

Seawater Ca²⁺ concentration influences solar orientation in *Talitrus saltator* (Crustacea, Amphipoda)

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SUMMARY

The role of salinity in the ecophysiology of many intertidal invertebrates has been extensively investigated. Calcium (Ca²⁺), magnesium (Mg²⁺), potassium (K⁺) and sodium (Na⁺) are the major constituents of seawater and it has been demonstrated that sandhoppers tested under the sun in diluted seawater (3.5‰) head seaward, instead of going landward as expected. Therefore, the variation in seawater salinity (from 35‰ to 3.5‰) influences their directional choice. This paper investigates the contribution of different cations to the sea–land directional choice of *Talitrus saltator* (Crustacea, Amphipoda) by the sun compass orientation mechanism. Results of releases carried out in basic seawater selectively deprived of Ca²⁺, Mg²⁺ or K⁺ and containing the same concentration of Na⁺ indicate that only the reduction in Ca²⁺ concentration affects the capacity of solar orientation. The pH does not influence the directional choice of sandhoppers and nor do small variations in salinity in the range 32–39‰. Moreover, the clear photopositive tendency registered in experiments of phototaxis in Ca²⁺-deprived seawater indicates that the absence of Ca²⁺ does not affect the normal functioning of the visual cells. Therefore, our results show that Ca²⁺ seawater concentration is important for the correct functioning of one of the principal mechanisms of orientation in supralittoral amphipods and it could affect their survival in the field.

Key words: calcium, sandhopper, *Talitrus saltator*, orientation, sun compass.

INTRODUCTION

The fundamental role played by calcium (Ca²⁺), alongside the role of other essential elements such as magnesium (Mg²⁺), potassium (K⁺) and sodium (Na⁺), is well known in many organisms (Wilkins and Wilkins, 2003). All these elements are also major constituents in terms of cations in seawater, and the role of salinity in the ecology and ecophysiology of many littoral and intertidal invertebrates has been extensively investigated (Kinne, 1963a; Kinne, 1963b; Calosi et al., 2007). Salt (or NaCl) concentration also influences the behavioural traits of many invertebrates, mainly of those species that must maintain or return to some zones in which they live. For example, to maintain their ecological optimum in the water column, estuarine crustacean larvae modify their behavioural response if exposed to salinity changes (Harges and Forward, 1982; Forward, 1989). Upon exposure to higher salinity, these larvae show a clear positive phototaxis and a negative geotaxis, while the opposite pattern is evoked by a salinity decrease. A change in NaCl concentration is the main environmental cue for detection of salinity variation (Harges and Forward, 1982; Forward, 1989). It is also known that many species of sandhoppers are able to return to their ecologically relevant zone (the damp belt of sand near the seawater) following the shortest route possible, therefore theoretically coincident with the sea–land axis of their home beach (Pardi and Papi, 1952; Pardi and Ercolini, 1986). Sandhoppers use the sun and other astronomical and celestial factors to determine the correct direction of orientation. Of course, the directional choice also depends on motivational status: if the sandhopper is in the sea, it orients landward; if in dry conditions, it orients towards the sea (i.e. towards the damp belt of sand) (Pardi and Pardi, 1953; Pardi and Papi, 1953). It has also been demonstrated

that when sandhoppers are released in deionized or diluted seawater (at a salinity of 3.5‰) they head seaward instead of going landward (Terracini-De Benedetti, 1963; Scapini, 1979). Therefore, the salt concentration of water influences the directional choice; however, the contribution of the different ions to the directional choice has never been investigated.

MATERIALS AND METHODS

Animal collection and experimental procedures

We used adults of *Talitrus saltator* (Montagu) collected on a sandy beach near the Morto Vecchio river mouth (Regional Natural Park of Migliarino San Rossore Massaciuccoli, Pisa, Italy). In the laboratory they were raised in conditions of natural temperature and with a light:dark (L:D) cycle in phase with natural conditions. Food was constantly available and the sand was kept well aired and wet.

