Open access • Journal Article • DOI:10.1007/S00227-012-2059-6

Ocean acidification and temperature rise: effects on calcification during early development of the cuttlefish Sepia officinalis — Source link

Narimane Dorey, Narimane Dorey, Frank Melzner, Sophie Martin ...+10 more authors

Institutions: International Atomic Energy Agency, University of Gothenburg, Pierre-and-Marie-Curie University, Centre national de la recherche scientifique ...+1 more institutions

Published on: 19 Aug 2013 - Marine Biology (Springer)

Topics: Sepia and Cuttlefish

Ocean acidification and temperature rise: effects on calcification during early development of the cuttlefish Sepia officinalis

Narimane Dorey, Frank Melzner, Sophie Martin, François Oberhänsli, Jean-Louis Teyssié, Paco Bustamante, Jean-Pierre Gattuso, Thomas Lacoue-Labarthe

Ocean acidification and temperature rise: effects on calcification during early development of the cuttlefish *Sepia officinalis*

Narimane Dorey[a,h], Frank Melzner[b], Sophie Martin[a,e,f], François Oberhänsli[a], Jean-Louis Teyssié[a], Paco Bustamante[c], Jean-Pierre Gattuso[d,g], Thomas Lacoue-Labarthe[a,c,d]*

a. International Atomic Energy Agency – Environment Laboratories (IAEA-EL), 4 Quai Antoine I[er], MC-98000 Principality of Monaco

b. Biological Oceanography, Helmholtz Centre for Ocean Research (GEOMAR), 24105 Kiel, Germany

c. Littoral, Environnement et Sociétés (LIENSs), UMR 7266, CNRS-Université de La Rochelle, 2 rue Olympe de Gouges, F-17042 La Rochelle Cedex 01, France

d. Université Pierre et Marie Curie-Paris 6, Observatoire Océanologique de Villefranche, 06230 Villefranche-sur-Mer, France

e. CNRS, Laboratoire Adaptation et Diversité en Milieu Marin, Station Biologique de Roscoff, Place Georges Teissier, 29682 Roscoff Cedex, France

f. Université Pierre et Marie Curie, Paris 06, Laboratoire Adaptation et Diversité en Milieu Marin, Station Biologique de Roscoff, Place Georges Teissier, 29682 Roscoff Cedex, France

g. CNRS-INSU, Laboratoire d'Océanographie de Villefranche, BP 28, 06234 Villefranche-sur-Mer Cedex, France.

h. Department of Biological and Environmental Sciences, The Sven Lovén Center for Marine Science, University of Gothenburg, Kristineberg, 45034 Fiskebäckskil, Sweden

Correspondence to: Ms Narimane Dorey

Address: Department of Biological and Environmental Sciences,
The Sven Lovén centre for Marine Sciences – Kristineberg,
University of Gothenburg,
45034 Fiskebäckskil, Sweden

Phone: +46 (0) 766-22 95 29

Fax: +46 (0) 523-185 02

E-mail: narimane.dorey@gu.se

*present address: tlacouel@gmail.com

Abstract:

This study investigated the effects of seawater pH (i.e. 8.10, 7.85 and 7.60) and temperature (16 and 19°C) on (i) the abiotic conditions in the fluid surrounding the embryo (viz. the perivitelline fluid), (ii) growth, development and (iii) cuttlebone calcification of embryonic and juvenile stages of the cephalopod *Sepia officinalis*. Egg swelling increased in response to acidification or warming, leading to an increase in egg surface while the interactive effects suggested a limited plasticity of the swelling modulation. Embryos experienced elevated pCO_2 conditions in the perivitelline fluid (> 3-fold higher pCO_2 than that of ambient seawater), rendering the medium under-saturated even under ambient conditions. The growth of both embryos and juveniles was unaffected by pH, whereas ^{45}Ca incorporation in cuttlebone increased significantly with decreasing pH at both temperatures. This phenomenon of hypercalcification is limited to only a number of animals but does not guarantee functional performance and calls for better mechanistic understanding of calcification processes.

Keywords: early-life stages; calcification; cephalopod; *Sepia officinalis*; ocean acidification; global warming; Ca-45

INTRODUCTION

Marine calcifying organisms were expected to be particularly impacted by ocean acidification-driven changes in marine carbonate chemistry (Seibel and Fabry 2003; Orr et al. 2005; Langdon and Atkinson 2005; Kleypas et al. 2006). Our current knowledge supports the hypothesis that a broad range of them indeed undergo reduced calcification or increased dissolution rates in response to predicted atmospheric pCO_2 increases (see review by Kroeker et al. 2010): corals (see review by Hoegh-Guldberg et al. 2007), planktonic foraminifera (e.g. Spero et al. 1997; Bijma et al. 2002), coccolithophores (e.g. Riebesell et al. 2000; Zondervan et al. 2001), bivalves (e.g. Gazeau et al. 2010, 2011; Waldbusser et al. 2010; Thomsen et al. 2010), pteropods (e.g. Comeau et al. 2011) or echinoderms (reviewed by Dupont et al. 2010). Some studies however reported unchanged or increased calcification rates under high seawater pCO_2 in echinoderms (Wood et al. 2008; Gooding et al. 2009; Ries et al. 2009), decapod crustaceans (Ries et al. 2009), juvenile cephalopods (Gutowska et al. 2010a,b), and teleost fish (otholiths: Checkley et al. 2009; Munday et al. 2011). Seibel and Walsh (2001, 2003) and Seibel and Fabry (2003) hypothesized that adults or juveniles of active species with high metabolic rates are not impaired as negatively by ocean acidification as species with lower metabolic rates. Highly mobile and active marine organisms (teleost fish, cephalopods and many brachyuran crustaceans) need a powerful ion regulatory apparatus to maintain constant blood pH despite fluctuations in blood / haemolymph and seawater pCO_2. This ability to efficiently regulate extracellular pH could be one explanation for the increased tolerance of some organisms to projected increases in future pCO_2 (Melzner et al. 2009). These processes, controlled by ion-transporter fueled by ATP-dependent pumps, may be energetically costly to maintain in acidified conditions (Hu et al. 2011a). Yet, ocean acidification will act simultaneous with other climate-related variables, including a global temperature rise. The interaction between these two co-occurring stressors could have a larger effect on animals by accumulating metabolic additional requirements (Pörtner 2008; Blackford 2010). As an example, regarding cephalopods, Rosa and Seibel (2008) showed that the effects of hypercapnia (~1000 µatm, pH_{NBS} ~7.62) on the jumbo squid metabolism were more pronounced at elevated temperatures (-15 to -20% of oxygen consumption during resting periods at 20-25°C respectively vs. no effect of hypercapnia at 10°C).

In organisms possessing complex life-cycles, it is often assumed that early-life stages may be the most vulnerable to environmental perturbations, including high pCO_2 (Pörtner and Farell 2004; Pörtner 2008; Kurihara 2008), especially in invertebrates (e.g. Kurihara 2008; Dupont and Thorndyke 2009). Even though this view is discussed controversly (Dupont and Thorndyke 2009; Byrne 2011), projected changes in seawater abiotic conditions could have strong effects on the development of embryos, larvae and juveniles and their calcification capacities. Until now however,

studies on the effects of high pCO_2 on the calcification capacities of water-breathing marine organisms' early life stages are still scarce. On the common cuttlefish *Sepia officinalis* for instance, Gutowska et al. (2008) showed maintained calcification and growth in juveniles after 40 days of exposure to high pCO_2. In a second study, Gutowska et al. (2010b) showed a 20-50% increase of the $CaCO_3$ fraction in juvenile's cuttlebones along with a structural change of the calcified matrix. It should be noted that both studies were done at much higher pCO_2 than the IPCC predictions for 2100 (i.e. ~4000 µatm, pH_{NBS} ~7.23 and ~6000 µatm, pH_{NBS} ~7.10; compared to 900 µatm, 7.70 pH units predicted for 2100; IPCC 2007). Additionally, very few studies have considered the impacts of global change on several successive life-stages (e.g. Parker et al. 2012, Dupont et al. 2012), while the transition from one stage to another (e.g. metamorphosis, hatching) can be radical. It has been demonstrated that, for example, juvenile performances can be impaired by stressful experiences during larval or embryonic life (Pechenik 2006).

Among the class Cephalopoda, the Sepiida (or cuttlefish) are characterized by an internal cuttlebone (Rodhouse 1998), i.e. an aragonitic-organic composite structure used as a structural skeleton that also serves as a buoyancy control device (Denton and Gilpin-Brown 1959). It is composed of a calcareous phragmocone containing several septae (i.e. delimiting lamellae), separated by small vertical pillars and walls forming chambers (Birchall and Thomas 1983). These chambers contain gas (Denton and Taylor, 1964) and are used to regulate the animal's vertical position in the water column. Cuttlefish buoyancy is adjusted by moving liquid in or out of the shell chambers via an osmotic pump (Denton and Gilpin-Brown 1961, Denton et al. 1961). The first chambers of the cuttlebone are synthesized during the embryonic phase within the egg case. Cuttlebone and associated buoyancy mechanism have to be functional upon hatching, as these necto-benthic predators immediately start to move and feed in complex three-dimensional environments (Boletzky 2003). The cuttlebone is produced rapidly, in accordance with the high, exponential growth rates observed in embryonic and juvenile cephalopods (*ca.* 3.5% of the bodyweight per day in 1.5 g cuttlefish at 17°C, Forsythe et al. 2002, Melzner et al. 2005).

Among Sepiida, *S. officinalis* is one of the most abundant cephalopods along the European coasts, including the Mediterranean sea (Boletzky 1983). This species is commercially important in this area with landings 50,000 tons per year in Europe (Pierce et al. 2010). After a relative short life (one to two years), adults spawn and lay their eggs in shallow waters (< 30-40 m, Guerra, 2006) from late winter to early summer (Rodhouse 1998). In the Ligurian Sea (NW Mediterranean), water temperatures fluctuate around 15-17°C when cuttlefish spawning reaches its peak (March-May, 0-50 m, DYFAMED: http://www.obs-vlfr.fr/sodyf/). The lecithotrophic embryos then develop for a relatively long time inside the eggs (two months at 16°C). Development ends with hatchlings that

resume a necto-benthic life-style and are essentially isometric copies of adults (Melzner et al. 2007). These juveniles experience warmer waters from May to July although temperature does not yet exceed 20°C. Regarding carbonate chemistry conditions, recent data in Ligurian Sea estimated a monthly average of surface seawater pCO_2 varying throughout the year in a limited range from 300 µatm (January-May) to 420 µatm (July-September; Bégovic and Copin-Montégut 2002; Touratier and Goyet 2011).

In the egg, the embryo bathes in the perivitelline fluid (PVF) and is protected by a flexible eggshell, which serves as a protective layer against the surrounding environment (Boletzky 1986). Exchange between seawater and PVF is limited and selective in terms of water and molecules (Gomi et al. 1986; de Leersnyder and Lemaire 1972), including pollutants (Bustamante et al. 2002, 2004, 2006, Lacoue-Labarthe et al. 2009, 2010a, 2010b, 2011a, 2011b). Due to the egg case serving as a diffusion barrier for respiratory gases (CO_2, O_2), embryos are exposed to low PVF pO_2 (pO_2 < 6 kPa, Cronin and Seymour 2000, Gutowska and Melzner 2009) and to high pCO_2 in turn (pCO_2 > 0.4 kPa i.e. > 2000-4000 µatm, Gutowska and Melzner 2009). PVF is also slightly hypertonic when compared to seawater (de Leersnyder and Lemaire 1972), which enables the characteristic egg swelling process (Cronin and Seymour 2000). In addition, the eggshell was shown to display a trace element selective permeability (Bustamante et al. 2002, 2004, 2006; Lacoue-Labarthe et al. 2008, 2009, 2010a, 2011a). Consequently, the embryo grows under abiotic conditions in the PVF that are very different from those that hatchlings and juveniles encounter in the seawater.

pCO_2-driven ocean warming and seawater carbonate chemistry change are likely to differently affect embryonic cuttlebone formation inside the PVF and juveniles living in seawater. To examine the effects of pH and temperature on the calcification capacity of *S. officinalis* successive early life stages (embryo and juveniles), this study investigated (1) the incorporation of the radiotracer ^{45}Ca into the cuttlebone in relation to (2) the abiotic conditions surrounding embryo and juveniles. Cuttlefish were reared during embryonic development and a significant part of early juvenile life (19 days) both at current pH (8.10) and at two lower pH treatment levels (7.85 and 7.60, as predicted for 2100); all three pH conditions were studied at two temperatures (16 and 19°C).

