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S. ZERVOUDAKI, C. FRANGOULIS, L. GIANNOUDI, E. KRASAKOPOULOU

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Effects of low pH and raised temperature on egg production, hatching and metabolic rates of a Mediterranean copepod species (*Acartia clausi*) under oligotrophic conditions

S. ZERVOUDAKI[1], C. FRANGOULIS[1], L. GIANNOUDI[1] and E. KRASAKOPOULOU[1,2]

[1] Hellenic Centre for Marine Research, Institute of Oceanography, P.O. Box 712, 19013 Athens, Greece
[2] Department of Marine Sciences, University of the Aegean, Greece

Corresponding author: tanya@hcmr.gr

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Abstract

This study includes the first information on the combined effect of low pH and raised temperature on egg production rate (EP), hatching success (HS), excretion and respiration of the Mediterranean copepod *Acartia clausi.* Adult individuals of *A. clausi* and fresh surface seawater were collected at a coastal station in Saronikos Gulf during April 2012. Four different conditions were applied: two different pH levels (present: 8.09 and future: 7.83) and two temperature values (present: 16°C and present+4°C= 20°C). EP and HS decreased significantly over the duration of exposure at future pH under both temperature conditions. However, the analysis of the combined effect of pH, T, chlorophyll α and the duration of the EP and HS experiments revealed that ocean acidification had no discernible effect, whereas warming, food and the duration of exposure were more significant for the reproductive output of *A. clausi.* Temperature appeared to have a positive effect on respiration and excretion. Acidification had no clear effect on respiration, but a negative effect on excretion was observed. Acidification and warming resulted in an increase of the excretion rate and the increase was higher than that observed by warming only. Our findings show that acidification does not have an obvious direct effect on the vital rates of the copepod, with the exception of excretion possibly. Therefore, the combination of acidification, ambient oligotrophic conditions and warming could affect the ability of the species to allocate resources for coping with multiple stressors.

Keywords: acidification, egg production, respiration, excretion, *Acartia clausi*, Mediterranean Sea.

Introduction

Rising atmospheric carbon dioxide (CO_2) is causing global warming and ocean acidification (Caldeira & Wickett, 2003, 2005; Feely *et al*., 2004; Orr *et al*., 2005), which are increasingly recognized as important drivers of change in biological systems (Lovejoy & Hannah, 2005). The potential impact of the anticipated rapid reduction of seawater pH on marine organisms, and their ability to adapt, will determine future marine biodiversity and ecosystem functioning. So far, the impact of ocean acidification on different groups of marine organisms remains under debate (e.g. Dupont *et al*., 2010; Hendriks *et al*., 2010; Kroeker *et al*., 2010).

The Mediterranean Sea is one of the most nutrient-poor regions of the global ocean (Dugdale & Wilkerson, 1988), with a trophic status ranging from mesotrophic in the northwest to extremely oligotrophic in the east (e.g., Ignatiades *et al*., 2009). Both acidification and warming are expected to alter the ecology of the Mediterranean significantly, although the evidence to date is sparse. Israel & Hophy (2002) found that acidifying seawater to pH 7.8 with CO_2 did not adversely affect growth and photosynthesis in a wide range of Mediterranean chlorophyte, rhodophyte and phaeophyte algae. Investigations into the effects of acidification at a natural volcanic CO_2 vent off Ischia in Italy showed that around 30% of the coastal biodiversity was lost at the mean pH levels predicted for 2100 (Hall-Spencer *et al*., 2008). Also, studies on the effect of acidification on Mediterranean pteropod species (*Cavolinia inflexa* and *Creseis acicula)* showed that ocean acidification will impact pteropod populations as well as the ecosystems in which they play a critical role (Comeau *et al*., 2010; 2012). To our knowledge, the combined effects of warming and acidification on specific marine organisms such as copepods, inhabiting the Mediterranean Sea have never been investigated.

Copepods are one of the most important components of zooplankton and play major roles in the structure and functioning of marine planktonic food webs. *Acartia clausi* is distributed throughout the Mediterranean basin and maximal abundances are found during spring (Siokou-Frangou *et al*., 2004). Given its omnivorous nature, this species can enhance phytoplankton growth, both by releasing the grazing pressure from their most important predators and by contributing to nutrient recy-

cling. Moreover, since heterotrophs are also a common component in its diet they can potentially affect microbial food webs and thus carbon export (Saiz *et al*., 2007; Zervoudaki *et al*., 2007). Thus, the study of the effect of low pH on the secondary production and metabolism of this keystone Mediterranean copepod species in relation to the oligotrophic conditions would help us gain a better understanding of the response of zooplankton to ocean acidification in the Mediterranean Sea.