The experimental apparatus used in sun compass orientation tests is similar to that used by Pardi and Papi (Pardi and Papi, 1953), slightly modified by Ugolini and Macchi (Ugolini and Macchi, 1988). It consisted of a goniometer over a circular transparent Plexiglas plate (diameter 30 cm). A transparent Plexiglas bowl (height 5 cm, diameter 20 cm) was placed on the goniometer, and animals were released inside the bowl to be tested. The bowl was covered with a sheet of transparent acetate to prevent animals from escaping. A cylindrical opaline white Plexiglas screen (height 5 cm, diameter 30 cm) was placed around the bowl to block out the view of the surrounding landscape but allow individuals to see the sun and the sky.

The experiments were conducted, around midday, in Florence from May to September 2007 and 2008. Sandhoppers were released

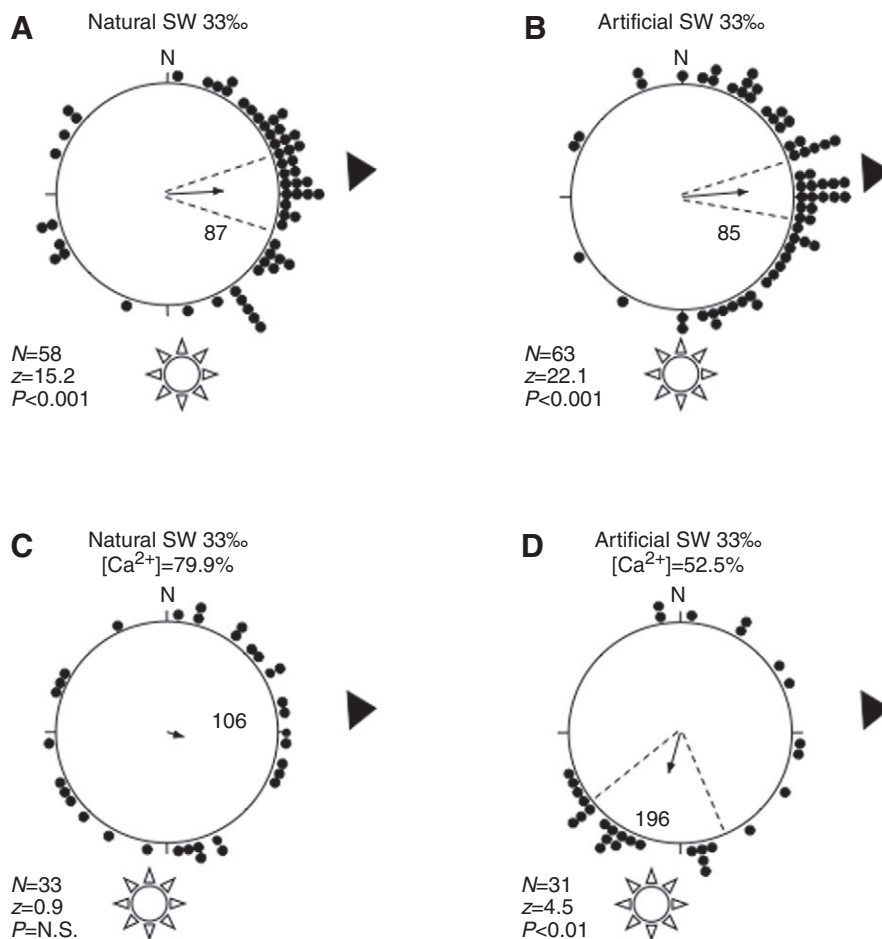


Fig. 1. Solar orientation test in natural and artificial seawater (A,B) and in natural and artificial seawater after modification by adding a solution of $0.1 \text{ mol l}^{-1} \text{ Na}_2\text{CO}_3$ (C,D). The heading above each distribution summarizes the treatment used in each test. The arrow inside each distribution represents the mean vector with the mean angle; dots represent sandhopper directions (each dot represents one individual); N, magnetic north; sun, azimuth of the sun at the time of the experiments; filled triangle, expected landward direction. In each distribution, the number of samples (N) and the Rayleigh test value (z) with probability level (P) are also reported. The broken lines inside each distribution show the confidence limits at 95%. See text for further explanations.

in groups of about 10 individuals at a time, under the sun and blue sky. Since the sandhoppers were released directly into the water, the expected direction of orientation was 84° (expected landward direction of the sea–land axis of their home beach). One direction for each individual was recorded, after 5–7 min of permanence in the solution, by a freeze-frame image using a video camera placed under the bowl.