MATERIALS AND METHODS

Animals:

Eight *S. officinalis* adults were obtained from a fisherman in the Principality of Monaco in both April 2008 ("*Experiment 1*") and February 2009 ("*Experiment 2*"). Male and female cuttlefish were

acclimated by pairs and kept in flow-through seawater aquarium systems (600L; temperature from 16 to 18°C) in the IAEA-EL premises. They were daily fed on living shore crabs *Carcinus maenas* or frozen fish. After mating, fertilized eggs laid by one single female were immediately separated to optimize oxygenation and randomly distributed to the appropriate experimental treatments (2 temperature x 3 pH treatments). All animals - adults, eggs and juveniles - were reared in 0.45 µm filtrated, UV sterilized and continuously aerated Mediterranean seawater (salinity: 38; light/dark cycle: 12h/12h).

Experimental design:

Experiment 1: approximately 300 newly laid eggs were randomly assigned in 6 plastic containers (one bottle per treatment) containing 5L of seawater being maintained under controlled conditions of temperature and pH through a crossed (2x3) experiment. Three incubation containers were kept in a bath maintained at 16°C and three in a bath at 19°C, based on the average warming of 3°C of the ocean surface waters expected for the end of the century (Levitus et al. 2005). Within each temperature treatment, one container was maintained at ambient pH (8.10; pCO_2 ~390 µatm) while the two others were maintained at pH values predicted to occur following optimistic and pessimistic future scenarios (IPCC, 2007): 7.85 (pCO_2 ~800 µatm) and 7.60 (pCO_2 ~1400 µatm). Water was renewed weekly during the first week and then every second day to maintain water quality constant ($[NH_4^+] < 0.1$ mg L^{-1}, $[NO_2^-] < 0.1$ mg L^{-1}, $[NO_3^-] < 5$ mg L^{-1}). Eggs from this incubation were used to follow egg mass accretion and embryonic growth (see below *Embryonic and juvenile development*), cuttlebone weight and ^{45}Ca accumulation (see below *^{45}Ca radiolabelling and sample treatment*).

Experiment 2: egg batches were maintained during the whole embryonic development under similar conditions as described above (2 temperature x 3 pH treatments). A sub-sample was used for abiotic conditions measurements in the PVF (see below *Egg perivitelline fluid (PVF) abiotic conditions*) while the remaining eggs were left to hatch. Ten days after hatching, juveniles were transferred and reared in plastic 4-L flat-bottom tanks in the same treatments as the eggs. Seawater was changed every day in all containers to maintain constant water quality and animals were fed *ad libitum* twice per day with live brine shrimp (*Artemia salina*), and juvenile shore crabs (*C. maenas*).

***Culture maintenance and seawater carbonate chemistry assessment**:*

Temperatures were controlled in each bath to within ± 0.5°C (Table 1) using temperature controllers connected to 300 W submersible heaters. In each container, pH was controlled within ± 0.05 pH units (Table 1) by bubbling pure CO_2, using a continuous pH-stat system (IKS, Karlsbad). The pH values of the pH-stat system were adjusted every two days from measurements of pH on the

total scale (pH_T). pH was measured in each bottle using a pH meter (Metrohm, 826 pH mobile) with a glass electrode (Metrohm, electrode plus) calibrated on the total scale using Tris/HCl (TRIS) and 2-aminopyridine/HCl (AMP) buffer solutions with a salinity of 38 and prepared according to Dickson et al. (2007). Total alkalinity (A_T) shifts between two seawater renewals were assessed in non-radiolabelled seawater of similar conditions (pH, temperature, number of living individuals). A_T was measured on seawater samples filtered through 0.7µm membranes, immediately poisoned with mercuric chloride and stored in a cool dark place pending analyses. A_T was determined potentiometrically using a home-made titration system, an Orion 8103SC pH electrode calibrated on the National Bureau of Standards scale and a computer-driven Metrohm 665 Dosimat titrator. A_T was calculated using a Gran function applied to pH values ranging from 3.5 to 3.0 as described by Dickson et al. (2007). The pCO_2 and the aragonite saturation state (Ω_{ar}) were determined from pH_T and A_T using the R package Seacarb (Lavigne and Gattuso 2009), with dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

***Egg perivitelline fluid (PVF) abiotic conditions**:*

Eggs used for pO_2, pH_{SWS} (SWS scale) and total dissolved inorganic carbon (CT) measurements inside the egg fluid were reared in the same temperature and pH cross treatments as previously described. During *Experiment 2*, at the end of development (stage 30 according to Lemaire 1970; i.e. days after spawning = 65 at 16°C and 47 for 19°C, Fig. 1), eggs (n = 8 to 12 per conditions) were gently lifted out of the tank, weighted and measurements started immediately afterwards. PVF sampling was realized within 15s, thus minimizing the artificial increase of the pCO_2 values caused by stressed embryos. Measurements were obtained using a 1 mL plastic syringe equipped with fiber optic micro-sensors (optodes, tip diameter 140 µm, Presens GmbH, Regensburg, Germany). Stable PVF pO_2 values were obtained within 10s in the syringe filled with 200–300 µL of PVF (as previously described in Gutowska and Melzner 2009). PVF pH (SWS scale) values were measured in 0.5 mL plastic tubes using a pH electrode (WTW Mic and WTW pH340i pH meter), with stable values reached within 1min. During the measurement period, the syringe and sensors were placed in a thermostatted water bath at the appropriate temperature (i.e. 16 or 19°C). The oxygen optode was calibrated according to the manufacturer's instructions with water vapor saturated air and a Na_2SO_3 solution. The pH optode was calibrated using TRIS and AMP as described above. CT was measured in 100 µL of PVF with a Corning 965 CO_2 analyzer in triplicates. Carbonate system parameters, i.e. pCO_2, Ω_{ar}, were calculated from CT and pH_{SWS} using CO2SYS software (Lewis and Wallace 1998), with dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

Embryonic and juvenile development:

During embryonic development, egg growth due to egg swelling was determined measuring the whole egg fresh weight, in both *Experiment 1* and *2*. The development course was followed by determination of the embryonic stages according to Lemaire (1970). At stage 30 during *Experiment 1*, embryos (n = 10) body dry weights (65°C overnight) were measured on a fine-scale balance (i.e. days after spawning = 61 and 42, at respectively 16°C and 19°C, Fig. 1). Similarly, during *Experiment 2*, 19-days old juveniles (n = 5 to 12; see Fig. 1) were immediately frozen at -20°C after sampling and dry body masses were measured after 12 h at 65°C.

^{45}Ca radiolabelling and samples treatment:

Experiment 1: Seawater in each bottle was spiked with ^{45}Ca (10 kBq L^{-1}). Radiotracers were purchased from Radioisotope Centre Polatum, Poland, ^{45}Ca [as $^{45}CaCl_2$; $T_{½}$ = 163 d]. Stock solutions were prepared in H_2O to obtain radioactivities that allowed the use of spikes of only a few microliters (typically 5-10 µL). The radiotracer spikes were renewed at each water change, in order to maintain radiotracer concentrations. Radiotracer activities in seawater were checked before and after each water renewal in 5-mL samples, in order to determine the time-integrated radiotracer activities (Rodriguez y Baena et al. 2006). When stage 30 was reached, 5 eggs were weighted and sampled to determine the radiotracer incorporation in the embryo. Eggs exposed to dissolved ^{45}Ca were dissected and the different egg compartments were separated among the eggshell, the vitellus, the PVF and the embryo. The cuttlebone was then separated from the embryo flesh, dried at 65°C for 24 h and weighted.

Experiment 2: to follow the incorporation of ^{45}Ca in the juvenile cuttlebone, 10-days old juveniles were exposed to ^{45}Ca (25 kBq L^{-1}) after the transfer from bottles to tanks for a maximum of three weeks. The radioactive exposure procedure was carried out as described above (see *Experiment 1*). After 9 days of exposure, 5 to 12 19-days old juveniles were sampled in each condition to determine the radiotracer concentration in their cuttlebone. Juveniles were sampled, frozen at -20°C and dried for one night at 65°C. In order to individualize cuttlebones from flesh, dried juveniles bodies were individually dissolved with 8 mL of NaOH (2N), heated at 40°C (5 hours) and continuously agitated until cuttlebones were completely free from flesh. Cuttlebones were collected, rinsed into clear distilled water and dried (24 h at 65°C) before dry mass was measured.

Radioactive sample treatment: Radioactive isolated calcareous structures were dissolved adding 300 µL of hydrochloric acid (HCl, 37%) at 80°C. After evaporation, the residues were dissolved in 1 mL of distilled water. Biological and seawater samples were counted after adding 10 mL of scintillation liquid, Ultima GoldTM XR (Perkin Elmer). Emissions were measured with a liquid

scintillation analyzer (Tri-Carb, Packarb 1600 TR or Perkin Elmer 2900 TR) calibrated with an appropriate standard for each counting that was used. Counting times were adapted to obtain relative propagated errors less than 5% (from 10 min to 24 h). Corrections for the physical half-life time and background noise were done in order to determine the ^{45}Ca concentrations at the sampling time (Bq). Uptake of ^{45}Ca in the cuttlebone was expressed as the amount of Ca incorporated (Q_{Ca}, in μmol ^{45}Ca cuttlebone^{-1}) according to Martin et al. (2011) and following the equation:

$$Q_{Ca} = [(A_{cut} / A_{sw}) \times C_{sw}] \times 10^3$$

where A_{cut} is the total ^{45}Ca activity in each cuttlebone (in Bq), A_{sw} is the time-integrated activity (in Bq g^{-1}) in seawater during the time of exposure and C_{sw} is the total Ca concentration in Mediterranean seawater (0.0114 mmol g^{-1}).

Data analyses:

Results are presented for both the end of embryonic development (stage 30) and day 19 of juvenile life and are given as mean ± SD. Statistical analyses were conducted using the software R (R Development Core Team, 2008). To test the effect of pH and temperature treatments and their interaction, two-ways ANOVAs were performed. Prior to analyses, the data was checked for normality distribution and homogeneity of variances. When necessary, post-hoc test and interaction plots were used to determine the factors' interactions. All the test decisions were taken at a threshold of α=0.05.

RESULTS

Seawater carbonate chemistry conditions: seawater carbonate chemistry parameters in the different pH and temperature conditions are reported in Table 1. Mean A_T of the seawater was 2.597 ± 0.012 mmol kg^{-1} and 2.549 ± 0.065 mmol kg^{-1} in the egg and juvenile experiments, respectively; in both experiments, A_T changed by less than 0.030 mmol kg^{-1} between two seawater renewals. Seawater temperature and pH were stable over the experiments, showing variations lower than 2.5% from the mean of each measured parameter. Both eggs and juveniles were maintained at pCO_2 of ca. 380 μatm (pH_T = 8.09), ca. 750 μatm (pH_T = 7.84) and ca. 1430 μatm (pH_T = 7.60) irrespective of the temperature (16 or 19°C), consistently with the actual and predicted pH values for the end of the century (IPCC 2007). In all experimental conditions, eggs and juveniles were reared in super-saturated seawater with respect to aragonite (Ω_{ar} > 1.15; Table 1).

***PVF abiotic conditions* (*Experiment 2*):** the egg development duration was logically shortened by temperature increase, and hatchling delay caused by increasing pCO_2 was not observed in our experimental conditions (stage 30 reached 65 days and 47 days after spawning, at respectively 16°C and 19°C, irrespectively of the pCO_2 treatment). The embryos develop inside the protective eggshell, bathing in the PVF which abiotic conditions (i.e. carbonate chemistry parameters and pO_2) were determined at the end of the development (stage 30). At this stage, PVF pO_2 was very low with ~25% of air saturation, all conditions merged (Fig. 2). At 16°C, the PVF pO_2 was 25.28 ± 2.95% vs. 23.73 ± 2.45% air sat. at 19°C, revealing a slightly but significantly higher oxygen consumption of the embryo at warmer temperature (Table 2; $P = 0.02$), especially visible in control pH conditions (8.10: 27.1 ± 2.4% at 16°C vs. 22.9 ± 3.0% air sat. at 19°C). Seawater pH did not significantly influence PVF pO_2 in eggs close to hatching ($P = 0.285$) but had a significant combined effect with the temperature ($P = 0.012$), revealing a slender decrease of the PVF pO_2 with decreasing pH only at 16°C (27.1 ± 2.4%, 25.4 ± 3.1% and 23.4 ± 2.2% air sat. in pH conditions of respectively 8.10, 7.85 and 7.60).

