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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 Teysse^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 1^{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 $p\text{CO}_2$ conditions in the perivitelline fluid (> 3-fold higher $p\text{CO}_2$ 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 $p\text{CO}_2$ 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 $p\text{CO}_2$ 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 (otoliths: 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 $p\text{CO}_2$. This ability to efficiently regulate extracellular pH could be one explanation for the increased tolerance of some organisms to projected increases in future $p\text{CO}_2$ (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 ($\sim 1000 \mu\text{atm}$, $\text{pH}_{\text{NBS}} \sim 7.62$) 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 $p\text{CO}_2$ (Pörtner and Farrell 2004; Pörtner 2008; Kurihara 2008), especially in invertebrates (e.g. Kurihara 2008; Dupont and Thorndyke 2009). Even though this view is discussed controversially (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 $p\text{CO}_2$ 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 $p\text{CO}_2$. 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 $p\text{CO}_2$ than the IPCC predictions for 2100 (i.e. $\sim 4000 \mu\text{atm}$, $\text{pH}_{\text{NBS}} \sim 7.23$ and $\sim 6000 \mu\text{atm}$, $\text{pH}_{\text{NBS}} \sim 7.10$; compared to $900 \mu\text{atm}$, 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 ($< 30\text{-}40 \text{ m}$, Guerra, 2006) from late winter to early summer (Rodhouse 1998). In the Ligurian Sea (NW Mediterranean), water temperatures fluctuate around $15\text{-}17^\circ\text{C}$ when cuttlefish spawning reaches its peak (March-May, $0\text{-}50 \text{ m}$, 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 $p\text{CO}_2$ 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 $p\text{O}_2$ ($p\text{O}_2 < 6$ kPa, Cronin and Seymour 2000, Gutowska and Melzner 2009) and to high $p\text{CO}_2$ in turn ($p\text{CO}_2 > 0.4$ kPa i.e. $> 2000\text{-}4000$ 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.

$p\text{CO}_2$ -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; $p\text{CO}_2 \sim 390 \mu\text{atm}$) while the two others were maintained at pH values predicted to occur following optimistic and pessimistic future scenarios (IPCC, 2007): 7.85 ($p\text{CO}_2 \sim 800 \mu\text{atm}$) and 7.60 ($p\text{CO}_2 \sim 1400 \mu\text{atm}$). Water was renewed weekly during the first week and then every second day to maintain water quality constant ($[\text{NH}_4^+] < 0.1 \text{ mg L}^{-1}$, $[\text{NO}_2^-] < 0.1 \text{ mg L}^{-1}$, $[\text{NO}_3^-] < 5 \text{ mg L}^{-1}$). 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 $\pm 0.5^\circ\text{C}$ (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 0.7 μ m 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 μ m, Presens GmbH, Regensburg, Germany). Stable PVF pO_2 values were obtained within 10s in the syringe filled with 200–300 μ L 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 μ L 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.

⁴⁵Ca radiolabelling and samples treatment:

Experiment 1: Seawater in each bottle was spiked with ⁴⁵Ca (10 kBq L⁻¹). Radiotracers were purchased from Radioisotope Centre Polatum, Poland, ⁴⁵Ca [as ⁴⁵CaCl₂; T_{1/2} = 163 d]. Stock solutions were prepared in H₂O 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 ⁴⁵Ca 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 ⁴⁵Ca in the juvenile cuttlebone, 10-days old juveniles were exposed to ⁴⁵Ca (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 Gold™ 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 $\mu\text{mol } ^{45}\text{Ca cuttlebone}^{-1}$) according to Martin et al. (2011) and following the equation:

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

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 ($0.0114 \text{ mmol g}^{-1}$).

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 \pm 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 $\alpha=0.05$.

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 $2.597 \pm 0.012 \text{ mmol kg}^{-1}$ and $2.549 \pm 0.065 \text{ mmol kg}^{-1}$ in the egg and juvenile experiments, respectively; in both experiments, A_{T} changed by less than $0.030 \text{ mmol kg}^{-1}$ 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 $p\text{CO}_2$ of ca. $380 \mu\text{atm}$ ($\text{pH}_{\text{T}} = 8.09$), ca. $750 \mu\text{atm}$ ($\text{pH}_{\text{T}} = 7.84$) and ca. $1430 \mu\text{atm}$ ($\text{pH}_{\text{T}} = 7.60$) 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 ($\Omega_{\text{ar}} > 1.15$; Table 1).