A different apparatus was also employed to evaluate the possible influence of Ca^{2+} on simple phototactic behaviour, since it has already been demonstrated that this element affects visual capacity in invertebrates (Dorloechter and Stieve, 1997) and phototactic orientation (Morel-Laurens, 1987; Wilkins and Wilkins, 2003). In summary, this device consists of a Plexiglas tube (length 60 cm, diameter 4.5 cm), horizontally placed, half-filled with artificial seawater, distilled water or artificial seawater deprived of Ca^{2+} (see below). One beam of white light (intensity 6.8 mW) generated by a Xenon lamp (150 W) penetrates into the tube by an extremity (diameter 20.7 mm). Since these experiments were carried out in a dark room, the binary choice was between light and dark. Groups of 10–12 sandhoppers were confined in a vertical cylinder inserted into the middle of the tube and given free choice after 10 min of permanence in the tube. After release, we counted the number of amphipods present at the two ends after only 30 s.

Test solutions

We used different solutions depending on the type of trial. All the solutions were prepared 24 h before each test. In every test, the control solution was made by dissolving 33 g of artificial sea salt

(Instant Ocean[®], Aquarium Systems, Mentor, OH, USA) in 1 litre of double-distilled water in order to achieve a salinity of 33‰ and pH 8.20. The saline concentration was checked before the experiments with a hand refractometer (Salt refractometer, Sperm Scientific 106 ATC, Scottsdale, AZ, USA).

In the test with a reduction of Ca^{2+} concentration, we added a small quantity of a solution of $0.1 \text{ mol l}^{-1} \text{ Na}_2\text{CO}_3$ to natural and artificial seawater in order to obtain two solutions with a lower concentration of Ca^{2+} : 79.9% and 52.5% of the initial Ca^{2+} concentration (Table 1).

The sandhoppers' orientation was also tested in a solution of seawater (pH 7.87, salinity 39‰) composed of 30 g of NaCl (0.513 mol l^{-1}), 1.078 g of CaCl_2 (9.71 mmol l^{-1}), 10.16 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ($49.97 \text{ mmol l}^{-1}$), 0.717 g of KCl ($9.617 \text{ mmol l}^{-1}$) and 0.84 g NaHCO_3 (10 mmol l^{-1}), which are the same concentrations as the main cations present in natural seawater (referred to as 'basic' seawater from now on) (Atkinson and Bingman, 1998). To evaluate the possible influence of three of these ions, solutions were also prepared without CaCl_2 (pH 7.95, salinity 37‰), without MgCl_2 (pH 7.41, salinity 32‰) and without KCl (pH 7.90, salinity 39‰) but containing NaCl and NaHCO_3 at the same concentrations. Salinity values of basic seawaters are not referred to in terms of NaCl concentration but to the ion chloride in solution and cannot be considered in order to evaluate the effect of salinity on sandhopper behaviour.

In fact, in order to exclude the possible influence of differences in salinity, the orientation of sandhoppers was tested in solutions of artificial seawater at a salinity range of 33–39‰. In the pH