At stage 30, the embryo bathed in an environment with lower pH than that of the surrounding seawater ($7.05 \leq$ PVF $pH_{SWS} \leq 7.51$ units, Fig. 2). The lowest values were reached in eggs exposed to pH 7.60 treatment (PVF $pH_{SWS} = 7.07 \pm 0.01$ at 16°C and 7.10 ± 0.03 at 19°C). Besides, PVF pH was significantly lower at 16°C ($pH_{SWS} = 7.07 \pm 0.01$, 7.21 ± 0.02 and 7.35 ± 0.03 units, in pH treatments of 8.10, 7.85 and 7.60 respectively) than at 19°C (7.10 ± 0.03, 7.22 ± 0.03 and, 7.45 ± 0.03 units, respectively, $P < 0.001$, Table 2).

In control pH condition, embryos were exposed to a ca. 5 to 6 times higher pCO_2 compared to external conditions, whatever the temperature (PVF vs. seawater: 2311 µatm vs. 376 µatm at 16°C and 1822 µatm vs. 370 µatm at 19°C). PVF pCO_2 increased with lower seawater pH (Fig. 2), reaching maximum values of ca. 4700 µatm in the lowest pH treatment (i.e. seawater pCO_2 of ca.1400 µatm). CO_2 gradient between PVF and seawater (ΔpCO_2 - i.e. pCO_2 inside the PVF minus seawater pCO_2) thus increased with higher seawater pCO_2 (Table 2, $P < 0.001$). Respective ΔpCO_2 values in pH treatments of 8.10, 7.85 and 7.60 were of 1863 ± 142, 2391 ± 192 and 2852 ± 178 µatm at 16°C and 1458 ± 164, 2217 ± 231 and 2794 ± 249 µatm at 19°C. The pCO_2 gradient and hence the difference of pCO_2 between the exterior and the interior of the egg, was also diminished under warmer treatments ($P < 0.001$). Alongside with the extremely high PVF pCO_2, the PVF was always under-saturated with respect to aragonite, even under control conditions (Fig. 2: $\Omega_{ar} = 0.644 \pm 0.038$ units at 16°C and 0.886 ± 0.039 units at 19°C). As expected, the Ω_{ar} values dropped with decreasing pH (Table 2, $P < 0.001$), leading to extremely low values in seawater pH treatment of 7.60 ($\Omega_{ar} = 0.335 \pm 0.011$ units at 16°C and $\Omega_{ar} = 0.418 \pm 0.032$ units at 19°C). Our results revealed

an interaction of both seawater pH and temperature with respect to PVF carbonate chemistry. Acidification rapidly worsen conditions advantageous to calcium precipitation in the embryonic fluid ($\Omega_{ar\ PVF}$ = 0.771 ± 0.130, 0.487 ± 0.062 and 0.372 ± 0.047 units at respectively 8.10, 7.85 and 7.60), while this effect appeared counterbalanced by seawater warming (Table 2: pH_{PVF}: $P < 0.001$; pCO_{2PVF}: $P < 0.01$; $\Omega_{ar\ PVF}$: $P < 0.001$; $\Delta pCO_{2\ PVF}$: $P < 0.05$) leading to more under-saturated fluids at low temperature.

Embryonic and juvenile development: the egg swelling was followed in *Experiment 1* (2008) and *2* (2009) by measuring the whole egg weight. Because egg size is a female-dependent property, the egg weights were clearly different between the two experiments. However, it is noteworthy that egg weights within each group were affected by temperature at the end of development (Fig. 3; Table 2; $P < 0.001$ for both years), with heavier eggs at 19°C than at 16°C (respectively 2.28 ± 0.10 g vs. 1.71 ± 0.16 g in 2008 and 3.69 ± 0.33 g vs. 2.95 ± 0.25 g in 2009). pH treatment also influenced significantly the egg weight ($P < 0.001$ in 2008 and $P = 0.007$ in 2009; Table 2) highlighting an enhanced egg swelling phenomenon under acidified conditions. This pH effect was however weaken at warmer temperature, with similar egg weights for all pH at 19°C (Fig. 3; Table 2: $P < 0.001$ in 2008 and $P = 0.028$ in 2009).

At the end of the development (*Experiment 1*), the whole body dry weight of the embryo was only affected by the temperature treatment ($P < 0.001$; Table 3). Embryos raised at 16°C during 61 days were significantly heavier than those that developed at 19°C during 42 days (Fig. 4; 32.39 ± 1.38 mg vs. 26.30 ± 1.65 mg respectively). Hatching success has not been significantly affected by treatments (< 5% of eggs were undeveloped, as usually observed in similar conditions). Survival rates of juveniles reared during the *Experiment 2* was > 90 % in all conditions during the first 3 weeks of incubation. The health of animal strongly decreased posterior to this date and high mortality was observed along the following days (~80% mortality reached by 36 and 30 days post-hatching in 16 and 19°C treatments respectively). Therefore, we focused on measures of ^{45}Ca uptake collected in healthy juveniles (showing hunting activities when fed, standing on the bottom of the tank) sampled after 19 days of incubation in the six treatments. According to these data, neither temperature nor pH had an effect on body dry weight after 19 days of incubation (see above: 10 days of maintenance post-hatching and 9 days of exposure; Fig. 4).

Cuttlebone growth and calcification: at the end of development, the weight of embryo's cuttlebone was not significantly affected by the pH treatment (*Experiment 1*, $P = 0.59$; Table 3 and Fig. 4) but was heavier in animals maintained at 19°C compared to 16°C (3.24 ± 1.28 mg vs. 1.43 ± 0.30 mg respectively; $P < 0.001$). In 19-days old juveniles, the cuttlebones were slightly heavier at 19°C than at 16°C (*Experiment 2*, 3.66 ± 0.86 mg vs. 3.00 ± 0.44 mg respectively; $P < 0.05$). Cuttlefish

calcification was assessed through ^{45}Ca incorporation in cuttlebones both during the whole embryonic development (42 or 61 days) and 9 days of juvenile life. This difference of incubation time explains the contrasting quantity of calcium (Q_{Ca}: in µmol) precipitated in embryo and juvenile calcareous structure (Fig. 5). Temperature also had an effect on ^{45}Ca incorporation, however, only at the embryonic stage: a seawater warming of 3°C reduced Q_{Ca} by a factor of 1.6 irrespective of pH treatment (Fig. 5; $P < 0.001$). Comparisons within each experiment indicated that accumulation of $CaCO_3$ (Q_{Ca}) was greater with decreasing pH both in embryo and juvenile stages (Fig. 5; $P < 0.001$). The lowest pH treatment led to an increase of calcium incorporation in cuttlebone from ca. 17% in embryo to up 80% in juvenile in our experimental conditions.

DISCUSSION

According to our results, a decrease of seawater pH by 0.25 to 0.50 units, as expected in average for the end of the century in global oceans, would increase the accumulation of calcium in the internal calcareous structure by 17% to 80% in embryonic and juvenile cuttlefish respectively. This study corroborates with the observation of Gutowska et al. (2010b) where cuttlebones of *Sepia officinalis* juveniles displayed a significant increase in $CaCO_3$ mass (*hypercalcification)* under very high pCO_2 (~6000 µatm for 40 days). Here, using ^{45}Ca ß-emitting radiotracer, a very sensitive nuclear detection method that allows for calcification rate estimation in early life stages (Fabry and Balch 2010), we demonstrate that such hypercalcification also occurs under realistic scenarios of increased temperature and pCO_2 in early life stages of *Sepia*.

In cephalopods, the requirements in essential elements are poorly known and only few studies have examined the elemental content of early stages (Craig and Overnell 2003, Villanueva and Bustamante 2006, Miramand et al. 2006). Calcium reserves needed for metabolic processes could be contained in the rich yolk of the cuttlefish egg (Boletzky 1974, 1989; e.g. Lacoue-Labarthe et al. 2009). In the octopod *Octopus vulgaris* however, calcium concentration increases during the oocyte development, suggesting that this essential element could be obtained from seawater intake (Villanueva and Bustamante 2006). It has been previously demonstrated that the eggshell displays a selective permeability to dissolved trace elements, independently of their essential or non-essential character (Bustamante et al. 2002, 2004, 2006, Lacoue-Labarthe et al. 2008, 2009, 2010a), and that changes in seawater pCO_2 conditions could affect these properties (Lacoue-Labarthe et al. 2009). In this study, it is worth noting that, whatever the treatment, ^{45}Ca was detected in embryos' cuttlebones revealing that 1) the eggshell is permeable to calcium and 2) the cuttlebone formation is dependent on calcium uptake from seawater. If external seawater is the only source of calcium, approximately

2.7 mL of seawater ($[Ca^{2+}]$ = 411 mg L_{SW}^{-1}) would need to be depleted of calcium in order to build a cuttlebone at the end of development at 19°C (dry weight = 3.0 mg, composed of 95% $CaCO_3$).

Both embryos and juveniles hypercalcified when exposed to elevated pCO_2, although the surrounding medium became less favorable to $CaCO_3$ precipitation (i.e. decreasing Ω_{ar}). Numerous studies on bivalve molluscs showed reduced rates of calcification in response to ocean acidification (e.g. Miller et al. 2009; Thomsen et al. 2010). Marine organisms display a species-specific ability to calcify (Ries et al. 2009, Findlay et al. 2011), probably linked to their ability to maintain favorable chemical condition for $CaCO_3$ precipitation at the calcification site when exposed to high pCO_2 (e.g. Venn et al. 2011). Protective organic layers covering the calcareous structure are a key to determine species vulnerability against corrosive seawater that can lead to external $CaCO_3$ dissolution (Tunnicliffe et al. 2009, Ries et al. 2009; Rodolfo-Metalpa et al. 2010, Thomsen et al. 2010). The cuttlefish is a unique mollusc model as it is characterized by a fully internal calcareous structure. This latter is bathed in extracellular fluids with their particular controlled carbonate chemistry and surrounded by the shell forming an epithelium (Appellöf 1893). Similarly, hypercalcification has also been observed in the internal otoliths of fish (Checkley, 2009), a structure bathing inside a fluid chamber and regulating fish equilibrium. Shiao et al. (2005) postulate that calcification conditions are maintained around the otolith by the ion-transporters discovered on the external membrane. The specificity of an internal structure in *S. officinalis* could thus be one reason for the ability to calcify even under the drastic abiotic conditions within the PVF as observed in this study.