Although their exoskeleton is non-calcified (Fitzer *et al*., 2012), the physiological response of copepods to acidification has been tested initially in fresh water ecosystems mainly (e.g. Hessen & Nilssen, 1983; Stenson *et al*., 1993). Recently, copepod response to ocean acidification has been studied only during limited laboratory studies (Kurihara *et al*., 2004; Mayor *et al*., 2007; Kurihara & Ishimatsu, 2008; Zhang *et al*., 2011; Fitzer *et al*., 2012; Li & Gao, 2012; Mayor *et al*., 2012; Weydmann *et al*., 2012) demonstrating that the elevated CO_2 concentration in seawater has sub-lethal effects on copepods mainly, thus influencing vital rates, i.e. egg production, hatching success. However, most studies were conducted under extreme CO_2 concentrations and only a few studies investigated the effects of predicted, near future conditions (Fitzer *et al*., 2012; Mayor *et al*., 2012; Weydmann *et al*., 2012). The need for studies of physiological mechanisms to predict climate effect on ecosystems at species and community level has been stressed (Portner & Farrell, 2008). Although the effect of temperature on copepod metabolism is well known (e.g. Ikeda *et al*., 2001), very few studies have focused on the combined effect of temperature and pH on copepods (Mayor *et al*., 2012; Vehmaa *et al*., 2012).

Considering the observed variation in physiological response, even among congeners (Kurihara *et al*., 2004), the results from this study include the first information on the combined effect of low pH and raised temperature on egg production, hatching success, excretion and respiration of the copepod *Acartia clausi,* in an oligotrophic environment of the eastern Mediterranean Sea.

Materials and Methods

Sampling was performed during April 2012 at a coastal station (about 12 m depth) in the eastern Saronikos Gulf (Aegean Sea, eastern Mediterranean Sea), which is considered to be a meso-oligotrophic region (Friligos, 1984). Surface temperature peaks in August (26.5°C) and the lowest temperatures are recorded in February (14.2°C). Salinity ranges between 38 and 39 (Christou, 1998) depending on the variability of the inflow of Aegean water (Kontoyiannis, 2010). In winter and spring, *Acartia clausi* almost exclusively dominates the copepod community (Christou, 1998; Siokou-Frangou *et al*., 2004).

Experiments on the reproductive output (Egg Production and Hatching Success) and metabolism measurements were performed under four different conditions roughly representing the current and year 2100 atmospheric CO_2 concentrations. Experiments were carried out in two temperature controlled rooms set at ambient temperature (T1= 16°C) and at ambient temperature incremented by 4°C (T2= 20°C), while the nominal experimental pH values were 8.09 (pH1) and 7.83 (pH2), which correspond respectively to the ambient seawater pH and to the surface pH value predicted for the Mediterranean Sea for year 2099 and have resulted from model simulations run during the MedSeA project employing the A1B SRES scenario (Vichi & Orr, pers. Comm.)

Temperature was measured continuously from surface down to the bottom of the station using a SeaBird Electronics SBE 9/11 plus CTD. Copepods were collected by oblique hauls using a 200 μm WP-2 net equipped with a large non filtering cod-end (5 l). The content of the cod end was diluted in a 15 l bucket containing seawater collected from the surface and transported within 1 h to the lab. Within the next two hours, adult females of *Acartia clausi* were sorted under a dissecting microscope (for the metabolic experiments males were also used). Fresh surface sea water (~90 l) used for copepod incubation was collected from the same station every day using a 2 l Hydrobios water sampler and transported within one hour to the lab. There the water was distributed in four (4) 20 l polycarbonate bottles placed in each of two temperature controlled rooms. Before copepod incubation and after pH regulation of water, chlorophyll α (Chl α) samples were taken from each of the above 20 l bottles. The water (1.5 l) was filtered onto GF/F (Whatman: diameter 47mm; nominal pore size 0.7 μm) filters, which were then extracted in 90% acetone for 24 h, and Chl α was determined using a TURNER 00-AU-10 fluorometer (Holm-Hansen *et al*., 1965).

Total scale seawater pH (pH_T) and dissolved inorganic carbon (C_T) were measured in the collected surface water. A pH meter (Metrohm, 827 pH lab) fitted with a glass electrode (Metrohm, Aquatrode Plus) calibrated on the total hydrogen ion concentration scale using a Tris/HCl buffer solution with a salinity of 38.0 (Dickson *et al*., 2007) was used to determine pH_T at the experimental temperatures. C_T was measured in triplicate seawater samples immediately after collection using a flow injection analysis (FIA) system comprising a gas-permeable membrane to remove CO_2 from the acidic reagent stream into a receiving stream and a conductivity detector (Hall & Aller, 1992). The reagent concentrations and flow rates, as well as the injected sample volume, were modified to increase analytical accuracy and repeatability and lower the detection limit. The flow rates of both the carrier (HCl) and receiver (NaOH) stream were 1.2 ml/min while the adjusted concentrations of HCl and NaOH were 20 mM and 5 mM, respectively, allowing a minimum detection of 0.5 mM of reagent grade $NaHCO_3$. The

size of the injection valve loop for all solutions and samples was 100 µl.