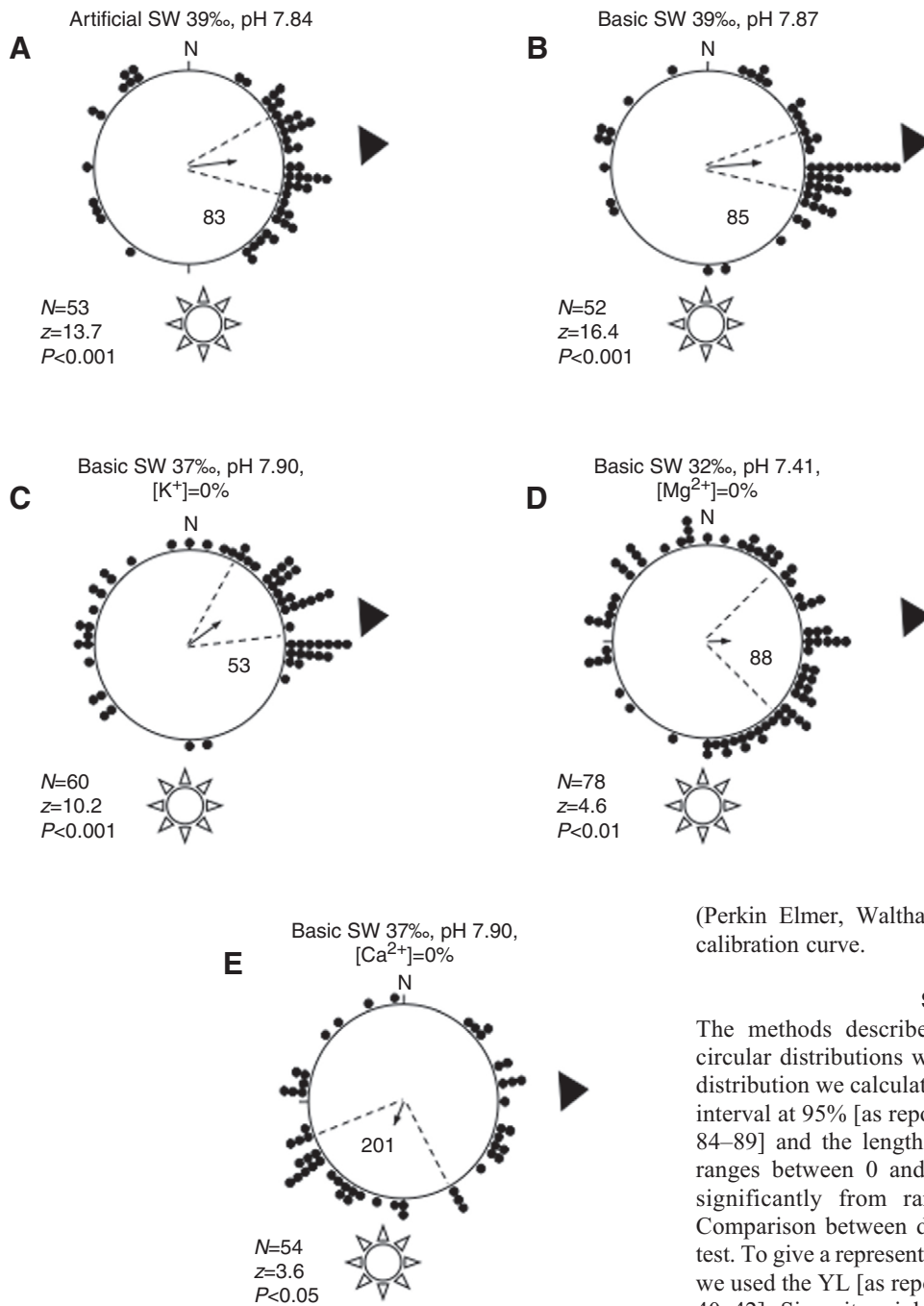


Fig. 2. Solar orientation test in artificial seawater (A) and basic seawater characterized by the presence of the main cations (B) and without K^+ (C), Mg^{2+} (D) and Ca^{2+} (E). See Fig. 1 and text for further explanations. Salinity values of basic test solutions are not in terms of NaCl concentration but in terms of chloride ions in solution.

test, the solution of artificial seawater (33‰ of salinity) was modified to obtain two solutions with differing pH: pH 6.50 (modified by adding a small quantity of $0.01 \text{ mol l}^{-1} \text{ CH}_3\text{COOH}$) and pH 9.20 (modified by adding a small quantity of $0.1 \text{ mol l}^{-1} \text{ NaOH}$). All chemicals were purchased from Sigma Aldrich (Milan, Italy).

pH measurement and Ca^{2+} ion determination

The determination of pH in both natural and synthetic seawaters was performed with a pH meter (pH340i; WTW, Weilheim, Germany). The instrument was calibrated before use with two buffer solutions (pH values of 4.01 and 7.00).