The higher calcification observed under acidified conditions of both embryos and juveniles should be considered in relation to the abiotic conditions in which the animals developed, i.e. the PVF vs. seawater. Within the egg at the end of development, PVF pO_2 was low due to the metabolic requirements of the growing embryo, as previously demonstrated (Cronin and Seymour 2000; Gutowska and Melzner 2009). In turn, aerobic metabolism produces CO_2, ultimately resulting in a very high PVF pCO_2, even in ambient conditions (Gutowska and Melzner 2009; this study). PVF pCO_2 was affected by increasing seawater pCO_2, with PVF pCO_2 rising up to ca. 4000 µatm when eggs were incubated at a seawater pCO_2 of ca. 1400 µatm (see Fig. 2). This additive effect of ocean acidification on the PVF hypercapnic conditions is necessary to maintain rates of CO_2 excretion from the PVF to the seawater (Hu et al. 2011a). Diffusive flux of metabolic CO_2 out of the egg (MCO_2) is proportional to the surface of the eggshell (A), the pCO_2 gradient between PVF and seawater (ΔpCO_2) and inversely proportional to the thickness (d) of the eggshell. It also depends on the specific material properties of the barrier (Krogh's gas diffusion coefficient, K):

$$MCO_2 = \Delta pCO_2 \times A/d \times K$$

While maintenance of the ΔpCO_2 seems to be crucial for cuttlefish embryos exposed to elevated seawater pCO_2, our work also suggests that other responses are facilitating CO_2 (O_2) diffusion from the egg to the seawater (and vice versa). A temperature increase of 3°C induces an egg weight increase of *ca.* 20-30%, resulting in an estimated egg surface area increase of 15-20% (Lacoue-Labarthe et al. 2009; this study). Although the mechanism controlling the swelling process is not fully understood (see Lacoue-Labarthe et al. 2009), the osmotic gradient driven water entry in the perivitelline space (de Leersnyder and Lemaire 1972) might be closely coupled to physiological processes of the developing embryo. The enhanced swelling of the egg, as shown in Fig. 3, directly increases the surface area (A) of the eggshell and reduces its thickness (d) (see Cronin and Seymour 2000). This process therefore contributes to facilitate CO_2 exchanges in order to maintain low PVF pCO_2, especially when metabolic rates are increased with elevated temperature and pCO_2 (Melzner et al. 2006). For example, at pH 8.10, the PVF pCO_2 was lower at 19°C than at 16°C whereas a higher value would be expected at warmer temperature, due to increased metabolic activities. We postulate that the enhanced egg swelling at 19°C and the subsequent increased surface / reduced thickness of the eggshell allows a ΔpCO_2 decrease and hence a lower PVF pCO_2. This higher gas exchange at higher temperature could also explain the slightly lower pO_2 at 19°C than at 16°C at the end of development. Nevertheless, the effect of pCO_2 on egg volume was weaken at high temperature, suggesting that swelling plasticity could be limited and that, at the end of development, the egg capsule probably reached its maximum stretching capacity at 19°C, irrespective of the pH treatment. Under such conditions, additive effects of pCO_2 and temperature might be hidden by structural limitation of the eggshell.

These adverse abiotic conditions in the PVF have implications regarding 1) the calcification capacities of the embryo and 2) the developmental conditions for the subsequent juvenile life. First, despite the under-saturation of the PVF, even under normal pH conditions ($0.58 \leq \Omega_{ar} \leq 0.96$), embryos are able to precipitate aragonite. They furthermore increased calcium accumulation in a PVF medium extremely under-saturated with $CaCO_3$ ($0.32 \leq \Omega_{ar} \leq 0.47$ when seawater pH = 7.60). In juvenile animals exposed to 6000 µatm of pCO_2, the higher rates of calcification were hypothetically linked to their efficient acid-base regulation capacities (Gutowska et al. 2010a). More precisely, cuttlefish were observed to rapidly increase blood bicarbonate (HCO_3^-) concentrations from 3.3 to 10.4 mM in the blood to compensate hypercapnia-induced extracellular acidosis (Gutowska et al. 2010b). Increases in extracellular [HCO_3^-] raises the calcium carbonate saturation state in extracellular fluids, potentially facilitating calcium precipitation at the site of biomineralization, or increasing HCO_3^- transport into calcifying epithelial cells (Gutowska et al. 2010a). Such process could also be at work in embryos since Hu et al. (2010, 2011a) demonstrated that the gills of late cuttlefish embryos are characterized by an active ion regulatory machinery

capable of acid-base regulation. In addition, Hu et al. (2011b) could establish the presence of ionocytes on skin and yolk epithelium even in earlier embryonic stages that lack gills. These authors revealed net proton excretory fluxes from the embryo to the surrounding medium, suggesting that even early cephalopod embryos are able to regulate body fluid pH by means of bicarbonate accumulation / net proton excretion. Hypermineralization was observed predominantly in species that are characterized by efficient blood / haemolymph accumulation capacities (crustacea: Ries et al. 2009, teleost fish: Checkley et al. 2009, Munday et al. 2011). In echinoderms, hypercalcification under acidified conditions has only been noted in adults (Wood et al. 2008 and Ries et al. 2009), stage at which abilities to regulate the internal coelomic fluid pH by modulating bicarbonate accumulation were observed (sea urchin: Stumpp et al. 2012). In this same group (Beniash et al. 1997, 1999, Politi et al. 2004) and recently in bivalves (Weiss et al. 2002, Jacob et al. 2011), it was shown that the initial stages of calcification are intracellular processes: an amorphous calcium carbonate (ACC) precursor phase is formed in vesicles and later exocytosed to be incorporated into the skeleton (see Addadi et al. 2006 for a review of current concepts). It is not unlikely that elevated blood bicarbonate concentrations, as encountered during hypercapnia in cuttlefish, increase the rates of import of this substrate for biomineralization into calcifying epithelial cells: ACC formation and calcification in general could be increased this way. The mechanisms behind cuttlefish calcification will be a beneficial topic for further research.

The ability of cuttlefish embryos to calcify under extremely high seawater pCO_2 raises the question of whether this high physiological performance is associated with increased metabolic costs. Recent studies have measured increased metabolic costs associated with experimentally elevated seawater pCO_2 (Wood et al. 2008; Thomsen and Melzner 2010; Stumpp et al. 2011), more particularly suggesting energetic trade-offs that impact growth rate - a situation similar to that encountered in many echinoderm larval stages (see Dupont et al. 2010 for a review, Stumpp et al 2011). Active compensation for acidosis and hypercapnia did not induce measureable additive costs in subadult *S. officinalis* exposed to a pCO_2 of 6000 µatm (Gutowska et al. 2008). However, PVF hypercapnia led to a decrease of embryo weight and a delay in embryonic development when eggs were incubated at 3700 µatm (Hu et al. 2011a) or at 1500 µatm (Lacoue-Labarthe et al. 2009). In this study, the pCO_2 did not significantly impact embryos weight before hatching (Fig. 4 and Table 3) contrasting with previous observations (Lacoue-Labarthe et al. 2009a; Hu et al. 2011a). Without obvious explanation, these results have to be considered with caution and would need confirmation in the future. Nevertheless, the lowest weight value, recorded at the highest temperature and pCO_2, suggests synergetic effects of both parameters as previously demonstrated in corals and molluscs (e.g. Rodolfo-Metalpa et al. 2011) and that combined ocean acidification and warming may reduce the efficiency of yolk utilization through an energy budget modulation (Pörtner 2008). Therefore,

on one side, under elevated seawater pCO_2, combined hypoxia and hypercapnia in the PVF would slow down the developmental rate, explaining the delay in hatching time observed by Hu et al. (2011a), and on the other side, extreme seawater and PVF pCO_2 would lead to an increased allocation of energy to acid-base and other cellular homeostatic processes (including ion movements associated with ACC formation for example) at the expense of animal growth. Enhanced egg swelling with increasing hypercapnia and temperature could as well worsen the energy loss for the embryo if the osmotic gradient maintenance between the PVF and seawater required active metabolic processes (Gomi et al. 1986).

The hatching event constitutes a harsh shock for the animal, as the embryo leaves the medium protecting it against direct predation and microbial attacks (Barbieri et al. 1997); however, the juvenile encounters a higher pH and lower pCO_2, resulting in a lower requirement for blood pH regulation. Still, ^{45}Ca incorporation in the cuttlebone was found to be increased even in juveniles under acidified conditions, indicating that similar mechanisms as described must be operative in juveniles as well. In contrary to Gutowska et al. (2010b), we were not able to measure a difference in cuttlebone mass in response to pCO_2 or temperature, however our animals were younger and smaller. The cuttlebone is mainly composed of $CaCO_3$ (92 to 95 % mass) embedded in an organic matrix, giving the organ structure; therefore, in this study, it is not possible to infer conclusion on the impact of the experimental treatments on the organic matrix. Nevertheless, it should be mentioned that a high seawater pCO_2 could modify the cuttlebone size, its internal structure, its organization or composition, resulting in an increase of calcium incorporation but no alteration of the weight. For example, under acidified conditions, along with 20-55% increase in the cuttlebone mass, Gutowska et al. (2010b) observed a strong increase in the number of shell-chambers constituting the cuttlebone, including an increase in the number of $CaCO_3$ structure such as pillars and walls. This last study also demonstrated that the mass of the non-acid-soluble organic matrix (or chitin), was significantly decreased under high pCO_2. In other words: even if cuttlebones were heavier, organic material synthesis in the cuttlebone was decreased. Possibly, and in contrast to calcium carbonate precipitation, synthesis of organic components is complex and energetically costly (Palmer 1992). In conclusion, both higher calcium and lower chitin incorporation could result in altering cuttlebone properties like its buoyancy, by increasing its density, or implosion resistance properties (Sherrard 2000). Further studies should be carried out to determine the consequences of enhanced calcification under increased pCO_2 in the cuttlefish early life stages on the microstructure of the cuttlebone and on the animal swimming abilities.

The results of this study demonstrate the particularities of the response to acidification in both in embryos and juveniles cuttlefish, in comparison to other invertebrates. Calcification processes

under elevated pCO_2 in cephalopods need to be better characterized in order to clarify the mechanisms leading to hypercalcification in this group. Although the combined effects of ocean acidification and temperature did not decrease calcium accumulation during the formation of the calcareous endoskeleton in *S. officinalis* embryos and juveniles, the functional properties of the cuttlebone as buoyancy device could be affected. The effect of raised pCO_2 should be investigated on the animal entire life cycle considering possible carry-over effects (e.g. Parker et al. 2012, Dupont et al. 2012) and not only from the calcification point of view. Linked to buoyancy abilities, further studies on feeding behavior under projected scenarios of future environmental change would be required in order to assess CO_2 impacts on population dynamics of the cuttlefish *S. officinalis*.

ACKNOWLEDGMENTS

Authors thank the reviewers and the editor of Marine Biology for their comments that strongly improved this paper. The International Atomic Energy Agency is grateful to the Government of the Principality of Monaco for the support provided to its Environment Laboratories. This work was supported by the IAEA. This work is a contribution to the "European Project on Ocean Acidification" (EPOCA) which received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 211384. Ms. André and Eric Rinaldi of Monaco are thanked for the collection of Sepia specimens. Mr Antoine and Mrs José Lacoue-Labarthe are thanked for providing juveniles of crabs and shrimps.

REFERENCES

Addadi L, Joester D, Nudelman F, Weiner S (2006) Mollusk shell formation: A source of new concepts for understanding biomineralization processes. Chem Eur J 12:980–987. doi: 10.1002/chem.200500980

Appellöf A (1893) Die Schalen von Sepia, Spirula, and Nautilus. Studien über den Bau und das Wachstum. Kongl Svenska Vetensk Acad Handl 25:1-106. ISSN 0023-5377

Barbieri E, Barry K, Child A, Wainwright N (1997) Antimicrobial activity in the microbial community of the accessory nidamental gland and egg cases of *Loligo pealei* (Cephalopoda: Loliginidae). Biol Bull 193(2):275-276

Bégovic M, Copin-Montégut C (2002) Processes controlling annual variations in the partial pressure of CO_2 in surface waters of the central northwestern Mediterranean Sea (Dyfamed site). Deep-Sea Res PT II 49:2031–2047

Beniash E, Aizenberg J, Addadi L, Weiner S (1997) Amorphous calcium carbonate transforms into calcite during sea urchin larval spicule growth. Proc Roy Soc B 264:461-465. doi: 10.1098/rspb.1997.0066

Beniash E, Addadi L, Weiner S (1999) Cellular control over spicule formation in sea urchin embryos: A structural approach. J Struct Biol, 125, 50-62. doi: 10.1006/jsbi.1998.4081

Bijma J, Honisch B, Zeebe RE (2002) The impact of the ocean carbonate chemistry on living foraminiferal shell weight: Comment on ‘‘Carbonate ion concentration in glacial-age deep waters of the Carribbean Sea’’ by W. S. Broecker and E. Clark, Geochem. Geophys. Geosyst. 3(11):1064. doi:10.1029/2002GC000388

Birchall JD, Thomas NL (1983) On the architecture and function of cuttlefish bone. J Mater Sci 18:2081-2086. doi: 10.1007/BF00555001

Blackford JC (2010). Predicting the impacts of ocean acidification: Challenges from an ecosystem perspective. J Marine Syst 81(1-2):12-18. doi: 10.1016/j.jmarsys.2009.12.016

Boletzky SV (1974) The “larvae” of Cephalopoda: a review. Thalassia Jugoslavica 10:45-76

Boletzky SV (1983) *Sepia officinalis* cephalopod life cycles species account. In: Boyle PR (ed) Cephalopod Life Cycle. Academic Press, London pp 31-52

