variations of the corresponding proton shifts (Hunter *et al.*, 2005). Therefore a comparison between the measured and calculated proton chemical shifts enables us to assess if the calculated conformations are in agreement with the protonation state conformations present in solution. For most protons of GHK II (RMSD: 0.43 ppm) and GHK III (RMSD: 0.68 ppm) the experimental and calculated values agreed within an error margin of 0.7 ppm. Only two protons differed by 0.9 ppm and 1.6 ppm for GHK II and GHK III respectively (see Table S1). This shows that there is still some need for refinement and we suggest that including solvent effects could help to explain the few observed deviations. However, the agreement between experimental and calculated proton chemical shift values suggests that the conformers obtained by our quantum chemical calculations are reasonably accurate models of the observed peptides and validates the chosen approach.

Note that the same approach is used for all protonation states, which also allows direct comparison between them. All three peptides consistently show similar trends and display less compact conformation and more uniformly distributed (positive) charge upon protonation. This stresses that there are considerable differences in conformation and MEP between protonation states present in today's oceans and those that will be present in future oceans.

405 *Effects of pH on peptide-mediated behaviour*

406 When tested in pH 8.1, a significant number of shore crabs increased the egg ventilation
407 frequency compared to the control in response to 5 x 10^{-8} M of cue. This corresponds to a
408 concentration of 10^{-10} M around the crab (50 mL) or 5 x 10^{-12} M in the bowl (1L). Future
409 oceanic pH conditions negatively affected the behavioural response to both cues. In pH 7.7,
410 an at least tenfold higher concentration (5 x 10^{-7} M, 10^{-9} M around the crab, 5 x 10^{-11} M in the
411 bowl) than at pH 8.1 was required for a significant number of crabs to respond to GHK and
412 GGR (Fig. 4). The natural concentration has not been reported in the literature as the natural
413 cue is unknown to date. However, studies with synthetic cue mimics triggering this behaviour
414 found similar or slightly higher threshold values for several shrimp species and mud crabs
415 (Forward Jr et al., 1987; Reinsel et al., 2014).
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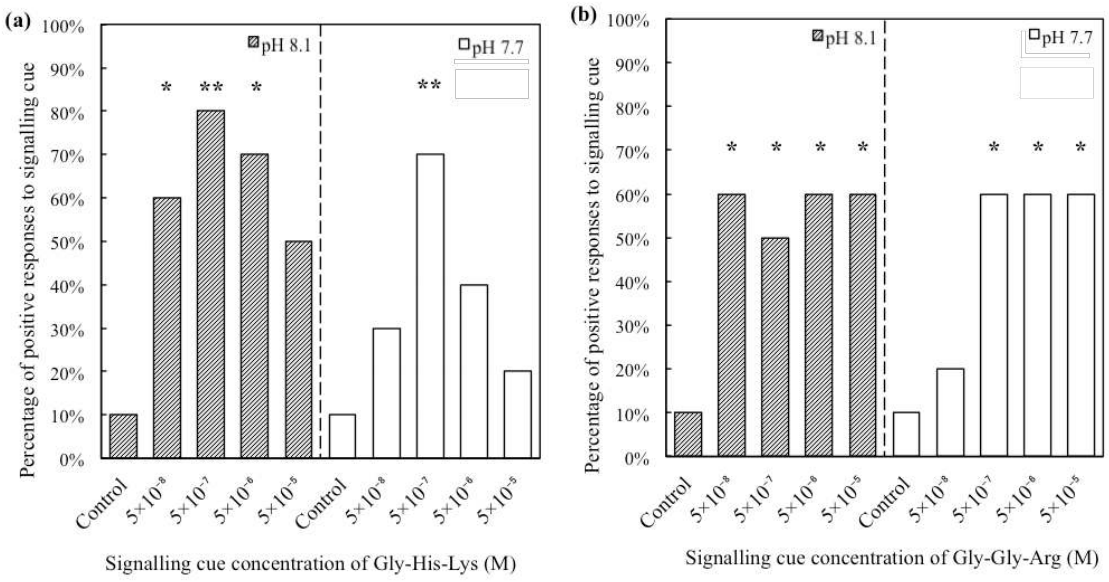

423

424 **Fig. 4** Effects of pH on peptide-mediated egg-ventilation behaviour of shore crabs. Percentage
425 of positive egg-ventilation responses of female *Carcinus maenas* to increasing concentrations
426 of the signalling cues glycyl-L-histidyl-L-lysine (GHK, a) and glycyl-glycyl-L-arginine
427 (GGR, b) in two different pH test conditions. Results for pH 8.1 are shown in grey (left) and
428 for pH 7.7 in white (right). Significant differences between the proportion of positive answers
429 at each concentration and the proportion of positive answers in controls with seawater are
430 indicated by asterisks with * for a significance level of $p < 0.05$ and ** for $p < 0.01$ (F-test,
431 n=10).

432

433

434 In order to further investigate the factors causing the observed change in threshold
435 concentration, bioassays were also performed after animals were left to acclimate in a pH 7.7
436 environment for one week. It was assumed that their metabolism and physiological processes
437 such as acid-base regulation would be clearly affected by then (Pörtner *et al.*, 2004; de la
438 Haye *et al.*, 2012; Henry *et al.*, 2012) and potentially cause inhibition of the behavioural
439 response if these were the main influencing factors. Crabs acclimated to low pH failed to
440 respond to the cue at any tested concentration in pH 7.7 test conditions. This highlights the
441 important role of physiology and metabolism in the inhibition of peptide-mediated behaviour.
442 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.