The concentration of Ca^{2+} ions in seawater samples was determined with an ICP-OES Optima 2000TM DV spectrometer

(Perkin Elmer, Waltham, MA, USA) by means of an external calibration curve.

Statistical analysis

The methods described by Batschelet (Batschelet, 1981) for circular distributions were used for statistical analysis. For each distribution we calculated the mean angle (α) with the confidence interval at 95% [as reported by Batschelet (Batschelet, 1981), pp. 84–89] and the length of the mean resultant vector (r) (which ranges between 0 and 1). To test if the distributions differed significantly from randomness we used the Rayleigh test. Comparison between distributions was made by the Watson U^2 test. To give a representation of the ‘goodness of orientation’ (GO), we used the YL [as reported by Batschelet (Batschelet, 1981), pp. 40–42]. Since it weights the cosine of difference between mean angle and expected direction for the mean vector length ($GO = r \times \cos \alpha - YL$) it ranges between +1 (best orientation and concentration of animals around the expected direction) and –1 (opposite best orientation), passing through 0 (directions uniformly distributed around 360°).

RESULTS

Fig. 1 clearly shows that although sandhoppers are well directed towards the expected direction when released in unmodified natural or artificial seawater (Fig. 1A,B), after precipitation of Ca^{2+} they originate random distributions (Fig. 1C) or they show a photopositive behaviour (Fig. 1D). This is true for a Ca^{2+} concentration corresponding to 79.9% of the total (Fig. 1C) and 52.5% of the total (Fig. 1D), respectively, as confirmed by the results of Watson U^2 test (Fig. 1C vs Fig. 1D; $U^2_{(33,31)} = 0.196$, $P < 0.05$).

Table 1. Concentrations of Ca^{2+} and pH values determined in natural and synthetic seawaters

Solution	pH	$[\text{Ca}^{2+}]$ (mg l^{-1})	Percentage variation
Natural seawater	8.2	443	
Natural seawater modified	8.7	354	-20.1%
Artificial seawater	8.2	440	
Artificial seawater modified	8.9	231	-47.5%

Natural and artificial seawaters were modified by adding a solution of $0.1 \text{ mol l}^{-1} \text{ Na}_2\text{CO}_3$.

The differing behaviour of sandhoppers observed in natural and artificial seawater before and after the addition of Na_2CO_3 could be due to the strong decrease of Ca^{2+} and the increase of Na^+ ions in the solution. In order to understand the reason for the sandhoppers behaviour, we prepared basic seawater solutions according to the 'test solutions' section of the Materials and methods; these solutions had a constant Na^+ concentration but differed in the absence of one of three major constituent cations (K^+ , Mg^{2+} and Ca^{2+}). The effects of these solutions on sandhopper orientation are reported in Fig. 2.

Releases carried out in basic seawater (Fig. 2B), i.e. in seawater where the main cations (Ca^{2+} , Mg^{2+} , K^+ , and Na^+) are present at the same concentrations as in natural seawater, show a good clustering around the expected direction, with only 2 deg. of difference with respect to the distribution obtained by releasing sandhoppers in unmodified artificial seawater (Fig. 2A). Therefore, the different concentration of chloride ions between the two test solutions does not affect sandhopper orientation. Distributions obtained releasing sandhoppers in basic seawater without K^+ (Fig. 2C) and without Mg^{2+} (Fig. 2D) show that sandhoppers are still able to maintain the correct landward direction by sun compass orientation. It is easy to note an increase in dispersion when sandhoppers are released in basic seawater without Mg^{2+} (Fig. 2D). However the distribution is statistically different from uniformity. Releases in basic seawater without Ca^{2+} originate a photopositive response (Fig. 2E), as observed previously with a reduction to 52.5% of the total Ca^{2+} concentration (Fig. 1D; see Fig. 2E vs Fig. 1D) ($U^2_{(54,31)}=0.111$, N.S.).

