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## Impact of ocean acidification and warming on the feeding behaviour of two gastropod species

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### Abstract

Increased atmospheric CO<sub>2</sub> produced by anthropogenic activities will be absorbed by the oceans over the next century, causing ocean acidification and changes in seawater carbonate chemistry. Elevated CO<sub>2</sub> causes sublethal physiological and behavioural responses of the locomotion and foraging behaviour of marine organisms. This study aims to investigate the independent and synergistic effects of long term exposure to low pH and increased temperature on the feeding behaviour of two gastropod species, *Hexaplex trunculus* and *Nassarius nitidus*, both in adults and juveniles. The gastropods were kept under controlled temperature (ambient = 20°C, increased = 23°C) and pH (ambient = 8, low = 7.6) conditions for 2.5 years. The percentage of animals that successfully reached their food, response time, total time required to reach their food (duration), and total distance covered, was measured. The speed and path index (i.e. how straightforward the movement is) were estimated as means of foraging efficiency. Increased temperature (under ambient pH) resulted in faster responses, a shorter duration until food was reached and a higher speed in *H. trunculus* adults. *H. trunculus* (both adults and juveniles) were less successful in reaching their food source under low pH and ambient temperature compared to all other treatments. The response time, duration, speed and path index were not affected by low pH (at ambient or increased temperature) for *H. trunculus* adults and juveniles, as well as for *N. nitidus*. The foraging performance of juveniles hatched and developed under low pH (either at ambient or increased temperature) was more effective than the performance of adults of the same species, thus indicating a degree of acclimation. Also, the scavenger *N. nitidus* was more successful and responded faster in reaching carrion than the predator *H. trunculus*, whereas no significant effects were observed for *N. nitidus* under low pH.

**Keywords:** Climate change; *Hexaplex trunculus*; *Nassarius nitidus*; experiment; high temperature; low pH.

### Introduction

Climate change is greatly modifying the structure and function of marine and coastal ecosystems (Cramer *et al.*, 2018). The increase of greenhouse gas emissions is responsible for more than half of the observed increase in global average surface temperature during the last 60 years (IPCC, 2014). A linear temperature increase trend of about 0.85°C has been observed since the end of the 20th century (IPCC, 2014). In addition, an increase of more than 120 ppmv in global annual atmospheric CO<sub>2</sub> concentration during the last 250 years is responsible for the acidification of the oceans (Dlugokencky, 2016). Elevated quantities of CO<sub>2</sub> in the atmosphere are absorbed by the oceans causing a reduction of carbonate ions (CO<sub>3</sub><sup>-2</sup>)

and pH (ocean acidification) (Parker *et al.*, 2013). The mean pH levels have already declined by 0.1 units compared with pre-industrial levels and a further reduction of 0.3-0.5 units is expected by the end of the century (IPCC, 2014). Environmental stressors can act synergistically, antagonistically or additively, affecting many physiological processes of marine organisms (Gooding *et al.*, 2009) and causing stronger (positive, offsetting or negative) biological effects (Harvey *et al.*, 2013).

Marine molluscs are keystone species of the ecosystem affecting both community structure and ecosystem functioning (Gazeau *et al.*, 2013), and they comprise economically important species as they constitute 21.4 % of the global marine aquaculture production (17.1 million metric tonnes; FAO, 2018). Ocean acidification affects

shell-forming marine organisms such as molluscs, since the decline of  $\text{CaCO}_3$  saturation states (reduction of  $\text{CO}_3^{2-}$ ) inhibits calcification rates (Doney *et al.*, 2009; Ries *et al.*, 2009), or may cause dissolution of the existing shell (Garrard *et al.*, 2014). Shell growth in *Nucella lamellosa* was inhibited after short term exposure to low pH (Nienhuis *et al.*, 2010), whereas in *Strombus luhuanus* even a very moderate pH decrease (0.04 units) caused significant reduction of growth over a 6-month period (Shirayama & Thornton, 2005). Shell thickness of *Littorina littorea* was significantly reduced following a 1.3 unit pH reduction and the organisms were unable to thicken their shells in the presence of predators (Bibby *et al.*, 2007). Similarly, the shell density of *Nassarius nitidus* and *Columbella rustica* was reduced under the combined effect of reduced pH (-0.4 units) and increased temperature (+3°C) for 3 months (Chatzinikolaou *et al.*, 2017). Altered physiological responses have been recorded in marine molluscs as the result of global warming and ocean acidification, such as depression of the metabolic rate (Michaelidis *et al.*, 2005; Bibby *et al.*, 2007), reduction of ammonia excretion (Liu & He, 2012), alterations of immune responses and enzyme production (Matozzo *et al.*, 2012; 2013). Climate change can also affect behavioural responses related to enhancement of predator avoidance (Manriquez *et al.*, 2013; Bibby *et al.*, 2007), reduction of feeding efficiency (Vargas *et al.*, 2013) and movement restriction (Ellis *et al.*, 2009). Although the impact of ocean acidification on the growth and shell structure of gastropods has been widely investigated by earlier studies, only a few studies up to now have dealt with effects on feeding behaviour. An investigation of feeding performance under elevated  $\text{pCO}_2$  conditions is essential, since it has been suggested that when food availability and consumption are sufficient, some marine calcifiers are able to overcome the negative effects of ocean acidification and cover their increased energy demands (Thomsen *et al.*, 2013). On the contrary, species in which feeding success is reduced under low pH are more likely to be significantly impacted (Clements & Darrow, 2018). A recent review summarizing the effects of elevated  $\text{CO}_2$  on the feeding rates of calcifying marine invertebrates indicates consistent negative impacts on grazing and suspension feeding molluscs (Clements & Darrow, 2018). However, there is a gap of knowledge regarding predatory and scavenging molluscs.