Boletzky SV (1986) Encapsulation of cephalopod embryos: a search for functional correlations. Am Malacol Bull 4:217–227

Boletzky SV (1989) Recent studies on spawning, embryonic development, and hatching in the Cephalopoda. Adv Mar Biol 6:86-109

Boletzky SV (2003) Biology of early life stages in cephalopod molluscs. Adv in Mar Biol 44:143-203. doi: 10.1016/S0065-2881(03)44003-0

Bustamante P, Cosson RP, Gallien I, Caurant F, Miramand P (2002) Cadmium detoxification processes in the digestive gland of cephalopods in relation to accumulated cadmium concentrations. Mar Environ Res 53:227-241. doi:10.1016/S0141-1136(01)00108-8

Bustamante P, Teyssié J, Danis B, Fowler SW, Miramand P, Cotret O (2004) Uptake, transfer and distribution of silver and cobalt in tissues of the common cuttlefish *Sepia officinalis* at different stages of its life cycle. Mar Ecol Prog Ser 269:185-195. doi: 10.3354/meps269185

Bustamante P, Teyssié J-L, Fowler SW, Warnau M (2006) Contrasting bioaccumulation and transport behaviour of two artificial radionuclides (^{241}Am and ^{134}Cs) in cuttlefish eggshell. Vie Milieu 56(2):153-156

Byrne M (2011) Impact of ocean warming and ocean acidification on marine invertebrate life history stages: Vulnerabilities and potential persistence in a changing ocean. Oceanogr Mar Biol Annu Rev 49:1-42

Checkley DM, Dickson AG, Takahashi M, Radich JA, Eisenkolb N, Asch R (2009) Elevated CO_2 enhances otolith growth in young fish. Science 324:1683–1683. doi: 101126/science1169806S

Comeau S, Gattuso J-P, Nisumaa A-M, Orr J (2011) Impact of aragonite saturation state changes on migratory pteropods. Proc Roy Soc B. doi: 10.1098/rspb.2011.0910

Craig S, Overnell J (2003) Metals in squid, *Loligo forbesi*, adults, eggs and hatchlings. No evidence for a role for Cu- or Zn-metallothionein. Comp Biochem Phys C 134(3):311-317. doi: 10.1016/S1532-0456(02)00274-0
Cronin ER, Seymour RS (2000) Respiration of the eggs of the giant cuttlefish *Sepia apama*. Mar Biol 136:863-870. doi: 10.1007/s002270000274
Denton EJ, Gilpin-Brown JB (1959) Buoyancy of the cuttlefish. Nature 184:1330-1331. doi:10.1038/1841330a0
Denton EJ, Gilpin-Brown JB (1961) The buoyancy of the cuttlefish, *Sepia officinalis* (L.). J Mar Biol Assoc UK 41:319-342. doi: 10.1017/S0025315400023948
Denton EJ, Gilpin-Brown JB, Howarth JV (1961) The osmotic mechanism of the cuttlebone. J Mar Biol Assoc UK 41:351-363. doi: 10.1017/S0025315400023961
Denton EJ, Taylor DW (1964) The composition of gas in the chambers of the cuttlebone of *Sepia officinalis*. J Mar Biol Assoc UK 44:203-207. doi: 10.1017/S0025315400024747
Dickson AG, Millero F (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep-Sea Res 34:1733-1743. doi: 10.1016/0198-0149(87)90021-5
Dickson AG, Sabine CL, Christian JR (2007) Guide to best practices for ocean CO_2 measurements. PICES Spec Publ 3, 191pp
Dupont S, Thorndyke MC (2009) Impact of CO_2-driven ocean acidification on invertebrates early life-history – What we know, what we need to know and what we can do? Biogeosci Discussions 6:3109-3131. doi:10.5194/bgd-6-3109-2009
Dupont S, Ortega-Martínez O, Thorndyke MC (2010) Impact of near-future ocean acidification on echinoderms. Ecotoxicology 19:449-62. doi: 10.1007/s10646-010-0463-6
Dupont S, Dorey N, Stumpp M, Melzner F, Thorndyke M (2012) Long term and trans life-cycle effects of exposure to ocean acidification in the green sea urchin *Strongylocentrotus droebachiensis*. Mar Biol. doi: 10.1007/s00227-012-1921-x
Fabry VJ, Balch WM (2010) Direct measurments of calcification rates in planktonic organisms. In: Riebesell U, Fabry VJ, Hanson L, Gattuso JP (eds) Guide for best practices in ocean acidification research and data reporting. Publications Office of the European Unit, Luxembourg, pp 201-212
Findlay HS, Wood HL, Kendall Ma, Spicer JI, Twitchett RJ, Widdicombe S (2011) Comparing the impact of high CO_2 on calcium carbonate structures in different marine organisms. Mar Biol Res, 7:565–575
Forsythe JW, Lee P, Walsh L, Clark T (2002) The effects of crowding on growth of the European cuttlefish, *Sepia officinalis* (Linnaeus, 1758) reared at two temperatures. J Exp Mar Biol Ecol 269(2):173-185. doi:10.1016/S0022-0981(02)00006-0
Gazeau F, Gattuso JP, Dawber C, Pronker AE, Peene F, Peene J, Heip CHR, Middelburg JJ (2010) Effect of ocean acidification on the early life stages of the blue mussel *Mytilus edulis*. Biogeosciences 7:2051-2060. doi: 10.5194/bg-7-2051-2010
Gazeau F, Gattuso J-P, Greaves M, Elderfield H, Peene J, Heip CHR, Middelburg JJ (2011) Effect of carbonate chemistry alteration on the early embryonic development of the Pacific oyster (*Crassostrea gigas*). PLoS one 6:e23010. doi:10.1371/journal.pone.0023010
Gomi F, Masamich Y, Nakazawa T (1986) Swelling of egg during development of the cuttlefish, *Sepiella japonica.* Zool Sci 3(4):641-645
Gooding Ra, Harley CDG, Tang E (2009) Elevated water temperature and carbon dioxide concentration increase the growth of a keystone echinoderm. P Natl Acad Sci USA 106:9316–9321
Guerra A (2006) Ecology of *Sepia officinalis*. Vie milieu 56(2):97–107
Gutowska MA, Pörtner H-O, Melzner F (2008) Growth and calcification in the cephalopod *Sepia officinalis* under elevated seawater pCO_2. Mar Ecol Prog Ser 373:303-309. doi: 10.3354/meps07782
Gutowska MA, Melzner F (2009) Abiotic conditions in cephalopod (*Sepia officinalis*) eggs: embryonic development at low pH and high pCO_2. Mar Biol 156:515-519. doi: 10.1007/s00227-008-1096-7

Gutowska MA, Melzner F, Langenbuch M, Bock C, Claireaux G, PörtnerHO (2010a) Acid-base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia. J Comp Physiol B 180:323-335. doi: 10.1007/s00360-009-0412-y
Gutowska MA, Melzner F, Pörtner H-O, Meier S (2010b) Cuttlebone calcification increases during exposure to elevated seawater pCO_2 in the cephalopod *Sepia officinalis.* Mar Biol 157:1653-1663. doi: 10.1007/s00227-010-1438-0
Hoegh-Guldberg O, Mumby PJ, Hoote, AJ et al. (2007) Coral reefs under rapid climate change and ocean acidification. Science 318:1737-1742. doi: 10.1126/science.1152509
Hu MY, Sucré E, Charmantier-Daures M, Charmantier G, Lucassen M, Himmerkus N, Melzner F (2010) Localization of ion-regulatory epithelia in embryos and hatchlings of two cephalopods. Cell Tissue Res 339:571-83. doi: 10.1007/s00441-009-0921-8
Hu MY, Tseng Y-C, Stumpp M, Gutowska MA, Kiko R, Lucassen M, Melzner F (2011a) Elevated seawater pCO_2 differentially affects branchial acid-base transporters over the course of development in the cephalopod *Sepia officinalis.* Am J Physiol 300:R1100-R1114. doi: 10.1152/ajpregu.00653.2010
Hu MY, Tseng Y-C, Lin L-Y, Chen P-Y, Charmantier-Daures M, Hwang P-P, Melzner F (2011b) New insights into ion regulation of cephalopod molluscs: a role of epidermal ionocytes in acid-base regulation during embryogenesis. Am J Physiol 301:R1700–R1709. doi: 10.1152/ajpregu.00107. 2011
IPCC (2007) Fourth Assessment Report of the Intergovernmental Panel on Climate Change. In: Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M.Tignor and H.L. Miller (eds.) Climate Change 2007. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.
Jacob DE, Wirth R, Soldati AL, Wehrmeister U, Schreiber A (2011) Amorphous calcium carbonate in the shells of adult *Unionoida*. J Struct Biol 173:241-249. doi: 10.1016/j.jsb.2010.09.011
Kleypas JA, Feely RA, Fabry VJ, Langdon C, Sabine CL, Robbins LL (2006) Impacts of ocean acidification on coral reefs and other marine calcifiers: a guide for future research, report of a workshop held 18–20 April 2005, St Petersburg, FL, sponsored by NSF, NOAA, and the US Geological Survey http://wwwucaredu/communications/Final_acidificationpdf
Kroeker KJ, Kordas RL, Crim RN, Singh GG (2010) Review and Synthesis: Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecol Lett 13:1419-1434. doi: 10.1111/j.1461-0248.2010.01518.x
Kurihara H (2008) Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates. Mar Ecol Prog Ser 373:275-284. doi: 10.3354/meps07802
Lacoue-Labarthe T, Warnau M, Oberhänsli F, Teyssié J-L, Koueta N, Bustamante P (2008a) Differential bioaccumulation behaviour of Ag and Cd during the early development of the cuttlefish *Sepia officinalis.* Aquat Toxicol 86:437-46. doi: 10.1016/j.aquatox.2007.12.005
Lacoue-Labarthe T, Martin S, Oberhänsli F, Teyssié J-L, Markich S, Jeffree R, Bustamante P (2009) Effects of increased pCO_2 and temperature on trace element (Ag, Cd and Zn) bioaccumulation in the eggs of the common cuttlefish, *Sepia officinalis.* Biogeosciences 6:2561-73. doi: 10.5194/bg-6-2561-2009
Lacoue-Labarthe T, Warnau M, Oberhänsli F, Teyssié JL, Bustamante P (2010a) Contrasting accumulation biokinetics and distribution of ^{241}Am, Co, Cs, Mn and Zn during the whole development time of the eggs of the common cuttlefish, *Sepia officinalis*. J Exp Mar Biol Ecol 382:131-138. doi:10.1016/j.jembe.2009.10.008
Lacoue-Labarthe T, Le Bihan E, Borg D, Koueta N, Bustamante P (2010b) Variation of acid phosphatases and cathepsins activities in the cuttlefish (*Sepia officinalis*) eggs: specific activity and effects of Ag , Cd , Cu exposures. ICES J Marine Sci 7:1517-1523. doi: 10.1093/icesjms/fsq044
Lacoue-Labarthe T, Villanueva R, Rouleau C, Oberhänsli F, Teyssié J-L, Jeffree R, Bustamante P (2011a) Radioisotopes demonstrate the contrasting bioaccumulation capacities of heavy metals in embryonic stages of cephalopod species. Plos One 6(11):e27653. doi:10.1371/journal.pone.0027653