Fig. 3 summarises the effect of the reduction of Ca^{2+} on the goodness of orientation (GO). There is a strong reduction in GO value in the test carried out using the solution with 79.9% of total Ca^{2+} . GO values decrease further with decreasing Ca^{2+} concentration (Fig. 3).

The distributions obtained by releasing sandhoppers in artificial seawater with different levels of pH (Fig. 4) (pH from 9.20 to 6.50)

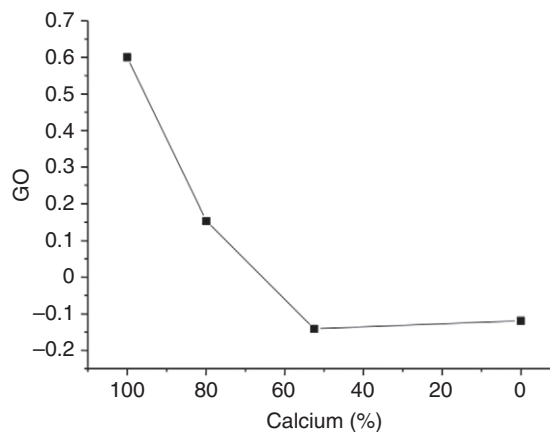


Fig. 3. Goodness of orientation (GO) values for each concentration of Ca^{2+} . See text for further explanations.

are all significantly well directed towards the expected landward direction. The mean vectors of the distributions shown in Fig. 4A and Fig. 4B are deflected by a maximum of 33 deg. with respect to the expected directions (see Fig. 1B as control test). However, despite the difference in pH, their mean angles differ by only 4 deg. (Fig. 4A vs Fig. 4B, $U^2_{(39,44)}=0.077$, N.S.). Moreover, no comparison between the distribution of controls (Fig. 1B; pH=8.00) and the others are statistically significant (Fig. 1B vs Fig. 4A; $U^2_{(63,39)}=0.172$, N.S.) (Fig. 1B vs Fig. 4B; $U^2_{(63,44)}=0.170$, N.S.).

Experiments investigating phototaxis using artificial seawater, double-distilled water and basic seawater without Ca^{2+} (Fig. 5) show a constant and clear preference for the illuminated part of the corridor independent of the salinity and presence or absence of Ca^{2+} .

DISCUSSION

Taking into account that in our tests the salinity of artificial and basic seawater is maintained at 32–39‰ and falls within the natural variation of seawater concentration near the shore, results clearly demonstrate that the absence (0%) or even slight reduction (20%) in concentration of Ca^{2+} in the seawater affects the sandhoppers' capacity to use the sun compass mechanism to orientate towards an ecologically efficient direction: the landward direction of the sea–land axis of their home beach. The influence on sandhoppers' behaviour, however, seems to depend on the quantity of calcium dissolved in the water. In fact, the 20% reduction in concentration results in a random distribution of sandhoppers' individual directions, while greater reductions of Ca^{2+} (47.5% or 100%) determine a photopositive behaviour (orientation towards the sun) that could be interpreted as an avoiding response to

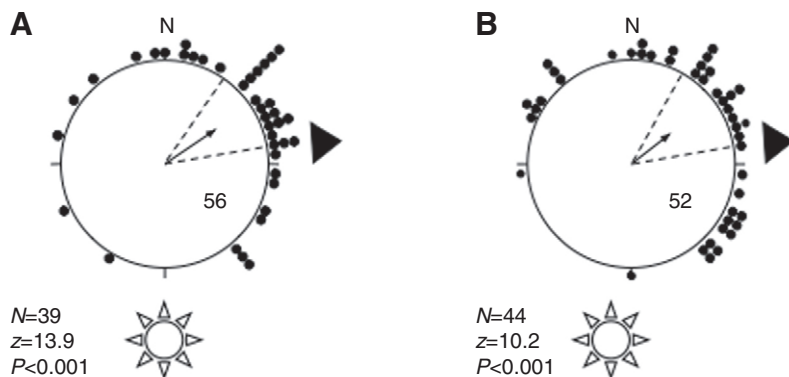


Fig. 4. Solar orientation test at different pH values: (A) pH 9.20; (B) pH 6.50. See Fig. 1 and text for further explanations.