The aim of this study was to investigate the independent and synergistic long term effects of low pH and increased temperature (in accordance with future IPCC scenarios) on the feeding performance of two adult gastropod species, the predator *Hexaplex trunculus* and the scavenger *Nassarius nitidus*, in order to compare responses related to different feeding habits. In addition, the feeding behaviour of juvenile *H. trunculus*, hatched and developed under the specific treatments, was studied in order to reveal potential acclimatisation mechanisms during the very early stages of life.

## Materials and Methods

### Species selection

The individual and combined effects of low pH and increased temperature were tested on the feeding behaviour of the gastropods *H. trunculus* (Muricidae) and *N. nitidus* (Nassariidae). These two common Mediterranean species are found in muddy-sandy sublittoral areas and are well-adjusted to varying physical environmental factors since they inhabit transitional coastal systems (e.g. rock pools, lagoons) where temperature and pH fluctuations occur naturally (Wahl *et al.*, 2016). *N. nitidus* is an agile scavenger, while *H. trunculus* is a non-selective carnivore and an occasional scavenger. Adult gastropods *N. nitidus* (n = 71) were collected from subtidal areas (<1 m depth) in the lagoons of Amvrakikos Bay (Western Greece) (39°3'41"N, 20°48'14.3"E) and *H. trunculus* (n = 53) were collected from the coast of Gournes (Heraklion, North Crete) (35°20'06.0"N, 25°16'43.9"E). All individuals had been acclimatized and maintained under the specific experimental conditions for 2.5 years following standard aquarist practices (i.e. feeding *ad libitum* on fresh mussels twice a week, cleaning of tanks) before conducting the study experiment. Young *H. trunculus* (n = 42) (termed "juveniles" here), that were hatched and kept under the respective conditions for 2.5 years were also used in the feeding experiments.

### Experimental setup

The selected treatments were based on the Intergovernmental Panel on Climate Change models for the eastern Mediterranean scenario until the year 2100 (IPCC, 2014). Mean ambient temperature in the natural habitat where the organisms were collected was 20°C during the period of the experiment, while in the warm treatments the temperature was 23°C (future prediction - 3°C increase). The ambient pH was 8 and the low pH treatment was 7.6 (future prediction - decrease of 0.4 units). Therefore, the gastropods were placed in small individual tanks (2-4l) and subjected to four experimental treatments: a) one control with ambient seawater temperature (20°C) and ambient pH (pH = 8) (tank 8A), b) one with ambient seawater temperature (20°C) and low pH (pH = 7.6) (tank 7.6A), c) one with warm seawater temperature (23°C) and ambient pH (pH = 8) (tank 8W) and d) one with warm seawater temperature (23°C) and low pH conditions (pH = 7.6) (tank 7.6W). The number of *H. trunculus* (juveniles and adults) and *N. nitidus* used for the feeding experiments differed between treatments (see Table 1), since in some treatments fewer (or no) gastropods survived or hatched during this long term experiment. *H. trunculus* (juveniles and adults) were subjected to all four treatments, whereas *N. nitidus* were subjected to the ambient temperature treatments only (8A and 7.6A).

The semi-closed system used for the experimental

**Table 1.** Average values ( $\pm$ SE) of response time (min), duration (min), speed ( $\text{cm min}^{-1}$ ), path index, % success for adult and juvenile *Hexaplex trunculus* and for *Nassarius nitidus* in all treatments. Also time for righting response (sec) and number of foot flicks are presented for *N. nitidus*. N: number of gastropods. *n.a.* not applicable. \*  $\pm$ SE is not available because only one individual reached food.