Lacoue-Labarthe T, Réveillac E, Oberhänsli F, Teyssié JL, Jeffree R, Gattuso JP (2011b) Effects of ocean acidification on trace element accumulation in the early-life stages of squid *Loligo vulgaris*. Aquat Toxicol 105:166-76. doi: 10.1016/j.aquatox.2011.05.021
Langdon C, Atkinson MJ (2005) Effect of elevated pCO_2 on photosynthesis and calcification of corals and interactions with seasonal change in temperature/irradiance and nutrient enrichment. J Geophys Res 110:C09S07. doi:10.1029/2004JC002576
Lavigne H, Gattuso J-P (2009) seacarb: seawater carbonate chemistry with R. R package version 2.4. http://CRAN.R-project.org/package=seacarb
Leersnyder M De, Lemaire J (1972) Sur la composition minérale du liquide périembryonnaire de l'oeuf de *Sepia officinalis* L. Cah Biol Mar 13:429-43
Lemaire J (1970) Table de développement embryonnaire de *Sepia officinalis* L (Mollusque Céphalopode). B Soc Zool Fr 95(4):773-782
Levitus S, Antonov J, Boyer T (2005) Warming of the world ocean, 1955–2003. Geophys Res Lett 32:L02604. doi:10.1029/2004GL021592
Lewis E, Wallace DWR (1998) Program Developed for CO_2 System Calculations. ORNL/ CDIAC-105. U.S. Dept. Energy, Oak Ridge, Tennessee. http://cdiac.ornl.gov
Martin S, Richier S, Pedrotti M-L, Dupont S, Castejon C, Gerakis Y, Kerros M-E, Oberhänsli F, Teyssié J-L, Jeffree R, Gattuso J-P (2011) Early development and molecular plasticity in the Mediterranean sea urchin Paracentrotus lividus exposed to CO_2-driven acidification. The J Exp Biol 214:1357-68. doi: 10.1242/jeb.051169
Mehrbach C, Culberson CH, Hawley JE, Pytkowicz RM (1973) Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol Oceanogr 18(6):897-907
Melzner F, Forsythe J, Lee P, Wood J, Piatowski U, Clemmesen C (2005) Estimating recent growth in the cuttlefish: are nucleic acid-based indicators for growth and condition the method of choice? J Exp Mar Biol Ecol 317:37-51. doi: 10.1016/j.jembe.2004.11.011
Melzner F, Bock C, Pörtner H-O (2006) Temperature-dependent oxygen extraction from the ventilatory current and the costs of ventilation in the cephalopod *Sepia officinalis*. J Comp Physiol B 176:607-621. doi: 10.1007/s00360-006-0084-9
Melzner F, Bock C, Pörtner H-O (2007) Allometry of thermal limitation in the cephalopod *Sepia officinalis*. Comp Biochem Phys A 146:149-54. doi: 10.1016/j.cbpa.2006.07.023
Melzner F, Gutowska MA, Langenbuch M, Dupont S, Lucassen M, Thorndyke MC, Bleich M, Pörtner H-O (2009) Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6:2313-2331. doi: 10.5194/bg-6-2313-2009
Miller WA, Reynolds AC, Sobrino C, Riedel GF (2009) Shellfish face uncertain future in high CO_2 world: influence of acidification on oyster larvae calcification and growth in estuaries. PloS One 4:e5661. doi: 10.1371/journal.pone.0005661
Miramand P, Bustamante P, Bentley D, Koueta N (2006) Variation of heavy metal concentrations (Ag, Cd, Co, Cu, Fe, Pb, V, Zn) during the life cycle of the common cuttlefish *Sepia officinalis*. Sci Total Environ 361:132-143. doi: 10.1016/j.scitotenv.2005.10.018
Munday PL, Hernaman V, Dixson DL, Thorrold SR (2011) Effect of ocean acidification on otolith development in larvae of a tropical marine fish. Biogeosciences 8:1631-1641. doi: 10.5194/bg-8-1631-2011
Orr J, Fabry VJ, Aumont O, Bopp L, Doney SC, Feely RA et al. (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437:681-6. 10.1038/nature04095
Palmer AR (1992) Calcification in marine molluscs: how costly is it? P Natl Acad Sci USA 89:1379-1382. doi: 10.1073/pnas.89.4.1379
Parker LM, Ross PM, O'Connor WA, Borysko L, Raftos DA, Pörtner, H-O (2012) Adult exposure influences offspring response to ocean acidification in oysters. Glob Change Biol 18:82–92. doi: 10.1111/j.1365-2486.2011.02520.x

Pechenik JA (2006) Larval experience and latent effects: metamorphosis is not a new beginning. Integr Comp Biol 46:323-333. doi: 10.1093/icb/icj028
Pierce GJ, Allcock L, Bruno I et al. (2010) Cephalopod biology and fisheries in Europe. ICES Cooperative Research Report, 303. ICES, Copenhagen, Denmark. ISBN 978-87-7482-078-9. http://oceanrep.geomar.de/10074/Politi Y, Arad T, Klein E, Weiner S, Addadi L (2004) Sea urchin spine calcite forms via a transient amorphous calcium carbonate phase. Science 306:1161-4. doi: 10.1126/science.1102289
Pörtner H-O, Farrell AP (2004) Physiology and Climate Change. Science 322:690-692. doi: 10.1126/science.1163156
Pörtner H-O (2008) Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. Mar Ecol Prog Ser 373:203-217. doi: 10.3354/meps07768
R Development Core Team (2008) R: A language and environment for statistical computing R Foundation for Statistical Computing, Vienna, Austria ISBN 3-900051-07-0, URL http://wwwR-projectorg
Riebesell U, Zondervan I, Rost B, Tortell PD, Zeebe RE, Morel FMM (2000) Reduced calcification of marine plankton in response to increased atmospheric CO_2. Nature 407:364-367
Ries JB, Cohen AL, McCorkle DC (2009) Marine calcifiers exhibit mixed responses to CO_2-induced ocean acidification. Geology 37:1131-1134. doi: 10.1130/G30210A.1
Rodhouse PG (1998) Physiological progenesis in cephalopod molluscs. Biol Bull 195:17-20
Rodolfo-Metalpa R, Lombardi C, Cocito S, Hall-Spencer JM, Gambi MC (2010) Effects of ocean acidification and high temperatures on the bryozoan *Myriapora truncata* at natural CO_2 vents. Marine Ecol 31(3):447-456. doi: 10.1111/j.1439-0485.2009.00354.x
Rodolfo-Metalpa R, Houlbrèque F, Tambutté É. Et al. (2011) Coral and mollusc resistance to ocean acidification adversely affected by warming. Nature Clim Change 1:308–312
Rodriguez y Baena A, Metian M, Teyssié JL, Debroyer C, Warnau M (2006) Experimental evidence for ^{234}Th bioaccumulation in three Antarctic crustaceans: Potential implications for particle flux studies. Marine Chem 100:354-365. doi: 10.1016/j.marchem.2005.10.022
Rosa R, Seibel BA (2008) Synergistic effects of climate-related variables suggest future physiological impairment in a top oceanic predator. P Natl Acad Sci USA 105:20776-80. doi: 10.1073/pnas.0806886105
Seibel BA, Walsh PJ (2001) Potential impacts of CO_2 injection on deep-sea biota. Science 294:319-320
Seibel BA, Fabry VJ (2003) Marine biotic response to elevated carbon dioxide. Adv Appl Biodivers Sci 4:59-67
Seibel BA, Walsh PJ (2003) Biological impacts of deep-sea carbon dioxide injection inferred from indices of physiological performance. J Exp Biol 206:641-650. doi: 10.1242/jeb.00141
Sherrard K (2000) Cuttlebone morphology limits habitat depth in eleven species of *Sepia* (Cephalopoda: Sepiidae). Biol Bull 198:404-414
Shiao J-C, Lin L-Y, Horng J-L, Hwang P-P, Kaneko T (2005) How can teleostean inner ear hair cells maintain the proper association with the accreting otolith? J Comp Neurol 488:331–341
Spero HJ, Bijma J, Lea DW, Bemis BE (1997) Effect of seawater carbonate concentration on foraminiferal carbon and oxygen isotopes. Nature 390:497-500. doi:10.1038/37333
Stumpp M, Wren J, Melzner F, Thorndyke MC, Dupont S (2011) CO_2 induced seawater acidification impacts sea urchin larval development I: Elevated metabolic rates decrease scope for growth and induce developmental delay. Comp Biochem Phys A 160 (3):331-340. doi: 10.1016/j.cbpa.2011.06.022
Stumpp M, Trübenbach K, Brennecke D, Hu MY, Melzner F (2012) Resource allocation and extracellular acid-base status in the sea urchin Strongylocentrotus droebachiensis in response to CO_2 induced seawater acidification. Aquat Toxicol 110-111:194–207. doi:10.1016/j.aquatox.2011.12.020

Thomsen J, Melzner F (2010) Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. Mar Biol 157:2667-76. doi: 10.1007/s00227-010-1527-0
Thomsen J, Gutowska MA, Saphörster J, Heinemann A, Trübenbach K, Fietzke J, Hiebenthal C, Eisenhauer A, Körtzinger A, Wahl M, Melzner F (2010) Calcifying invertebrates succeed in a naturally CO_2-rich coastal habitat but are threatened by high levels of future acidification. Biogeosciences 7:3879-3891
Touratier F, Goyet C (2011) Impact of the Eastern Mediterranean Transient on the distribution of anthropogenic CO_2 and first estimate of acidification for the Mediterranean Sea. Deep-Sea Res PT I 58:1–15
Tunnicliffe V, Davies KTA, Butterfield DA, Embley RW, Rose JM, Chadwick Jr WW (2009) Survival of mussels in extremely acidic waters on a submarine volcano. Nature Geosci 2:344-8. doi: 10.1038/ngeo500
Venn A, Tambutté É, Holcomb M, Allemand D, Tambutté S (2011) Live Tissue Imaging Shows Reef Corals Elevate pH under Their Calcifying Tissue Relative to Seawater. PloS one, 6(5):e20013. doi:10.1371/journal.pone.0020013
Villanueva R, Bustamante P (2006) Composition in essential and non-essential elements of early stages of cephalopods and dietary effects on the elemental profiles of *Octopus vulgaris* paralarvae. Aquaculture 261:225-240. doi: 10.1016/j.aquaculture.2006.07.006
Waldbusser GG, Bergschneider H, Green MA (2010) Size-dependent pH effect on calcification in post-larval hard clam *Mercenaria spp*.. Mar Ecol Prog Ser 417:171-182
Weiss IM, Tuross N, Addadi L, Weiner S (2002) Mollusc larval shell formation: amorphous calcium carbonate is a precursor phase for aragonite. J Exp Zool 293:478-91. doi: 10.1002/jez.90004
Wood HL, Spicer JI, Widdicombe S (2008) Ocean acidification may increase calcification rates, but at a cost. Proc Roy Soc B 275:1767-73. doi: 10.1098/rspb.2008.0343
Zondervan I, Zeebe RE, Rost B, Riebesell U (2001) Decreasing marine biogenic calcification: A negative feedback on rising atmospheric pCO_2. Global Biogeochem Cy 15:507-516