Seawater pH was adjusted to the desired future pH_T value (7.83) by adding calculated amounts of HCl (1M), $NaHCO_3$ (1M) and/or Na_2CO_3 (1M) solutions using the ppH and pTA functions of the R package *seacarb* (Lavigne & Gattuso, 2011) successively. The changes in salinity due to the addition of the acid and salts were negligible. Salinity was checked with a portable refractometer (accuracy ±0.2). The combined addition of acid and bicarbonate and/or carbonate that was used to manipulate the carbonate chemistry closely mimics the on-going and future changes in seawater carbonate chemistry due to the invasion of anthropogenic CO_2 into the surface ocean (Gattuso & Lavigne, 2009; Schulz *et al*., 2009). The oceanic CO_2 uptake increases seawater CO_2 and C_T without changing the charge balance and therefore does not alter total alkalinity. The addition of carbonic acid salts increases C_T to the desired levels but also introduces alkalinity, which is then counterbalanced by the addition of HCl. The application of this manipulation approach allows the increase of dissolved inorganic carbon at constant alkalinity, a situation that resembles the changes in carbonate chemistry that have occurred since the beginning of the industrial revolution and are expected to continue during the coming years. Triplicate samples were taken to determine the carbonate chemistry parameters (Table 1) from pH_T, C_T, temperature and salinity using the R package *seacarb* (Lavigne & Gattuso, 2011). In total, six measurements of pH_T, C_T, temperature and salinity were performed corresponding to six experimental days: day 1 was the beginning of 5 egg production experiments, whereas the first metabolism experiment started on day 2 and the last one started on day 6. Therefore, seawater pH was regulated 6 times (i.e. from day 1 to day 6).

For the estimation of EP, 4 females of *Acartia clausi* were placed in each of eight 620 ml polycarbonate bottles per treatment containing well-mixed 60 µm filtered water adjusted to the above conditions. The females were incubated for 24 h under a 14:10 h light: dark cycle (spring daily cycle), after which the content of all the incubation bottles was filtered through a 40 µm mesh. Then the female individuals were checked for mortality and live animals were transferred to fresh collected surface sea water, which had been adjusted to the experimental conditions. The spawned eggs were counted under a dissecting microscope and all of them were gently transferred by micropipette into 75 ml culture flasks containing seawater with the same pH as the maternal incubation. The above procedure was performed for 5 days using the same animals and freshly collected and regulated seawater each day. The eggs produced daily were subsequently incubated for 48 h, after which the numbers of nauplii and unhatched eggs were recorded. HS (%) for the eggs produced each day was calculated as the number of nauplii present after 48 h / the number of eggs added at the start of the incubation x 100. Mortality over the whole experimental period (5 days) was <10% of the initial total number of animals introduced (32 animals).

To measure *A. clausi* metabolic rates (respiration and excretion), three independent experiments (expA, expB, expC) were performed, each time using freshly collected *A. clausi* adults. Before experimental incubation, animals were acclimated under the above described pH and temperature conditions for a period of 36-48h, during which pH and temperature regulated seawater was renewed every 24 hours. For the experimental incubation, a sealed chamber, single end-point method with filtered incubation seawater (i.e. non feeding conditions) and <0.2 individuals ml^{-1} were used (Ikeda *et al*., 2000). For this purpose, for each of the four treatments, approximately 40 acclimated individuals were transferred into each of 5 x 300 ml bottles (experimental) filled with GF/F filtered incubation seawater regulated at each of the previously stated pH and temperature conditions (4 x 300 ml bottles per treatment were used as control). Each incubation lasted 20 - 24 h, after which dissolved oxygen (O_2), ammonium (NH_4) and pH were measured in the bottles, and dead and live animals were counted. Mortality at the end of the metabolism experiments was <10% of the initial number of animals introduced. There was no significant difference in pH between control and experimental bottles at the end of incubation, nor between the beginning and the end of the experiment. Dissolved oxygen was determined using a Metrohm Dosimat 665, according to the Winkler method as modified by Carpenter (1965); method precision is approximately 2.2 mmol O_2 l^{-1}. Ammonium samples (40 ml) were analysed spectrophotometrically according to the standard method (Koroleff, 1970). Excretion and respiration rate calculations were made considering the concentration difference between experimental and control bottles, bottle volume and incubation time (Ikeda *et al*., 2000).