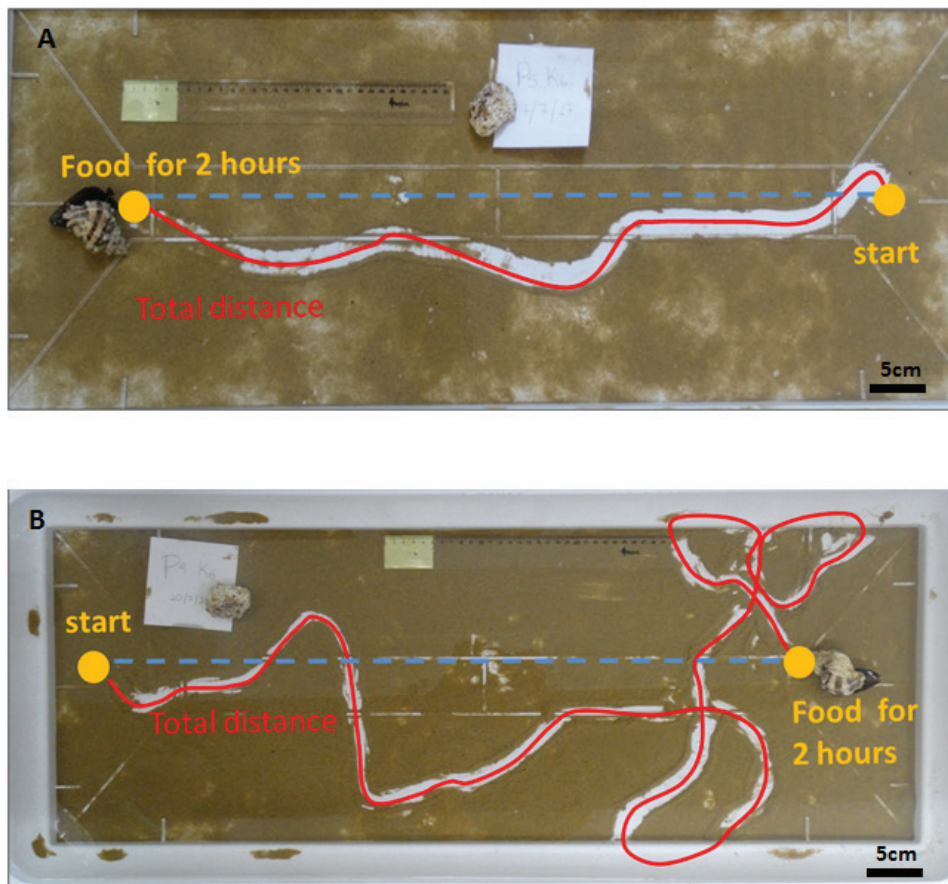
<i>Hexaplex trunculus</i> adults								
Treatment	N	Response time (min)	Duration (min)	Speed ( $\text{cm min}^{-1}$ )	Path index	Success %	Righting response (sec)	Foot flicks
7.6A	12	14.9 ( $\pm$ 4.4)	55.0*	2.38*	0.60*	8.3	<i>n.a.</i>	<i>n.a.</i>
8A	9	18.1 ( $\pm$ 9.1)	54.8 ( $\pm$ 20.6)	3.55 ( $\pm$ 0.86)	0.67 ( $\pm$ 0.15)	44.4	<i>n.a.</i>	<i>n.a.</i>
7.6W	17	6.5 ( $\pm$ 1.2)	67.9 ( $\pm$ 8.4)	2.91 ( $\pm$ 0.26)	0.46 ( $\pm$ 0.07)	52.9	<i>n.a.</i>	<i>n.a.</i>
8W	15	9.8 ( $\pm$ 2.7)	31.5 ( $\pm$ 5.5)	6.72 ( $\pm$ 1.08)	0.52 ( $\pm$ 0.09)	53.3	<i>n.a.</i>	<i>n.a.</i>
<i>Hexaplex trunculus</i> juveniles								
7.6A	18	4.9 ( $\pm$ 1.1)	49.3 ( $\pm$ 17.3)	4.02 ( $\pm$ 0.78)	0.51 ( $\pm$ 0.14)	16.7	<i>n.a.</i>	<i>n.a.</i>
8A	9	5.1 ( $\pm$ 1.4)	40.2 ( $\pm$ 10.8)	3.92 ( $\pm$ 0.65)	0.65 ( $\pm$ 0.09)	66.7	<i>n.a.</i>	<i>n.a.</i>
7.6W	9	4.7 ( $\pm$ 0.7)	23.1 ( $\pm$ 4.5)	5.20 ( $\pm$ 0.74)	0.83 ( $\pm$ 0.08)	88.9	<i>n.a.</i>	<i>n.a.</i>
8W	6	4.7 ( $\pm$ 1.4)	43.0 ( $\pm$ 17.8)	3.79 ( $\pm$ 1.12)	0.65 ( $\pm$ 0.13)	50.0	<i>n.a.</i>	<i>n.a.</i>
<i>Nassarius nitidus</i> adults								
7.6A	36	1.1 ( $\pm$ 0.4)	11.5 ( $\pm$ 1.8)	3.97 ( $\pm$ 0.44)	0.48 ( $\pm$ 0.04)	94.4	74.3 ( $\pm$ 25.6)	1.8 ( $\pm$ 0.1)
8A	35	2.9 ( $\pm$ 1.1)	11.1 ( $\pm$ 2.7)	4.13 ( $\pm$ 0.36)	0.52 ( $\pm$ 0.03)	97.1	129.5 ( $\pm$ 44.7)	2.6 ( $\pm$ 0.8)

treatments was set up at the Hellenic Centre for Marine Research (Cretaquarium) and its detailed operation is described in Chatzinikolaou *et al.* (2017). Submerged aquarium heaters (RESUN) were automatically turned on when necessary to increase the temperature to the desired level in the warm treatment tanks. A bubbling CO<sub>2</sub> system was used to adjust the low pH treatments to 7.6, which was continuously monitored using an automated pH controller system (Tunze 7070/2), calibrated twice every month with 5.00, 7.00 and 10.00 WTW NBS buffers, attached to the CO<sub>2</sub> bottles through a manometer (Gloor model 5100/10). Temperature and pH (on the National Bureau of Standards scale -pH<sub>NBS</sub>) were additionally measured daily using a 3420 WTW multi-meter, while salinity (salinometer) and oxygen (OxyGuard) were checked every other day. A submerged pump (Boyo WM-15) with additional air-stones was used to ensure sufficient water circulation. In addition, 50% of the water in the experimental tanks was renewed twice a week thus maintaining high water quality in the system (NO<sub>2</sub> < 0.1mg/l, NO<sub>3</sub> < 0.5mg/l, NH<sub>3</sub>/NH<sub>4</sub> < 0.2mg/l). Nitrogenous waste products were also assessed twice a week using photometric test kits (Tetra kit). Total alkalinity (TA) was measured every two weeks according to the Standard Operating Procedure (SOP 3b) described in Dickson *et al.* (2007), using open-cell titration (Metrohm Dosimat 765). Measured parameters (pH and TA) were used to calculate the additional parameters of the seawater carbonate system (Appendix I - Table 1) using the CO2SYS program with the dissociation constants of Mehrbach *et al.* (1973) as refitted by Dickson & Millero (1987).

#### Feeding experiment

Elongated plastic trays, measuring 90 cm x 33 cm for *H. trunculus* and 26 cm x 16 cm for the smaller-sized *N. nitidus* were filled with seawater from each of the respective experimental treatments. A thin layer of washed sand was added and equally spread on the tray in order to facilitate visual monitoring of the movement trails (Fig. 1). Prior to each feeding experiment, one gastropod was selected randomly across the different treatments, removed from the experimental tank and positioned individually at one edge of the plastic tray. Immediately after, at the start time of each feeding experiment, a mussel was manually opened (in order to avoid confounding responses due to prey size selection) and placed at the opposite edge of the tray as a food source for a maximum of 2 hours. The animals had not been fed for a period of about 10 days before the experiment. The tray was covered with cling film in order to eliminate evaporation and gas exchanges, and thus to keep pH constant. The experiments were performed in constant temperature rooms in order to eliminate seawater temperature changes during the experiments. Temperature and pH were monitored using a 3420 WTW multi-meter during the entire duration of the feeding experiment (120 minutes) to ensure that experimental conditions remained constant.