PVF abiotic conditions (Experiment 2): the egg development duration was logically shortened by temperature increase, and hatchling delay caused by increasing $p\text{CO}_2$ 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 $p\text{CO}_2$ treatment). The embryos develop inside the protective eggshell, bathing in the PVF which abiotic conditions (i.e. carbonate chemistry parameters and $p\text{O}_2$) were determined at the end of the development (stage 30). At this stage, PVF $p\text{O}_2$ was very low with ~25% of air saturation, all conditions merged (Fig. 2). At 16°C, the PVF $p\text{O}_2$ was $25.28 \pm 2.95\%$ vs. $23.73 \pm 2.45\%$ 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: $27.1 \pm 2.4\%$ at 16°C vs. $22.9 \pm 3.0\%$ air sat. at 19°C). Seawater pH did not significantly influence PVF $p\text{O}_2$ 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 $p\text{O}_2$ with decreasing pH only at 16°C ($27.1 \pm 2.4\%$, $25.4 \pm 3.1\%$ and $23.4 \pm 2.2\%$ 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 ($7.05 \leq \text{PVF pH}_{\text{SWS}} \leq 7.51$ units, Fig. 2). The lowest values were reached in eggs exposed to pH 7.60 treatment (PVF $\text{pH}_{\text{SWS}} = 7.07 \pm 0.01$ at 16°C and 7.10 ± 0.03 at 19°C). Besides, PVF pH was significantly lower at 16°C ($\text{pH}_{\text{SWS}} = 7.07 \pm 0.01$, 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 $p\text{CO}_2$ compared to external conditions, whatever the temperature (PVF vs. seawater: $2311 \mu\text{atm}$ vs. $376 \mu\text{atm}$ at 16°C and $1822 \mu\text{atm}$ vs. $370 \mu\text{atm}$ at 19°C). PVF $p\text{CO}_2$ increased with lower seawater pH (Fig. 2), reaching maximum values of ca. $4700 \mu\text{atm}$ in the lowest pH treatment (i.e. seawater $p\text{CO}_2$ of ca. $1400 \mu\text{atm}$). CO_2 gradient between PVF and seawater ($\Delta p\text{CO}_2$ - i.e. $p\text{CO}_2$ inside the PVF minus seawater $p\text{CO}_2$) thus increased with higher seawater $p\text{CO}_2$ (Table 2, $P < 0.001$). Respective $\Delta p\text{CO}_2$ values in pH treatments of 8.10, 7.85 and 7.60 were of 1863 ± 142 , 2391 ± 192 and $2852 \pm 178 \mu\text{atm}$ at 16°C and 1458 ± 164 , 2217 ± 231 and $2794 \pm 249 \mu\text{atm}$ at 19°C. The $p\text{CO}_2$ gradient and hence the difference of $p\text{CO}_2$ between the exterior and the interior of the egg, was also diminished under warmer treatments ($P < 0.001$). Alongside with the extremely high PVF $p\text{CO}_2$, the PVF was always under-saturated with respect to aragonite, even under control conditions (Fig. 2: $\Omega_{\text{ar}} = 0.644 \pm 0.038$ 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 ($\Omega_{\text{ar}} = 0.335 \pm 0.011$ units at 16°C and $\Omega_{\text{ar}} = 0.418 \pm 0.032$ 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 ($\Omega_{\text{ar PVF}} = 0.771 \pm 0.130$, 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}: $P < 0.01$; $\Omega_{\text{ar PVF}}$: $P < 0.001$; $\Delta p\text{CO}_2$ 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 weakened 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 ⁴⁵Ca 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 $p\text{CO}_2$ ($\sim 6000 \mu\text{atm}$ for 40 days). Here, using ^{45}Ca β -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 $p\text{CO}_2$ 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 $p\text{CO}_2$ 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 ($[Ca^{2+}] = 411 \text{ mg L}_{SW}^{-1}$) 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 $\Delta p\text{CO}_2$ seems to be crucial for cuttlefish embryos exposed to elevated seawater $p\text{CO}_2$, 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 $p\text{CO}_2$, especially when metabolic rates are increased with elevated temperature and $p\text{CO}_2$ (Melzner et al. 2006). For example, at pH 8.10, the PVF $p\text{CO}_2$ 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 $\Delta p\text{CO}_2$ decrease and hence a lower PVF $p\text{CO}_2$. This higher gas exchange at higher temperature could also explain the slightly lower $p\text{O}_2$ at 19°C than at 16°C at the end of development. Nevertheless, the effect of $p\text{CO}_2$ on egg volume was weakened 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 $p\text{CO}_2$ 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 ($0.58 \leq \Omega_{\text{ar}} \leq 0.96$), embryos are able to precipitate aragonite. They furthermore increased calcium accumulation in a PVF medium extremely under-saturated with CaCO_3 ($0.32 \leq \Omega_{\text{ar}} \leq 0.47$ when seawater pH = 7.60). In juvenile animals exposed to $6000 \mu\text{atm}$ of $p\text{CO}_2$, 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 $[\text{HCO}_3^-]$ 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 $p\text{CO}_2$ 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 $p\text{CO}_2$ (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 $p\text{CO}_2$ 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 $p\text{CO}_2$ 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 $p\text{CO}_2$, 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 $p\text{CO}_2$, 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 $p\text{CO}_2$ 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 $p\text{CO}_2$, 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 $p\text{CO}_2$ 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 $p\text{CO}_2$ 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 $p\text{CO}_2$. 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 $p\text{CO}_2$ 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 $p\text{CO}_2$ 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 $p\text{CO}_2$ 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*.