Statistical analysis. One-way ANOVA (95% confidence level) was performed in order to compare the mean values of Chl α, EP and HS for the 5 different levels of Day. Pair wise comparisons were made using the Kruskal-Wallis test. Multi-factor analysis of variance was performed for O_2 and NH_4 in order to determine which factors (number of experiment, pH and T) have a statistically significant effect on the above variables. The relationship of the temperature, pH, time of exposure (days) and Chl α variables on EP and HS were also analyzed using multiple regression analysis. This analysis also computed several multivariate ANOVA statistics for each of the effects in the model. These statistics are used to determine whether a particular effect has a significant relationship (95% confidence level) with the group of dependent variables being modelled. Data were transformed to $\log_{10}(x+1)$ in order to follow normal distribution. The above statistical analyses were performed with the Statgraphic Plus software package.

Results

The pH_T value of coastal station seawater was measured immediately after collection and ranged between 8.04 and 8.14 during the experiment. Experimental pH_T values were 8.05±0.04 and 7.83±0.02 at the present temperature (T1), and 8.01±0.02 and 7.83±0.02 at the elevated temperature (T2) treatments (Table 1). The carbonate chemistry conditions of the sampling site (pH_T 8.09±0.04) were roughly simulated in both pH1T1 and pH1T2 treatments as revealed by Table 1. Compared to control (pH1) conditions, C_T, HCO_3^- and CO_2 significantly increased in the acidified (pH2) conditions by 5.3%, 8.5% and 80% at present temperature and by 6%, 9.5% and 75%, respectively, at future temperature. Carbonate ion (CO_3^{2-}) concentration decreased by 34.8% and 30% in the T1 and T2 treatments, respectively, whereas alkalinity remained fairly constant (Table 1).

Concentrations of Chl α were low at all treatments (Fig. 1). The mean value for each treatment for the whole experimental period ranged between 0.028 ± 0.007 and 0.045 ± 0.008 μg l^{-1} and there is no statistical difference (F=1.830, P=0.182) between treatments regarding Chl α values.

EP in both the control and experimental treatments ranged from 1.5 to 5.2 egg ind^{-1} day^{-1} (Fig. 2). At the present pH, EP did not reveal any significant differences among days in both temperature treatments. This was not the case for the future pH where EP decreased significantly over the duration of the experiment at present temperature (F=9.3, df=4, P=0.0005) as well at future temperature (F=20.26, df=4, P=0.00001), indicating an effect of acidification and warming on the reproductive activity of this species.

HS in controls and experimental treatments varied between 16.6 to 94.4% (Fig. 3). With the present pH at both temperature treatments, HS did not show any statistical difference with the duration of the experiment. However, HS demonstrated significant reduction at the future pH (pH2-T1: F=8.96, df=4, P=0.0007, pH2-T2: F=3.55, df=4, P=0.034).

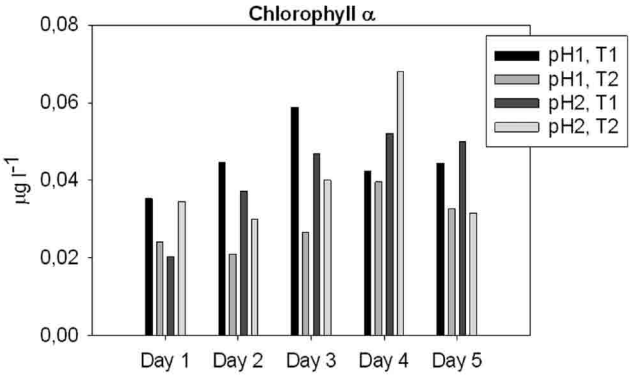

Fig. 1: Concentrations of Chlorophyll α in the incubation water for all treatments.

The performance of multiple regression between EP and the other variables (pH, T, Chl α and days) showed a strong negative correlation with the duration of exposure (days) (P=0.00001) and a positive relationship with Chl α (P=0.02). Warming (P=0.0001) and trophic conditions (P=0.04) positively affect egg hatching, whereas a negative relationship was detected with the duration of experiments (P=0.01).

Respiration (O_2 consumption) rate values ranged from 0.40 to 1.40 μl ind^{-1} d^{-1} at pH2T1 (expC) and pH1T2 (expC) respectively, and excretion (NH_4) rate values, ranged from 7 to 100 ngN ind^{-1} d^{-1} at pH2T1 (expC) at pH2T2 (expB) respectively (Fig. 4). There was no significant difference for excretion (F=2.45, df=2, P=0.098) and respiration (F=2.41, df=2, P=0.10) rates between the three independent experiments. In all three experiments, temperature had a significant positive effect on respiration (F=8.28, df=1, P=0.006) and excretion (F=21.72, df=1, P=0.00001). The effect of pH on respiration did not display a clear, significant trend. The effect of pH on excretion appeared to depend on the temperature, decreasing the excretion rate at present temperature (F=14.09, df=1, P=0.0009) and increasing it at future temperature (F=8.19, df=1, P=0.01).