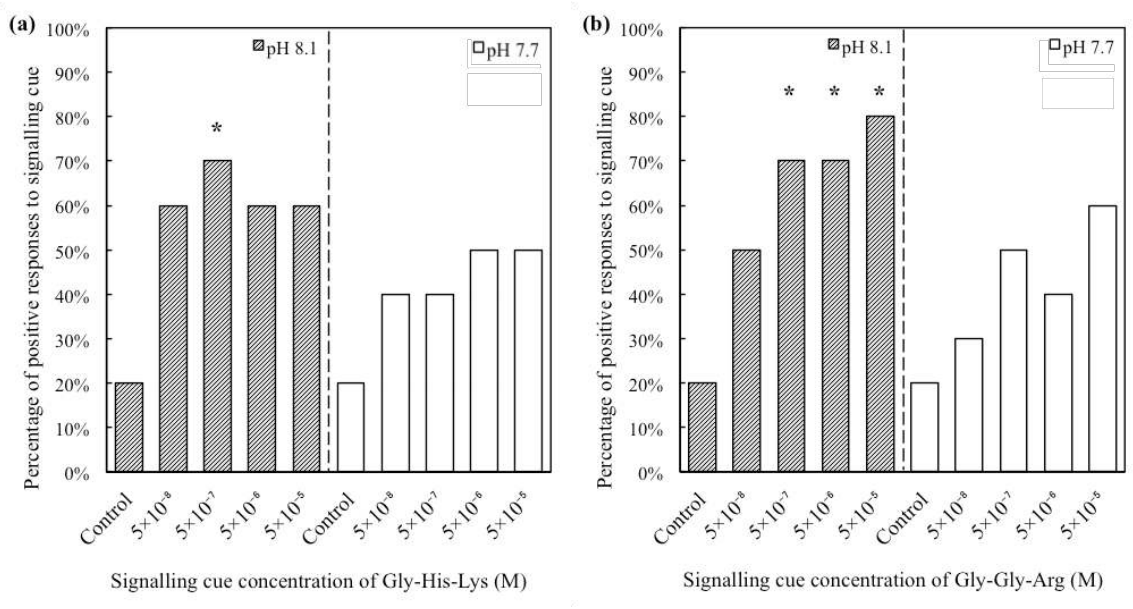

Fig. 5 Effects of pH on peptide-mediated behaviour of shore crabs after acclimation to pH 7.7. Percentage of positive egg-ventilation responses of female *Carcinus maenas* to increasing concentrations of the signalling cues glycyl-L-histidyl-L-lysine (GHK, a) and glycyl-glycyl-L-arginine (GGR, b) in 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).

Discussion

Chemical communication amongst organisms involves a sender, a receiver and signalling molecules that carry the information from one to the other. Successful signal reception by the receiver depends on the interaction of the signalling molecule and a receptor, which triggers a cellular response. Changes caused by pH to the components involved in signal reception, including the signalling cues as well as the receptors, could therefore significantly impair chemoreception and so alter the associated behaviour.

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

Our results show that the investigated peptide signalling molecules are likely to change reversibly with pH. They are not only susceptible to protonation with progressing ocean acidification but are also likely to change conformation and charge distribution. The exact receptor and binding site involved in mediating egg-ventilation behaviour in crustaceans are both currently unknown. However, according to Rittschof *et al.* (1990), the binding site likely resembles the catalytic site of a trypsin-like serine protease. Pettis *et al.* (1993) suggested that the binding site contains a hydrophobic component and a positively charged group a few amino acids away. The hydrophobic binding site component could interact with hydrophobic parts of the peptide molecules, especially at their N-terminal and central amino acids. The positively charged binding site group is likely to interact with the peptide carboxyl group. The authors also found that the length of the peptide's hydrophobic side chains and the partial charge of the L-arginine guanidinium side chain significantly affect binding affinity (Pettis *et al.*, 1993). The large neutral areas and distinct negatively charged patch at the carboxyl group found in the protonation states of GHK, GGR and LR at today's oceanic pH conditions provide a good match to this proposed receptor model. However, the protonated forms of the 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 pK_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 (≥ 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

normal pH. This could suggest that signal reception is not significantly hindered by physiological and metabolic acclimation to future pH conditions but by pH affecting the signal reception mechanism. For our system, physiological and metabolic effects associated with low pH conditions, such as the impact of acid-base regulation on signal transduction (Nilsson *et al.*, 2012) or changes to the organism's fitness level (Pörtner *et al.*, 2004), were therefore found to possess less significant influence.

The different mechanisms behind altered behaviour in high CO_2 conditions

Our results clearly identify the pH-induced change to peptide signalling molecules and the associated impairment of signal reception as important mechanistic components to explain the observed changes of chemically mediated behaviour in high CO_2 conditions.

This contrasts with the statements of Leduc *et al.* (2013) and Munday *et al.* (2009), who excluded pH-induced effects for signalling molecules as likely reason of reduced behavioural responses in marine organisms. However, as in most biological studies their experimental design uses conditioned water with an unknown composition and concentration of signalling cues. The use of compound mixtures poses the risk of synergistic or antagonistic effects and does not allow assessing the impact of pH on the specific chemical(s) that trigger the observed behaviour. It is important to note that many chemical cues in nature are actually bouquets of chemicals (multicomponent) that are received in combination (Wyatt, 2014a). However, peptide cues in particular are often single, unique cues due to their specific sequence (Wyatt, 2014a). Conditioning water, e.g. by exposure to a predator for several hours (Munday *et al.*, 2010), further inherits the risk of exceeding natural concentrations, which could affect the specificity of the cues and the corresponding behaviour (Wyatt, 2014a). In contrast, our choice of a test-system with known signalling molecules and concentrations 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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Supporting information caption:

- SI_OA affects signalling cues_Method-details.pdf