Elapsed time before the gastropods began moving (response time) and the total period until they successfully reached their food (duration) were measured with a stopwatch. The number of gastropods that reached their food source successfully, as well as the number of gastropods that did not move at all or failed to reach the food within the preset experimental time (i.e. 120 minutes) was recorded. Photos were taken at the end of each feeding



**Fig. 1:** A: Example of an adult *Hexaplex trunculus* that reached food successfully following a relatively straightforward movement. B: Example of an adult *Hexaplex trunculus* that reached food successfully following a non-straightforward movement. Solid line (red): trail of movement, Dotted line (blue): straight-line distance between start position and food source. Black bar at the right bottom indicates level of magnification.

experiment in order to measure the “total distance” covered by each gastropod that successfully reached the food source, using Image J (version 1.51j8) (Fig. 1). Also, the “direct distance” between the initial position of the gastropod and the food source was measured as a straight line. Each measurement was taken three times in order to eliminate possible deviations. The speed of movement was evaluated as the ratio of the total distance to duration. The path index was estimated as the ratio of “direct distance” to “total distance” and was used as an index to indicate whether the movement towards the food source was straightforward or not (i.e. path index  $\approx 1$  indicated a straightforward movement, Fig. 1). *N. nitidus* was placed on the experimental tray in an upside down starting position, in order to record an additional response index; namely, the elapsed time before the gastropods turned over to their normal crawling position (righting response). In addition, we measured the number of foot flicks, which are characteristic of this species, required to accomplish this righting movement.

### Statistical analysis

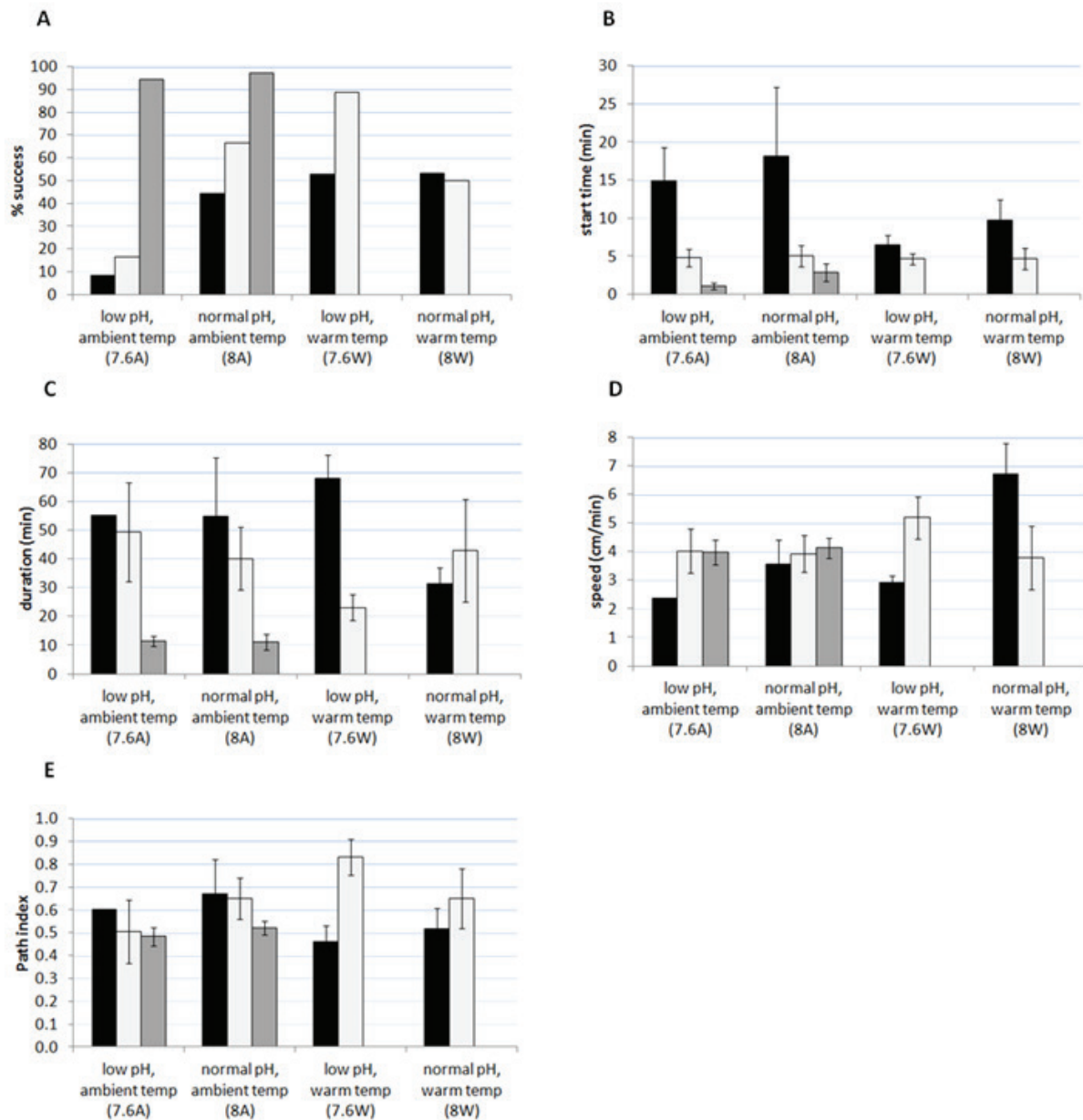
The data were tested for normality distribution (Anderson-Darling test) and homogeneity of variances (Bartlett’s test, Levene’s test). Parametric (One-way ANOVA)

and non-parametric (Kruskal-Wallis) tests were used to compare the data (response time, total duration, speed, path index, righting response time, number of foot flicks) obtained from the four different treatments. Comparisons were performed between the two different species (adults only), as well as between adults and juveniles of *H. trunculus*. When differences were significant, post-hoc analysis (Tukey’s test) was performed. Significant differences were considered at a threshold of  $\alpha = 0.05$ . The MINITAB software package (version 13.2) was used for the analysis. The results of the statistical analysis are presented in Appendix I - Table 2.