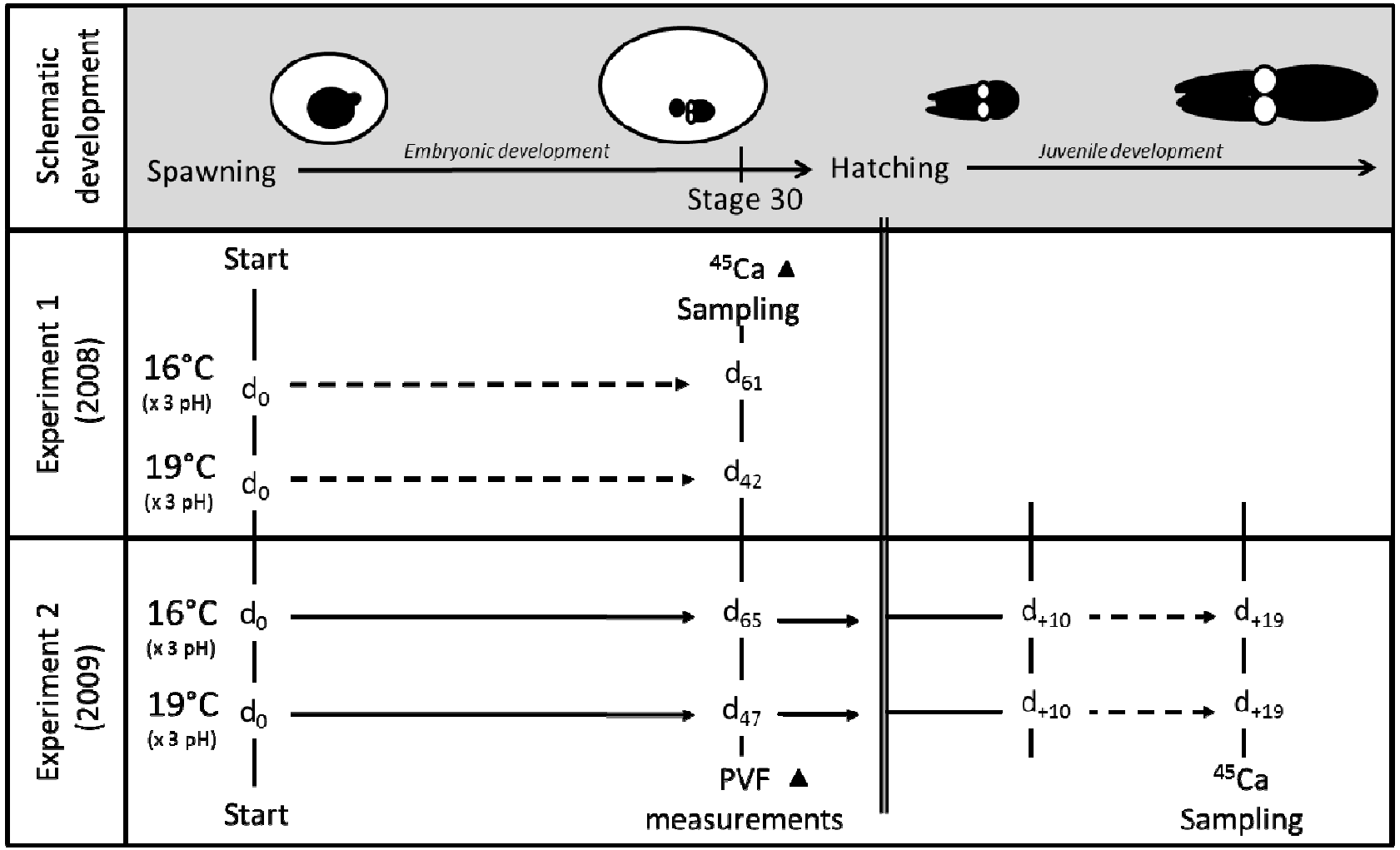

Fig. 1 Experimental design scheme (*Experiments 1* and *2*) set up to study the impact of temperature and pH along the embryonic and juvenile development of the cuttlefish (*Sepia offcinalis*). Dotted lines represent the exposure of animals to dissolved ^{45}Ca in seawater. For each "^{45}Ca sampling", measurements of the cuttlebone dry weight, body dry weight, ^{45}Ca content in the cuttlebone (Q_{ca}) have been carried out. Fresh eggs were weighted at the end of the embryonic development (▲; stage 30).

Table 1 Carbonate system parameters during the experimental set-up in both 2008 (*Experiment 1*) and 2009 (*Experiment 2*). Results are expressed as mean ± SD. The partial pressure of CO_2 (pCO_2) and aragonite saturation state (Ω_{ar}) were calculated for a seawater salinity of 38, using the measured parameters in bold.

Experiment	A_T ($\mu mol\ kg^{-1}$)	Temperature (°C)	pH_T	pCO_2 (μatm)	Ω_{ar}
Experiment 1	**2597 ± 12**	**16.0 ± 0.1**	**7.60 ± 0.08**	*1433 ± 208*	*1.21 ± 0.31*
			7.84 ± 0.05	*775 ± 83*	*1.97 ± 0.20*
			8.11 ± 0.09	*378 ± 65*	*3.35 ± 0.63*
		18.9 ± 0.3	**7.58 ± 0.08**	*1517 ± 273*	*1.30 ± 0.26*
			7.86 ± 0.12	*766 ± 181*	*2.32 ± 0.73*
			8.14 ± 0.09	*393 ± 83*	*3.56 ± 0.67*
Experiment 2	**2549 ± 65**	**16.0 ± 0.4**	**7.60 ± 0.09**	*1381 ± 30*	*1.16 ± 0.03*
			7.81 ± 0.04	*739 ± 15*	*1.97 ± 0.04*
			8.06 ± 0.08	*376 ± 11*	*3.17 ± 0.09*
		19.1 ± 0.5	**7.60 ± 0.03**	*1395 ± 38*	*1.30 ± 0.04*
			7.83 ± 0.02	*741 ± 22*	*2.19 ± 0.06*
			8.03 ± 0.06	*370 ± 7*	*3.46 ± 0.06*

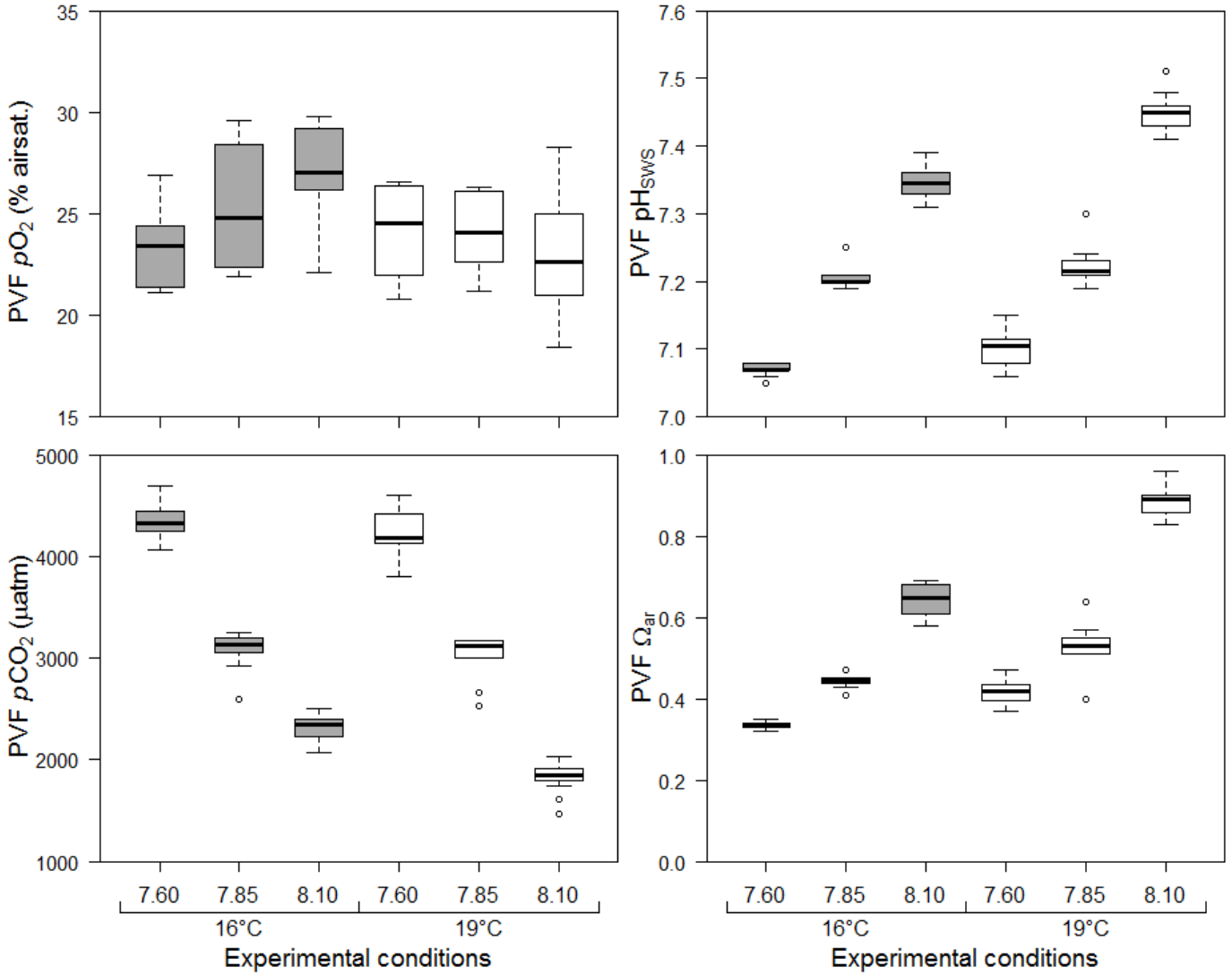

Fig. 2 Abiotic conditions in the perivitelline fluid (PVF) of *S. officinalis* eggs during *Experiment 2* (stage 30; n = 8 to 12) including pO_2 (% air saturation), pH (SWS scale), pCO_2 (µatm) and Ω_{ar} in the different experimental conditions (pH = 7.60, 7.85 and 8.10; temperature = 16°C and 19°C). Results of the statistical analyses are reported in Table 2.

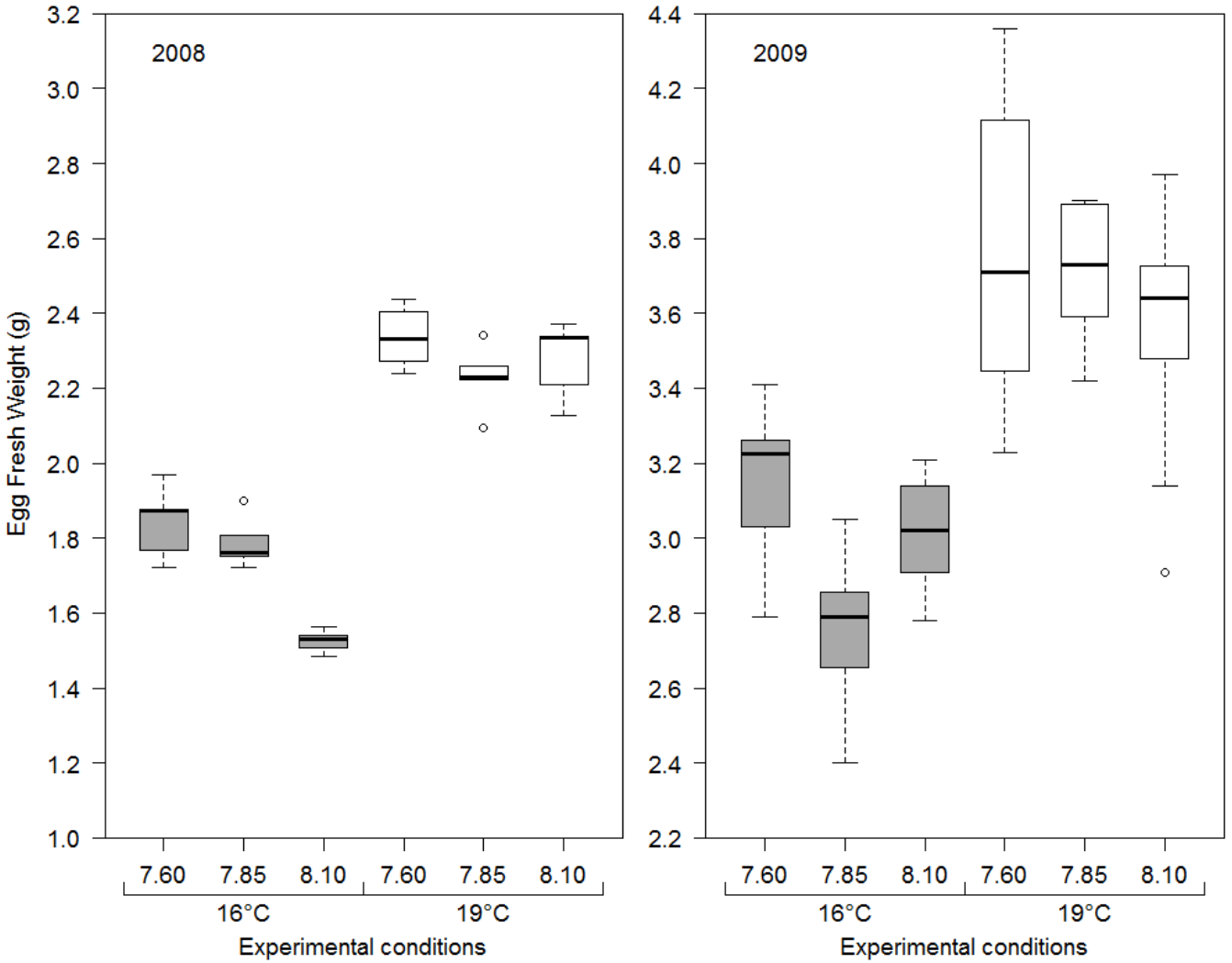

Fig. 3 Weights of *S. officinalis* nearly hatched eggs (g; stage 30; n = 10) in the different experimental conditions (pH = 7.60, 7.85 and 8.10; temperature = 16°C and 19°C). Data are issued from two experiments carried out in 2008 (*Experiment 1;* left) and 2009 (*Experiment 2;* right; note the different scales). Results of the statistical analyses are reported in Table 2.