Table 1. Seawater carbonate chemistry parameters in the different experimental conditions. Data are the means ± SD of the measurements of six experimental days. Carbonate system parameters were computed with the R package *seacarb* (Lavigne & Gattuso, 2011) based on the known values of C_T, pH and salinity at the two experimental values of temperature.

		measured			calculated				
		pH_T	C_T [μmol/kg]	Salinity	A_T [μmol/kg]	HCO_3^- [μmol/kg]	CO_3^{2-} [μmol/kg]	CO_2 [μmol/kg]	pCO_2 [μatm]
T1	pH1	8.05±0.04	2419±40	38.7 ± 0.2	2698±40	2199±44	204±14	16.3±1.4	463±14
	pH2	7.83±0.02	2548±71	38.7 ± 0.2	2690±60	2386±66	133±5	29.4±0.8	824±23
T2	pH1	8.01±0.02	2364±84	38.7 ± 0.2	2662±87	2122±74	227±23	14.5±1.3	466±21
	pH2	7.83±0.02	2508±82	38.7 ± 0.2	2660±72	2324±78	159±29	25.4±3.5	823±32
Sampling site		8.09±0.04	2374±42	38.7 ± 0.2	2681±76	2137±31	222±30	14.4±1.6	417±31

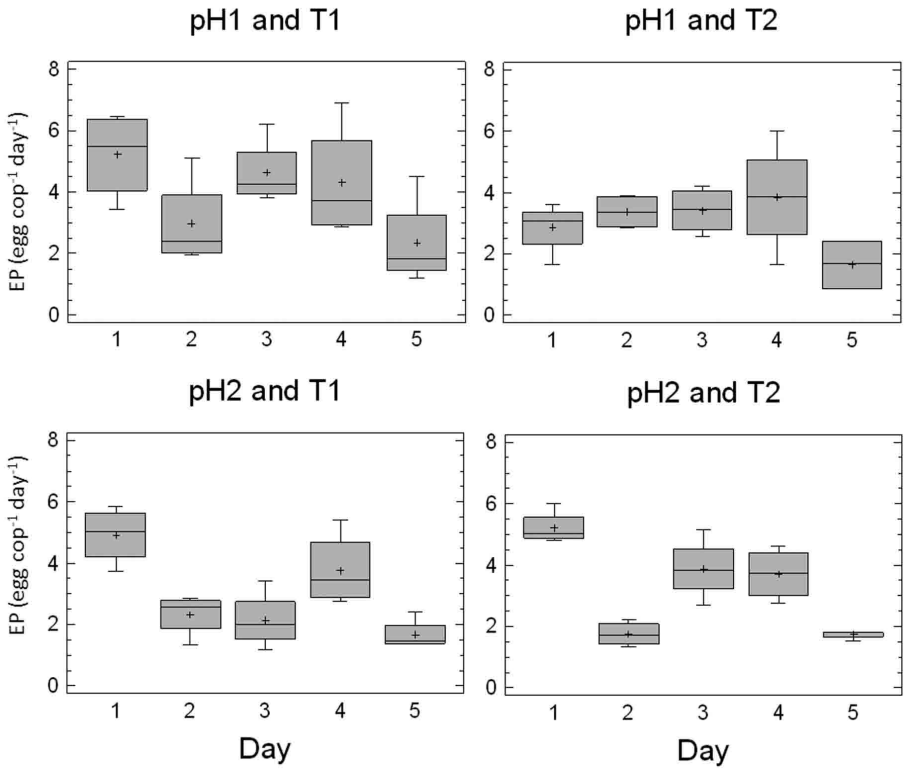

Fig. 2: Box and Whisker plots of egg production for *Acartia clausi* under two different temperature and pH conditions (T1=16°C, T2= 20°C, pH1=8.09, pH2=7.83). The box encloses the middle 50%, where the median is drawn as a vertical line inside the box. Horizontal lines, extending from each end of the box, represent the distribution of data (min and max values).