## Results

### Overall success

The percentage of juvenile *H. trunculus* that reached their food source successfully (Table 1, Fig. 2A) was higher than the respective percentage of their conspecific adults in all treatments, except for the one where the temperature was increased and pH was ambient (8W) where they were similar. Furthermore, *N. nitidus* were even more successful (>94%) than both *H. trunculus* juveniles (<89%) and adults (<54%) under ambient temperature treatments (both for ambient and low pH). While



**Fig. 2:** A: percentage (%) of gastropods that successfully reached their food, B: average response time (min) after which gastropods began moving, C: average total duration (min) until reaching food successfully, D: average speed (cm min<sup>-1</sup>) of gastropods that successfully reached their food, E: average path index of gastropods that successfully reached their food. Black bars: *Hexaplex trunculus* adults, White bars: *Hexaplex trunculus* juveniles, Grey bars: *Nassarius nitidus* adults. Error bars represent  $\pm$ SE (i.e. variation among individuals).

*N. nitidus* did not seem to be affected by low pH in ambient temperature (7.6A), *H. trunculus* (both juveniles and adults) were less successful in reaching their food under these conditions (7.6A) compared to the control treatment. In particular, only one adult *H. trunculus* (out of 12) managed to reach the food source successfully in the 7.6A treatment, while the rest did not move at all, moved out of the experimental tray or did not reach their food within the experimental time (120 minutes). The impact of low pH was more severe in ambient temperature for *H. trunculus*, while both adults and juveniles responded successfully in the increased temperature treatments (independently of the pH) where at least 50% of the gastropods were successful in reaching their food.

### Response time

Although adult *H. trunculus* seemed to respond faster when the temperature was increased (both for ambient and low pH) (Table 1, Fig. 2B), the differences were not statistically significant between treatments (Kruskal-Wallis:  $H = 2.06$ ,  $p = 0.560$ ). Juveniles also started moving towards their food after a similar time period ( $\approx 5$  minutes) in all treatments (Kruskal-Wallis:  $H = 0.84$ ,  $p = 0.839$ ). Low pH did not affect significantly the initial response time of *N. nitidus* (Kruskal-Wallis:  $H = 1.07$ ,  $p = 0.301$ ). However, a quicker reaction to food stimulus under low pH was observed for *N. nitidus*, which had a significantly faster righting response compared to the control (Krus-

kal-Wallis:  $H = 4.55$ ,  $p = 0.033$ ) and with fewer (although not significantly different) foot flicks (Kruskal-Wallis:  $H = 0.03$ ,  $p = 0.854$ ) (Table 1). When the three groups (i.e. *H. trunculus* adults, *H. trunculus* juveniles and *N. nitidus* adults) were compared for each treatment separately, it was observed that *H. trunculus* adults started moving significantly later than juveniles and than *N. nitidus* in the ambient temperature treatments, both for low pH (7.6A - Kruskal-Wallis:  $H = 35.63$ ,  $p < 0.001$ ) and ambient pH (8A - Kruskal-Wallis:  $H = 16.54$ ,  $p < 0.001$ ). The effect of increased temperature, either in combination with low pH (7.6W) or alone (8W), reduced the response time in *H. trunculus* adults and, therefore, the differences were not statistically significant compared to juveniles (7.6W - Kruskal-Wallis:  $H = 0.18$ ,  $p = 0.671$ ; 8W - Kruskal-Wallis:  $H = 1.26$ ,  $p = 0.261$ ).

### Duration

The time required (duration) (Table 1, Fig. 2C) for juvenile *H. trunculus* to reach their food was not significantly affected by low pH, increased temperature or the combination of both factors (Kruskal-Wallis:  $H = 3.34$ ,  $p = 0.342$ ). Similarly, no significant differences were observed for *N. nitidus* between the control and the low pH treatment (Kruskal-Wallis:  $H = 1.60$ ,  $p = 0.206$ ). However, adult *H. trunculus* reached their food in significantly less time under the increased temperature treatment (8W) compared to all other treatments (ANOVA:  $F = 4.28$ ,  $p = 0.03$ ). In addition, juvenile *H. trunculus* reached food in a significantly shorter time than adults (almost 3 times faster) in the treatment combining low pH and increased temperature (7.6W) (ANOVA:  $F = 20.45$ ,  $p < 0.001$ ).

### Speed

The speed of adult *H. trunculus* was significantly higher when the temperature was increased and pH was ambient (8W) (Kruskal-Wallis:  $H = 10.98$ ,  $p = 0.012$ ), whereas low pH treatments did not affect the speed of gastropods compared to the control (Table 1, Fig. 2D). The speed of juveniles was similar for all treatments (ANOVA:  $F = 0.79$ ,  $p = 0.519$ ), and the same was observed for *N. nitidus* (Kruskal-Wallis:  $H = 0.82$ ,  $p = 0.364$ ). In the control treatment (ambient temperature and ambient pH), the speed of *H. trunculus* adults and juveniles were similar when moving towards their food, as well as with *N. nitidus* (ANOVA:  $F = 0.16$ ,  $p = 0.849$ ), while juveniles were faster than adults under the combined effect of low pH and increased temperature (Kruskal-Wallis:  $H = 6.75$ ,  $p = 0.009$ ).