Table 2 Two-way ANOVA analyses testing the influence of temperature (T; 16 vs. 19°C), pH (pH; 7.60, 7.85 and 8.10) and their interactions (T×pH) on nearly hatched eggs (stage 30) regarding: *i.* the perivitelline-fluid (PVF) abiotic conditions (pO_2, pH, pCO_2, and Ω_{ar}) and the pCO_2 gradient (ΔpCO_2 = PVF pCO_2 - seawater pCO_2) during *Experiment 2* (n=8 to 12) and *ii.* eggs fresh weight (Eggs FW) both during *Experiments 1* (2008) and *2* (2009) (n=10). Results in bold are significant.

	T				pH				T×pH			
	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
PVF pO_2	1	35.88	**5.57***	0.02	2	8.27	1.28 ^{ns}	0.29	2	31.01	**4.81***	0.01
PVF pH	1	0.067	**112.70*****	1.10^{-14}	2	0.48	**816.45*****	$< 2.10^{-16}$	2	0.010	**16.81*****	2.10^{-6}
PVF pCO_2	1	1747381	**46.69*****	8.10^{-9}	2	23995501	**641.18*****	$< 2.10^{-16}$	2	281779	**7.53****	0.001
PVF Ω_{ar}	1	0.38	**283.46*****	$< 2.10^{-16}$	2	0.80	**596.65*****	$< 2.10^{-16}$	2	0.042	**31.34*****	1.10^{-9}
ΔpCO_2	1	1162398	**31.06*****	9.10^{-7}	2	6558119	**175.26*****	$< 2.10^{-16}$	2	153672	**4.11***	0.02
Eggs FW_{2008}	1	2.37	**346.51*****	9.10^{-16}	2	0.09	**13.02*****	1.10^{-4}	2	0.069	**10.06*****	6.10^{-4}
Eggs FW_{2009}	1	8.49	**125.31*****	7.10^{-16}	2	0.37	**5.43****	0.007	2	0.26	**3.80***	0.028

df= degree of freedom; MS= mean squares; F=F-value. $P < 0.001$ (***), $P < 0.01$ (**), $P < 0.05$ (*), $P < 0.1$ (†), ns= non-significant.

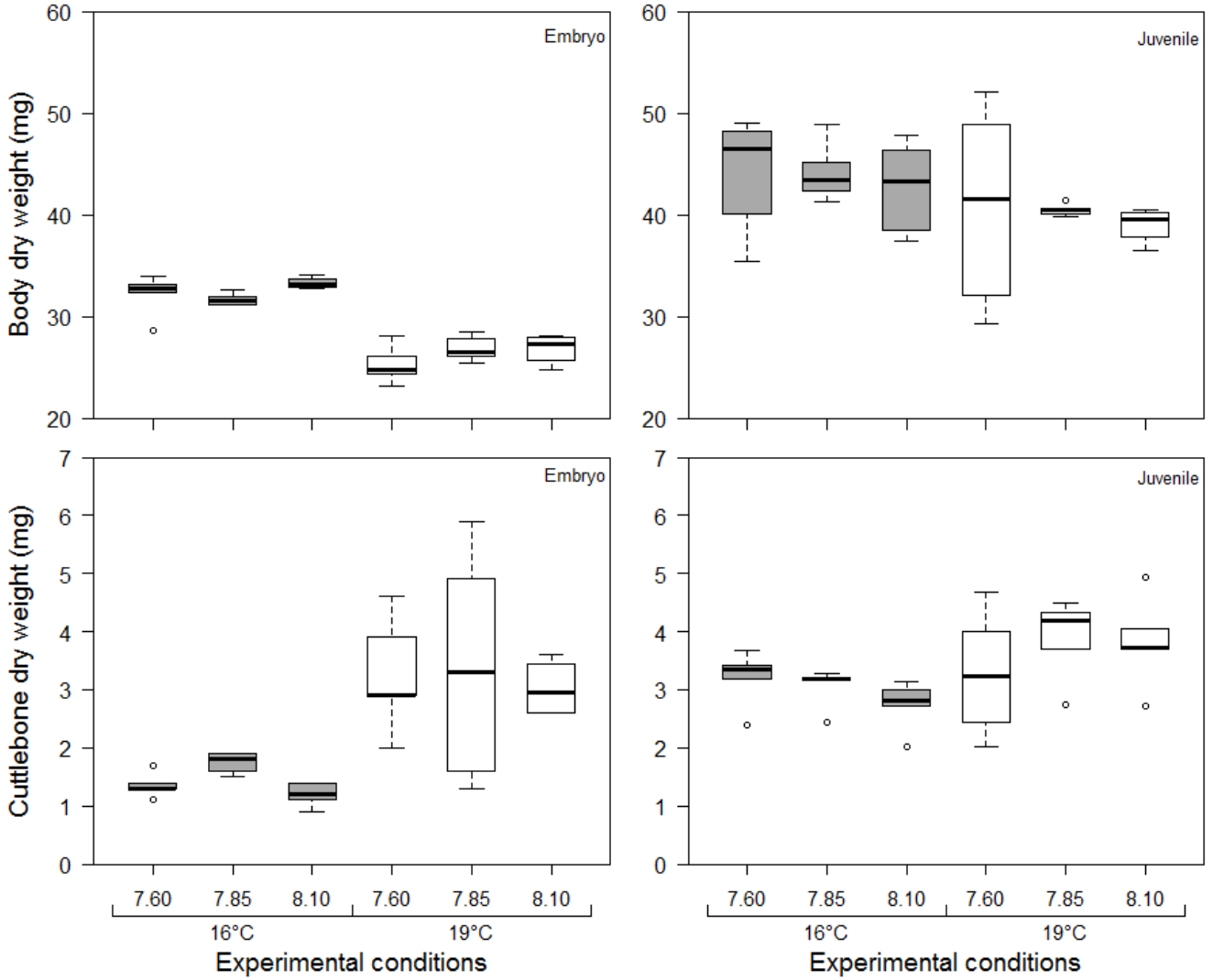

Fig. 4 Body (g; upper figures) and cuttlebone (mg; bottom figures) dry weight of *S. officinalis* embryos (left figures; stage 30; n = 5) and juveniles (right figures; 19-days; n = 5) in the different experimental conditions (pH = 7.60, 7.85 and 8.10; temperature = 16°C and 19°C). Results of the statistical analysis are reported in Table 3.

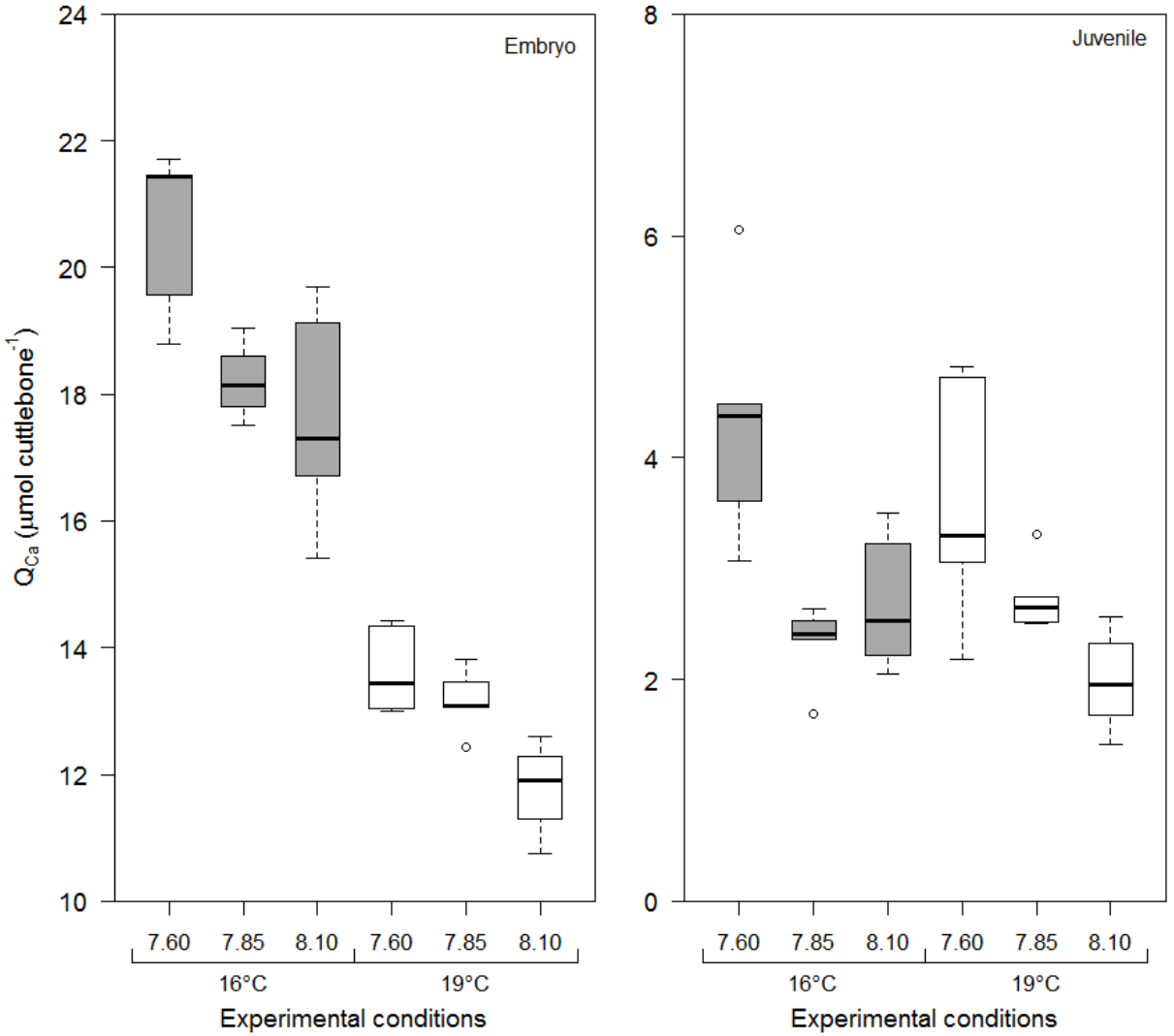

Fig. 5. Calcium-45 content (Q_{Ca}: µmol cuttlebone^{-1}; n = 5 to 12) in cuttlebones of *S. officinalis* embryos (left; stage 30) and juveniles (right; 19-days old) following incubation to ^{45}Ca in the different experimental con(pH = 7.60, 7.85 and 8.10; temperature = 16°C and 19°C). Note the different scales for embryos and juveniles. Results of the statistical analysis are reported in Table 3.

Table 3 Two-way ANOVA analyses testing the influence of Temperature (T), pH (pH) conditions and their interactions (T×pH) on body and cuttlebone dry weight and cuttlebone ^{45}Ca content (Q_{Ca}, µmol cuttlebone^{-1}) at both the end of egg development (*Embryo*, 61 d at 16°C and 42 d at 19°C) and after 19 days of juvenile life (*Juvenile*). Results in bold are significant.

		T				pH				T×pH			
		df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
Body Dry Weight	*Embryo*	1	269.22	**131.37*** **	5.10^{-11}	2	4.58	2.24 ^{ns}	0.13	2	2.92	1.43 ^{ns}	0.26
	Juvenile	1	93.92	3.34†	0.08	2	8.06	0.29 ^{ns}	0.75	2	0.38	0.013 ^{ns}	0.99
Cuttlebone Dry Weight	*Embryo*	1	23.71	**25.38*** **	4.10^{-5}	2	0.50	0.54 ^{ns}	0.59	2	0.038	0.040 ^{ns}	0.96
	Juvenile	1	3.29	**6.88***	0.015	2	0.16	0.32 ^{ns}	0.73	2	0.71	1.48 ^{ns}	0.25
Cuttlebone Q_{Ca}	*Embryo*	1	249.76	**224.66*** **	2.10^{-13}	2	13.93	**12.53*** **	2.10^{-4}	2	2.29	2.06 ^{ns}	0.15
	Juvenile	1	0.83	1.45^{ns}	0.24	2	7.85	**13.71*** **	1.10^{-4}	2	1.06	1.85 ^{ns}	0.18

df= degree of freedom; MS= mean squares; F=F-value. $P < 0.001$ (***), $P < 0.01$ (**), $P < 0.05$ (*), $P < 0.1$ (†), ns= non-significant.