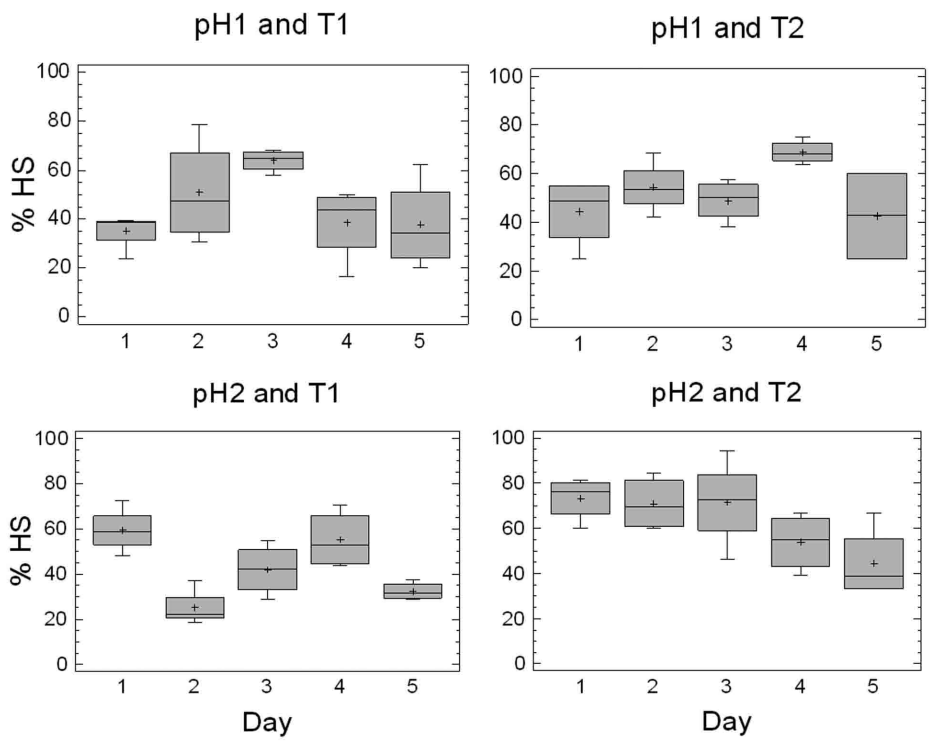

Fig. 3: Box and Whisker plots of Hatching Success (%) for *Acartia clausi* under two different temperature and pH conditions (T1=16°C, T2= 20°C, pH1=8.09, pH2=7.83). The box encloses the middle 50%, where the median is drawn as a vertical line inside the box. Horizontal lines, extending from each end of the box, represent the distribution of data (min and max values).

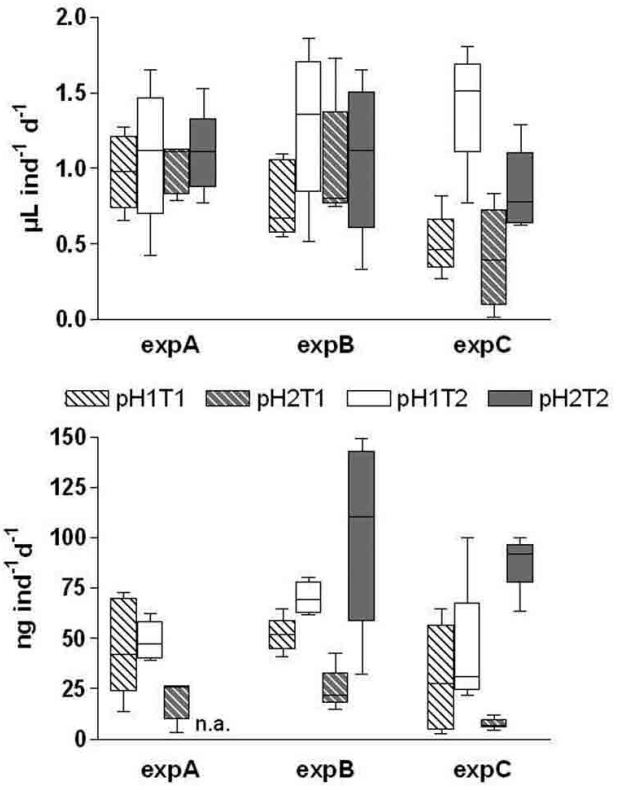

Fig. 4: Box and Whisker plots of oxygen consumption (top), and ammonia excretion (bottom) for *Acartia clausi* under two different temperature and pH conditions (T1=16°C, T2= 20°C, pH1=8.09, pH2=7.83), from three independently conducted experiments (expA, expB, expC, using freshly collected *A. clausi* adults). The box encloses the middle 50%, where the median is drawn as a vertical line inside the box. Horizontal lines, extending from each end of the box, represent the distribution of data (min and max values).

Discussion

This study includes the first information on the combined effect of ocean acidification and warming on specific vital rates (egg production, hatching, respiration and excretion) of a wild-caught Mediterranean copepod species under oligotrophic conditions.