### Path index

The independent or combined effect of low pH and increased temperature did not cause significant differences in the path index of *H. trunculus*, both for adults and juveniles, and *N. nitidus*, between the different treat-

ments (*H. trunculus* adults - Kruskal-Wallis:  $H = 2.16$ ,  $p = 0.539$ ; juveniles - Kruskal-Wallis:  $H = 5.48$ ,  $p = 0.140$ ; *N. nitidus* - ANOVA:  $F = 0.55$ ,  $p = 0.461$ ). Although no significant differences were observed, both in the control (8A) and the increased temperature treatment (8W), between *H. trunculus* adults and juveniles (8A-ANOVA:  $F = 1.85$ ,  $p = 0.170$ ; 8W-ANOVA:  $F = 0.66$ ,  $p = 0.439$ ), when low pH was combined with increased temperature (7.6W), juveniles moved in a more straightforward way towards their food (Kruskal-Wallis:  $H = 8.61$ ,  $p = 0.003$ ).

## Discussion

Warm temperatures generally trigger physiological and metabolic responses (e.g. respiration, excretion, filtration rates) in gastropods (Noisette *et al.*, 2016). Foraging behaviour, which is an energetically expensive activity, is also favoured in higher temperatures. The duration of a drilling attack and the subsequent ingestion time of the gastropod *Nucella lapillus* are accelerated in higher temperatures (Miller, 2013). In this study, increased temperature accelerated the response and the speed at which adult *H. trunculus* reached their food. However, low pH in ambient temperature had more detrimental effects than the combination of low pH and increased temperature. The majority of *H. trunculus* in treatment 7.6A did not move at all or did not manage to successfully reach their food source. A reduced or unsuccessful response to feeding stimulus may be the result of limited chemosensory function as observed in polychaetes, crabs and fish exposed to acidification (De La Haye *et al.*, 2012; Johannesen *et al.*, 2012; Schaum *et al.*, 2013). Low pH conditions may alter the chemical structure of the signalling cue itself, making food less detectable (Roggatz *et al.*, 2016), or may also interfere with neurotransmitter functioning and modify the ability of molluscs to detect chemical cues (Watson *et al.*, 2014; Clements *et al.*, 2018). Alternatively, metabolic depression may be the reason for reduced responsiveness under acidified conditions, as reported for *Nassarius festivus*, which was able to down-regulate its metabolic rate under low pH conditions (Leung *et al.*, 2015). *Nucella lapillus* decreased foraging cost with increasing acidification, thus coping with the higher energetic expenditure that could have been triggered by limited chemo-sensory function under these conditions (Queirós *et al.*, 2015). However, in other species, such as the whelk *Tenguella marginalba*, high  $pCO_2$  increased the standard metabolic rate and energy requirements as the gastropods attempted to maintain homeostasis, thus resulting in higher consumption of the prey *Crassostrea gigas* (Wright *et al.*, 2018). Nevertheless, increased prey consumption in this case might have been the result of weaker prey shells or of inhibition of the normally occurring metabolic rate suppression of *C. gigas* in the presence of a predator cue, thus making the prey more “visible” (Wright *et al.*, 2018).

Several studies have indicated negative effects of acidification on the feeding behaviour of gastropods. Low pH had negative effects on the locomotion and sensory performance of *Concholepas concholepas* in the presence

of a prey odour, thereby decreasing the overall ability of the gastropods to forage efficiently (Domenici *et al.*, 2017). Queirós *et al.* (2015) note that low pH affected the predatory response of *N. lapillus* by increasing foraging distance, indicating a decreased ability to locate food. Although in our study very few adult and juvenile *H. trunculus* reached their food successfully at low pH, for the ones that eventually managed to do so, low pH did not affect any of the evaluated feeding behaviour parameters (response time, duration, speed, path index). Similarly to our results, foraging duration did not increase with acidification in *N. lapillus* (Queirós *et al.*, 2015). The opposite was recorded for *C. concholepas*, for which movement duration and decision time increased, while route finding was not as efficient under high PCO<sub>2</sub> (Domenici *et al.*, 2017). However, besides the low foraging performance, *C. concholepas* located their prey eventually under conditions of low pH and increased temperature (Domenici *et al.*, 2017). The experiments on *C. concholepas* lasted 6 months, while the gastropods used in the current study were kept under the experimental conditions for 2.5 years, thus indicating a potential degree of acclimation, which is in accordance with the milder effects observed. Domenici *et al.* (2017) also indicated that the effects of elevated PCO<sub>2</sub> on the effective foraging behaviour of *C. concholepas* are complex and may be compensated by elevated temperatures, as also observed during the current study where the warmer treatments had a less severe impact. Similarly, Clements *et al.* (2018) supported that the effect of low sediment pH on the neurophysiology of marine bivalves can be adversely affected by elevated temperatures.

The negative effects of low pH on the successful foraging performance of *H. trunculus*, as observed in the current study, as well as the other species mentioned above, may be the result of a severe impact of acidification on the equilibrium sensory organ of gastropods. Statoliths provide the animal with information on the direction of gravity (Dorsett, 1986), and are also formed of aragonitic calcium carbonate deposited on a protein matrix (Radtke, 1983). The impact of ocean acidification on calcareous structures may well affect the statoliths, thus altering the equilibrium and locomotion ability of the animals. Changes in calcification rates due to reduced pH may affect the shape, size and mass of statoliths, thus disturbing the coordination and orientation ability of gastropods and affecting vital activities such as movement and foraging behaviour.

The early life stages of molluscs appear to be more sensitive to the effects of ocean acidification (Clements & Darrow, 2018). Manríquez *et al.* (2014) indicated that juvenile *C. concholepas* had poor orientation skills and reduced predator detection response under CO<sub>2</sub>-rich conditions, but were still able to perceive cues originating from prey items. However, in the current study, the foraging performance of juveniles hatched and developed under low pH (either at ambient or increased temperature) was more effective than that of adults of the same species, thus indicating a degree of acclimation during the early life stages. Juveniles were more successful than

adults when moving towards their food source in all treatments, while they reached almost 89% in the treatment combining low pH and increased temperature (7.6W). In addition, their speed and path index indicating a more straightforward movement were significantly higher than those of adults in the 7.6W treatment, while the time required to reach carrion was 3 times shorter. It should be noted that foraging performance (duration, speed, path index) was similar between adults and juveniles in the control treatment (8A) and in the increased temperature treatment (8W), thus indicating a triggering effect of the future climate change scenarios (i.e. acidification combined with warming, 7.6W) for individuals already acclimatised in such conditions throughout the duration of their lives.