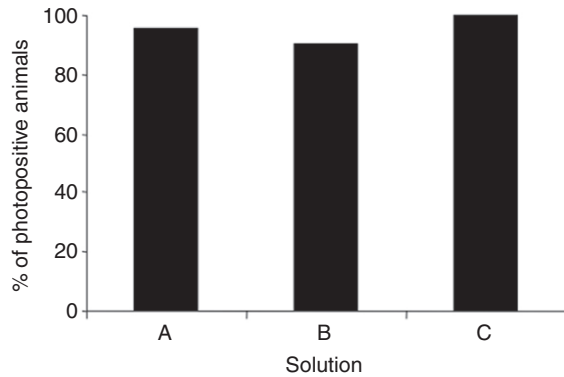


Fig. 5. Phototactic behaviour test. Percentage of photopositive animals tested in (A) artificial seawater, (B) double-distilled water and (C) basic seawater without Ca^{2+} . The number of animals tested is also reported. See text for further explanations.

exit from a stressful situation. The use of the sun compass mechanism does not depend on the pH (see Fig. 4A,B) nor on the salinity in the range adopted in our experiments (see Fig. 1B, Fig. 2A). It is also independent of the presence of the many others ions (e.g. Sr^{2+} , PO_4^{3-} , SO_4^{2-} , NO_3^- , NH_4^+) normally present in natural or artificial seawater (Atkinson and Bingman, 1998), since sandhoppers tested in basic seawater comprising only the main cations (Ca^{2+} , Na^+ , Mg^{2+} and K^+) are well orientated towards the direction of land and with no relevant difference with respect to controls nor mean angle (only 2 deg. of difference).

Some effect could also be noted for Mg^{2+} and K^+ , even though this is less noticeable compared with the effect of Ca^{2+} . In fact, in the absence of Mg^{2+} , despite causing an increase in dispersion, the mean vector is still well directed towards the expected direction, and the difference in mean angle compared with controls is only 5 deg. For K^+ we register a deviation of 30 deg. with respect to the controls. However, in both cases, the distributions are statistically different from uniformity and with the mean vectors directed towards the landward direction.

What is the effect of the absence (reduction) of Ca^{2+} in the seawater on the capacity of solar orientation? It is well known that Ca^{2+} is a very important ion for many aspects of cellular physiology (Wilkins and Wilkins, 2003); therefore, we can (trivially) hypothesize that in our experimental conditions its reduced concentration (1) influenced the visual system cells and/or (2) determined a general bad functioning at the cellular level owing to modification of the external and internal cellular Ca^{2+} concentrations.

The clear photopositive tendency seen in artificial seawater, basic Ca^{2+} -deprived seawater and double-distilled water should indicate that the absence of Ca^{2+} does not affect the normal functioning of the visual cells, at least in our experimental conditions. Moreover, it should be remembered that *T. saltator* is a quite strong hyper-hypo regulator of osmotic and ionic concentration (Morritt, 1988; Morritt, 1989; Calosi et al., 2007). Therefore, without excluding any hypothesis, and taking into account the reduced time spent in the modified seawater by *T. saltator* during the experiments (maximum of 7.15 min), we believe that neither of the above two hypotheses are likely. A third hypothesis could consider the important role of

Ca^{2+} on the regulation of nitric oxide (NO) production in the central nervous system level. In this case, an alteration of the Ca^{2+} concentration could affect the efficient functioning of the sun compass mechanism in one or both of its components: the compass direction and/or the chronometric mechanism. In fact, it is well known that NO production influences many important functions, such as neurotransmission, neuromodulation, learning and memory formation (Martinez, 1995; Jacklet, 1997; Müller, 1997; Colasanti and Venturini, 1998). In particular, in crustaceans it has been demonstrated that the NO/cGMP pathway is associated with the organization and production of motor behaviour (Scholz, 2001).

In conclusion, frankly speaking, we do not yet have any real explanation for our findings from the physiological point of view, but we can say that Ca^{2+} seawater concentration is important for the correct functioning of one of the principal mechanisms of orientation in supralittoral amphipods and therefore it is important for their survival in the field.

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