Egg production and hatching success decreased significantly over the duration of exposure to future pH at both temperature conditions; however, the analysis of the combined effect of pH, T, Chl α and duration of the experiments on EP and HS revealed that ocean acidification had no discernible effect, whereas warming, food and the duration of exposure are more significant for the reproductive output of *Acartia clausi*. Previous studies investigating the response of copepod reproduction to sea water acidification using the addition of CO_2, revealed that egg production and hatching success are significantly inhibited by elevated partial pressure of CO_2 (8 000 ppm and 10 000 ppm) (Kurihara *et al*., 2004; Mayor *et al*., 2007; 2012; Kurihara & Ishimatsu, 2008; Mc Conville *et al*., 2013). Eggs of *Acartia erythraea* incubated in seawater acidified with 2000 ppm CO_2 did not display reduced hatching success (Kurihara *et al*., 2004); only marginal effects have been observed in *A. tsuensis* (Kurihara & Ishimatsu, 2008) at the same pCO_2 level and no apparent effect on egg hatching success was found for *C. finmarchicus* at 1000 ppm CO_2 concentration (Mayor *et al.,* 2012). Recently, Weydmann *et al*. (2012) reported that egg production of *Calanus glacialis* females was unaffected by a pH level of 7.6 and 6.9 but reduced hatching success was observed among the eggs that were incubated at the lowest pH level. Similar findings were observed for *Calanus sinicus* where no effect on adult survival and egg production rate was observed during an eight-day incubation period in seawater with a CO_2 level of up to 10000ppm (Zhang *et al*., 2011).

In this study, experiments were performed by altering the carbonate system with the combined addition of strong acid and carbonic acid salts. Lately, Schulz *et al*. (2009) and Gattuso *et al*. (2010) declared that among the various manipulation approaches, three of them, namely gas bubbling, addition of high-CO_2 content seawater, and combined addition of acid and bicarbonate and/or carbonate, succeed in simulating on-going and future changes in seawater carbonate chemistry and exactly reproduce the changes of all parameters of the carbonate system expected in year 2100. However, each manipulation technique has its own benefits and drawbacks. For instance, although bubbling with CO_2-enriched air is a very efficient and relatively easy way of manipulating the carbonate system, the acid-addition combined with addition of bicarbonate and/or carbonate method is still preferable when working with species that are sensitive to the intense physical stress exerted by bubbling (Rost *et al*., 2008). Although our experiments were performed using a different manipulation technique, the results obtained in future conditions (calculated pCO_2 823±32 µatm) are in agreement with previous findings, i.e. that hatching success in marine copepods remains unimpaired by CO_2 concentrations <2000 ppm (Mayor *et al*., 2012; Mc Conville *et al*., 2013).

The egg production of *Acartia* species has been the subject of much research (e.g. Uye, 1981; Kiørboe *et al*., 1985; Saiz *et al*., 1992; Jónasdóttir & Kiørboe, 1996), and production rate was reported to be 10–50 eggs f^{-1} d^{-1}. EP of *A. clausi* found in this study was lower than the reported range or previous measurements during spring in the Aegean Sea (Zervoudaki *et al*., 2007). The response of egg production to increasing Chl α concentration was similar to the generally observed functional response of copepod egg production (e.g. Kiørboe *et al*., 1985). Low egg production measured in this study indicates that the females were food-limited during the incubations, due to the low concentrations of Chl α at the start of each of the daily incubations.

Temperature appeared to have a positive effect on hatching success (multiple regression analysis). The high

HS observed at 20°C (94%) in this study is in agreement with the rates obtained by Holste & Peck (2005) (92%) and by Chinnery & Williams (2004) (85%) for *A. tonsa* and other congeners at the same temperature.

The duration of experiments appeared to have a significant negative effect on both egg production and hatching success, in particular at future pH. To our knowledge, the information available so far indicates substantial stage- and interspecific difference with regard to sensitivity to elevated CO_2 levels as well as duration of exposure to acidified conditions. Short-term exposure of *Calanus* and *Acartia* species to high levels of CO_2 induced acidification revealed that egg production was severely reduced over the duration of incubation in acidified conditions (Kurihara *et al*., 2004; Mayor *et al*., 2007; Weydmann *et al*., 2012). However, recently, a long-term study on cohorts of *C. finmarchicus* eggs did not reveal an effect on survival after 28 days of exposure to seawater acidified with 3300ppm CO_2 (Pedersen *et al*., 2013). Moreover, a multigenerational study of the harpacticoid copepod *Tisbe battagliai* showed that naupliar production was negatively affected by pH levels as high as 7.82 (Fitzer *et al*., 2012). On the other hand, lately, Mc Conville *et al*. (2013) found that reproduction of *Centropages typicus* and *Temora longicornis* was sensitive during long term exposure to acute elevated seawater CO_2 (9830 ppm), whereas neither species was affected by exposure to CO_2 levels predicted for the year 2100 (750 ppm).