*N. nitidus* can move rapidly and crawl very fast downstream or upstream against the water current, in order to locate carrion even at a distance of up to several meters (Crisp *et al.*, 1978; Fretter & Graham, 1984). The chemical receptors on the epithelium of the anterior border of the foot, on the siphon and on the cephalic tentacles appear to be the cause of such rapid feeding responses (Crisp, 1971; Morton & Yuen, 2000). The scavenger *N. nitidus* was, in general, more successful in reaching carrion than the predator *H. trunculus*, even at ambient conditions (control). Although both species moved at a similar speed and had a similar path index, *H. trunculus* adults responded several minutes later to the food stimulus presented to them compared to *N. nitidus*. *N. nitidus* were equally successful in reaching their food source under low pH, as they were under ambient pH conditions. In addition, total duration until food was reached, the degree of straightforward movement (path index) and the speed of gastropods were not affected by more acidic pH than the current oceanic values. Although the response time after a food stimulus was presented to *N. nitidus* was similar under low and ambient pH, when the gastropods were positioned upside down, the time necessary for them to turn over to their normal crawling position was significantly less in low pH, thus indicating a quicker righting response. This behaviour is common to the Nassaridae that, following the presence of a food stimulus, can emerge immediately from the sediment with continuous thrusts of their foot (foot flicks), which is used as a lever to push the snail over in a series of somersaults (Gore, 1966).

In the current study, low pH had no significant impacts on the feeding behaviour of *N. nitidus* but had an impact on *H. trunculus*, thus supporting the hypothesis that the effects of ocean acidification on gastropods are species-specific. The differences in the degree of sensitivity to ocean acidification vary even among species of the same family. For example, the foraging behaviour of *N. festivus* under low pH was characterised by reduced speed and non-directional movement towards carrion, thus increasing time and energy costs (Leung *et al.*, 2015). Even when *N. festivus* managed to locate food, the reduced consumption rates and longer feeding times observed would require greater effort to fulfil energy needs



and, in addition, exposure to predation risk during feeding would be higher (Leung *et al.*, 2015). Leung *et al.* (2015) suggested that the reduced foraging success of *N. festivus* in low pH was more likely caused by direct damage to sensory organs (e.g. olfactory receptors and neurotransmitter receptors). According to a review published by Gazeau *et al.* (2013) the effects of decreased pH are highly species-specific and depend on species sensitivity and potential compensation mechanisms (Michaelidis *et al.*, 2005).

Low foraging performance in scavenging and predator gastropods may be explained by the additive effects of various components relevant to cognitive, sensory and locomotor performance (Domenici *et al.*, 2017). Inability to feed effectively may negatively impact the core functions of an organism, in turn crucially impacting survival (Pepin *et al.*, 2014). Therefore, foraging behaviour is an important indicator of individual performance, given that energy obtained from food is essential to fuel all biological functions (Leung *et al.*, 2015). Understanding the interactions of multiple stressors is a key in understanding the ecological repercussions of climate change (Nagelkerken & Munday, 2016). In addition, it is important to bear in mind that many organisms will be able to adapt to the negative effects of ocean acidification through acclimation since changes in seawater chemistry are gradual and take place on a scale of decades (Hendriks *et al.*, 2010). Therefore, this study attempted to shed some light on the feeding performance of two different gastropod species kept under low pH and increased temperature conditions during a long-term experiment. Valuable conclusions were also drawn regarding the responses at different life stages; specifically, for individuals hatched and developed throughout their lives under the specific conditions, thus indicating a significant ability for acclimation to adverse climate change scenarios.

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## APPENDIX I

**Table 1.** Mean values ( $\pm$ StDev) of seawater carbonate chemistry parameters as measured or calculated for all treatments during the experimental period. pH<sub>NBS</sub>: pH measured at the NBS scale using a 3420 WTW multi-meter; TA: total alkalinity measured according to SOP 3b using an open-cell titration (Metrohm Dosimat 765); pCO<sub>2</sub>: partial pressure of CO<sub>2</sub>; TC: total carbon; HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup>: carbonate and bicarbonate ion concentration;  $\Omega_{\text{Aragonite}}$ : aragonite saturation state;  $\Omega_{\text{Calcite}}$ : calcite saturation state. \* indicates parameters calculated using the CO2SYS program with the dissociation constants of Mehrbach *et al.* (1973) as refitted by Dickson & Millero (1987).

Treatment	Temperature (°C)	pH <sub>NBS</sub>	TA (μmol/kg)	pCO <sub>2</sub> * (μatm)	TC * (μmol/kg)	HCO <sub>3</sub> <sup>-</sup> * (μmol/kg)	CO <sub>3</sub> <sup>2-</sup> * (μmol/kg)	$\Omega_{\text{Aragonite}}$ *	$\Omega_{\text{Calcite}}$ *
7.6A	21.8 (±0.45)	7.60 (±0.08)	2701.6 (±72)	2044.6 (±273)	2643.4 (±80)	2498.9 (±77)	84.6 (±7)	1.98 (±0.17)	1.30 (±0.11)
8A	21.9 (±0.67)	8.13 (±0.05)	2590.2 (±36)	394.0 (±6)	2227.7 (±32)	1953.5 (±28)	262.6 (±4)	6.15 (±0.09)	4.04 (±0.06)
7.6W	24.8 (±0.36)	7.57 (±0.10)	2651.3 (±54)	2481.5 (±154)	2616.7 (±56)	2473.9 (±53)	74.0 (±3)	1.74 (±0.08)	1.05 (±0.05)
8W	24.7 (±0.38)	8.13 (±0.07)	2584.0 (±93)	381.3 (±39)	2188.2 (±90)	1891.9 (±85)	285.8 (±10)	6.71 (±0.23)	4.44 (±0.15)

**Table 2.** Results of statistical comparisons between different treatments (7.6A, 8A, 7.6W and 8W) or different groups (*Hexaplex trunculus* adults, *Hexaplex trunculus* juveniles and *Nassarius nitidus*) for response time, duration, speed and path index, as well as for righting response and number of foot flicks (for *N. nitidus* only).