The respiration (O_2 consumption) rates found in this study were higher than the ones described in literature for the same species in the western Mediterranean Sea (0.10 to 0.32 µl ind^{-1} d^{-1}, Gaudy *et al*., 2000), but close to the range described for this species in other seas (0.31 to 1.06 µl ind^{-1} d^{-1}; Ikeda *et al*., 2001). On the other hand, excretion (NH_4) rate values were lower than the range measured for *Acartia clausi* in the western Mediterranean Sea (90 to 270 ngN ind^{-1} d^{-1}, Gaudy *et al*., 2000) and in other seas (84 to 178 ngN ind^{-1} d^{-1}; Ikeda *et al*., 2001). The resulting, oxygen consumption to ammonia excretion ratio (O/N), i.e. the metabolic quotient (Ikeda *et al*., 2000), had an overall mean of 28.0, a value which is higher than that described for *A. clausi* in the western Mediterranean Sea (mean 21.2 in Gaudy *et al*., 2000) and that for copepods in general (mean 20.7), but still within the global range of O/N values found in the literature compilation of Ikeda *et al*. (2001). These lower ammonia excretion rate values and resulting high O/N ratio could be due, as for egg production rate, to the low food level (Chl α) during incubation, whereas the above literature range was obtained at higher food conditions (Ikeda *et al*., 2001).

Literature on the effect of temperature on marine copepod metabolism is quite extensive (reviews by Ikeda *et al*. 2001; Frangoulis *et al*. 2005). Concerning pH, its effect on zooplankton community structure and on cladocerans respiration rate has been studied in lakes (Potts & Fryer, 1979; review by Geelen & Leuven, 1986). The existing knowledge about the effect of pH on the metabolism of marine copepods is more limited (Marshall *et al*., 1935; Li & Gao, 2012). To our knowledge, at least in the Mediterranean Sea, the combined effects of warming and acidification on the metabolism of marine copepods have never been investigated.

In this study, for all three (independently performed) metabolic experiments, temperature increase had a positive effect on respiration, as already described by Ikeda *et al*. (2001). On the contrary, the effect of pH on *A. clausi* respiration rate did not display a clear significant trend in all experiments, such as the increase of respiration of *Calanus tenuiremis* described by Li & Gao (2012). This absence of a clear effect on the respiration rate might be due to the smaller pH difference applied in this study (pH 7.83-8.09), and/or the different copepod species and/or the lower food level in the present experiments, compared to the ones (pH 7.83-8.18) in the study of Li & Gao (2012). This is supported by the absence of a pH effect on the respiration rate of another *Calanus* species (*Calanus finmarchicus*) for an even wider variation of pH (pH 7.4–pH 8.5) observed by Marshall *et al*. (1935).

Warming had the known positive effect on excretion rate (Ikeda *et al*., 2001) in all the metabolic experiments performed. With acidification, on the contrary, the *A. clausi* excretion rate decreased at 16°C. However, the combination of acidification and warming not only increased the excretion rate, but the increase was higher than when only warming was applied. Although a stress effect due to the combination of both acidification and warming is possible, this should normally result in a further decrease of the excretion rate (review by Ikeda *et al*., 2000). A possible explanation could be a combined effect of acidification and warming on a physiological process such as enzymatic activity. In the oxidation of amino acids to ammonia, two types of enzymes play a role: transaminase and glutamate dehydrogenase (GDH) (review by Mayzaud, 1986). The pH and temperature effect on transminase has not been studied in marine zooplankton to our knowledge. The pH for optimum efficiency of GDH of a mysid shrimp has been found to be 8.5 (Bidigare & King, 1981), whereas GDH activity appears to increase with temperature at least until 22.4°C in a mixed population of marine zooplankton (Hernandez-Leon & Torres, 1997). If these pH and temperature effects on GDH are similar in the case of *A. clausi*, the combined effect of warming and acidification should result in an increase of excretion rate, but not above the level found when warming only is applied. The mechanism of the combined effect of warming and acidification on the excretion rate of *A. clausi* remains unclear and requires further investigation. This could be: different pH and T optima for the GDH of *A. clausi* than those found in literature; a shift of the GDH optimum under the combined effect of warming and acidification; an effect on other enzymes (transaminase), or another reason.

Consequently, available information from the literature showed that even when pH seems to be of major importance, it is unclear whether the pH effects act directly or indirectly, or only as an additional stress factor. Also, our findings revealed that a direct effect of ocean acidification on the vital rates of copepods was not obvious. However, the combination of low pH with the ambient oligotrophic conditions (Chl α <1 $\mu g\ l^{-1}$, Shushkina *et al*., 1997), where organisms were more vulnerable due to lack of food, and warming which affect physiological rates at the individual level perhaps reduces the ability of the species to allocate resources for coping with multiple stressors (Kletou & Hall-Spencer, 2012). In this respect, the effect of ocean acidification in the oligotrophic environment of the Mediterranean Sea could be more profound, altering the overall plankton community structure. Nevertheless, information on the ecological impact of ocean acidification and warming in the planktonic system of the Mediterranean Sea is very limited and requires further investigation in order to gain a better understanding of Mediterranean ecosystem dynamics under the influence of acidification and warming.

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