Response time					
Comparison of treatments for each group					
Groups	Treatments	Test	probability	test value	Significant
Hex adults	I5 - I6 - K5 - K6	Kruskal Wallis	P = 0.560	H = 2.06	no
Hex juveniles	I5 - I6 - K5 - K6	Kruskal Wallis	P = 0.839	H = 0.84	no
<i>N. nitidus</i>	I5 - I6	Kruskal Wallis	P = 0.301	H = 1.07	no
Comparison of groups for each treatment					
Treatments	Groups	Test	probability	test value	Significant
7.6A	Hex ad - Hex juv - Nas	Kruskal Wallis	P < 0.001	H = 35.63	yes
8A	Hex ad - Hex juv - Nas	Kruskal Wallis	P < 0.001	H = 16.54	yes
7.6W	Hex ad - Hex juv	Kruskal Wallis	P = 0.671	H = 0.18	no
8W	Hex ad - Hex juv	Kruskal Wallis	P = 0.261	H = 1.26	no
Duration					
Comparison of treatments for each group					
Groups	Treatments	Test	probability	test value	Significant
Hex adults	I5 - I6 - K5 - K6	Anova	P = 0.030	F = 4.28	yes
Hex juveniles	I5 - I6 - K5 - K6	Kruskal Wallis	P = 0.342	H = 3.34	no
<i>N. nitidus</i>	I5 - I6	Kruskal Wallis	P = 0.206	H = 1.60	no

Continued

Table 2 Continued

<b>Comparison of groups for each treatment</b>					
<b>Treatments</b>	<b>Groups</b>	<b>Test</b>	<b>probability</b>	<b>test value</b>	<b>Significant</b>
7.6A	----- only 1 Hex adult reached food successfully (no comparisons made)-----				
8A	Hex ad - Hex juv - Nas	ANOVA	P = 0.511	F = 0.47	no
7.6W	Hex ad - Hex juv	ANOVA	P < 0.001	F = 20.45	yes
8W	Hex ad - Hex juv	ANOVA	P = 0.417	F = 0.72	no
<b>Speed</b>					
<b>Comparison of treatments for each group</b>					
<b>Groups</b>	<b>Treatments</b>	<b>Test</b>	<b>probability</b>	<b>test value</b>	<b>Significant</b>
Hex adults	I5 - I6 - K5 - K6	Kruskal Wallis	P = 0.012	H = 10.98	yes
Hex juveniles	I5 - I6 - K5 - K6	ANOVA	P = 0.519	F = 0.79	no
<i>N. nitidus</i>	I5 - I6	Kruskal-Wallis	P = 0.364	H = 0.82	no
<b>Comparison of groups for each treatment</b>					
<b>Treatments</b>	<b>Groups</b>	<b>Test</b>	<b>probability</b>	<b>test value</b>	<b>Significant</b>
7.6A	----- only 1 Hex adult reached food successfully (no comparisons made)-----				
8A	Hex ad - Hex juv - Nas	ANOVA	P = 0.849	F = 0.16	no
7.6W	Hex ad - Hex juv	Kruskal-Wallis	P = 0.009	H = 6.75	yes
8W	Hex ad - Hex juv	ANOVA	P = 0.162	F = 2.32	no
<b>Path index</b>					
<b>Comparison of treatments for each group</b>					
<b>Treatments</b>	<b>Groups</b>	<b>Test</b>	<b>probability</b>	<b>test value</b>	<b>Significant</b>
Hex adults	I5 - I6 - K5 - K6	Kruskal Wallis	P = 0.539	H = 2.16	no
Hex juveniles	I5 - I6 - K5 - K7	Kruskal Wallis	P = 0.140	H = 5.48	no
<i>N. nitidus</i>	I5 - I6	ANOVA	P = 0.461	F = 0.55	no
<b>Comparison of groups for each treatment</b>					
<b>Treatments</b>	<b>Groups</b>	<b>Test</b>	<b>probability</b>	<b>test value</b>	<b>Significant</b>
7.6A	----- only 1 Hex adult reached food successfully (no comparisons made)-----				
8A	Hex ad - Hex juv - Nas	ANOVA	P = 0.170	F = 1.85	no
7.6W	Hex ad - Hex juv	Kruskal-Wallis	P = 0.003	H = 8.61	yes
8W	Hex ad - Hex juv	ANOVA	P = 0.439	F = 0.66	no
<b>Righting response (<i>N. nitidus</i> only)</b>					
<b>Comparison of treatments</b>					
<b>Group</b>	<b>Treatments</b>	<b>Test</b>	<b>probability</b>	<b>test value</b>	<b>Significant</b>
<i>N. nitidus</i>	I5 - I6	Kruskal-Wallis	P = 0.033	H = 4.55	yes
<b>Foot flicks (<i>N. nitidus</i> only)</b>					
<b>Comparison of treatments</b>					
<b>Group</b>	<b>Treatments</b>	<b>Test</b>	<b>probability</b>	<b>test value</b>	<b>Significant</b>
<i>N. nitidus</i>	I5 - I6	Kruskal-Wallis	P = 0.854	H = 0.03	no