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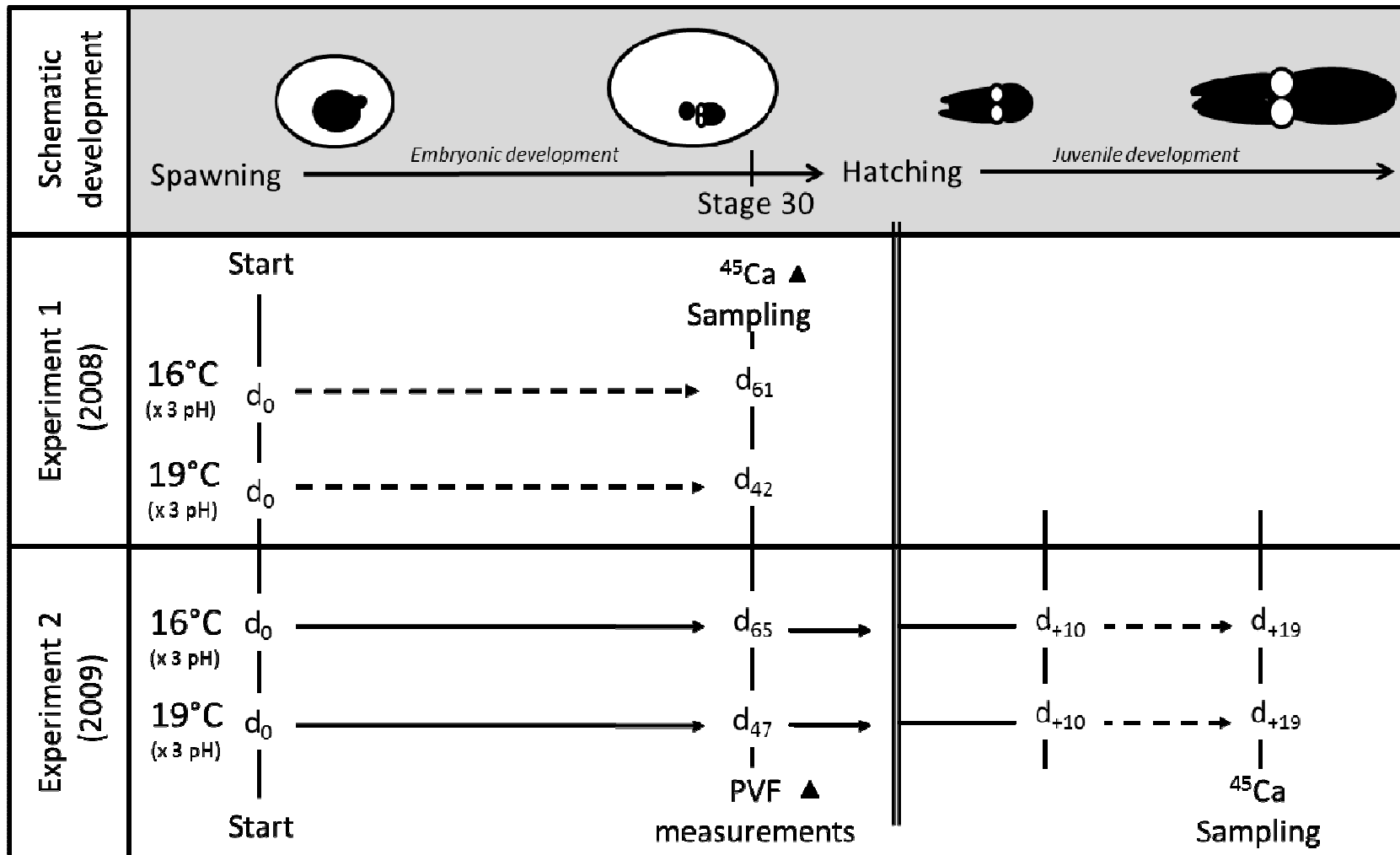


Fig. 1 Experimental design scheme (*Experiments 1* and *2*) set up to study the impact of temperature and pH along the embryonic and juvenile development of the cuttlefish (*Sepia officinalis*). Dotted lines represent the exposure of animals to dissolved ^{45}Ca in seawater. For each “ ^{45}Ca sampling”, 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 \pm SD. The partial pressure of CO₂ ($p\text{CO}_2$) and aragonite saturation state (Ω_{ar}) were calculated for a seawater salinity of 38, using the measured parameters in bold.

Experiment	A_T ($\mu\text{mol kg}^{-1}$)	Temperature ($^{\circ}\text{C}$)	pH_T	$p\text{CO}_2$ (μatm)	Ω_{ar}
<i>Experiment 1</i>	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
<i>Experiment 2</i>	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

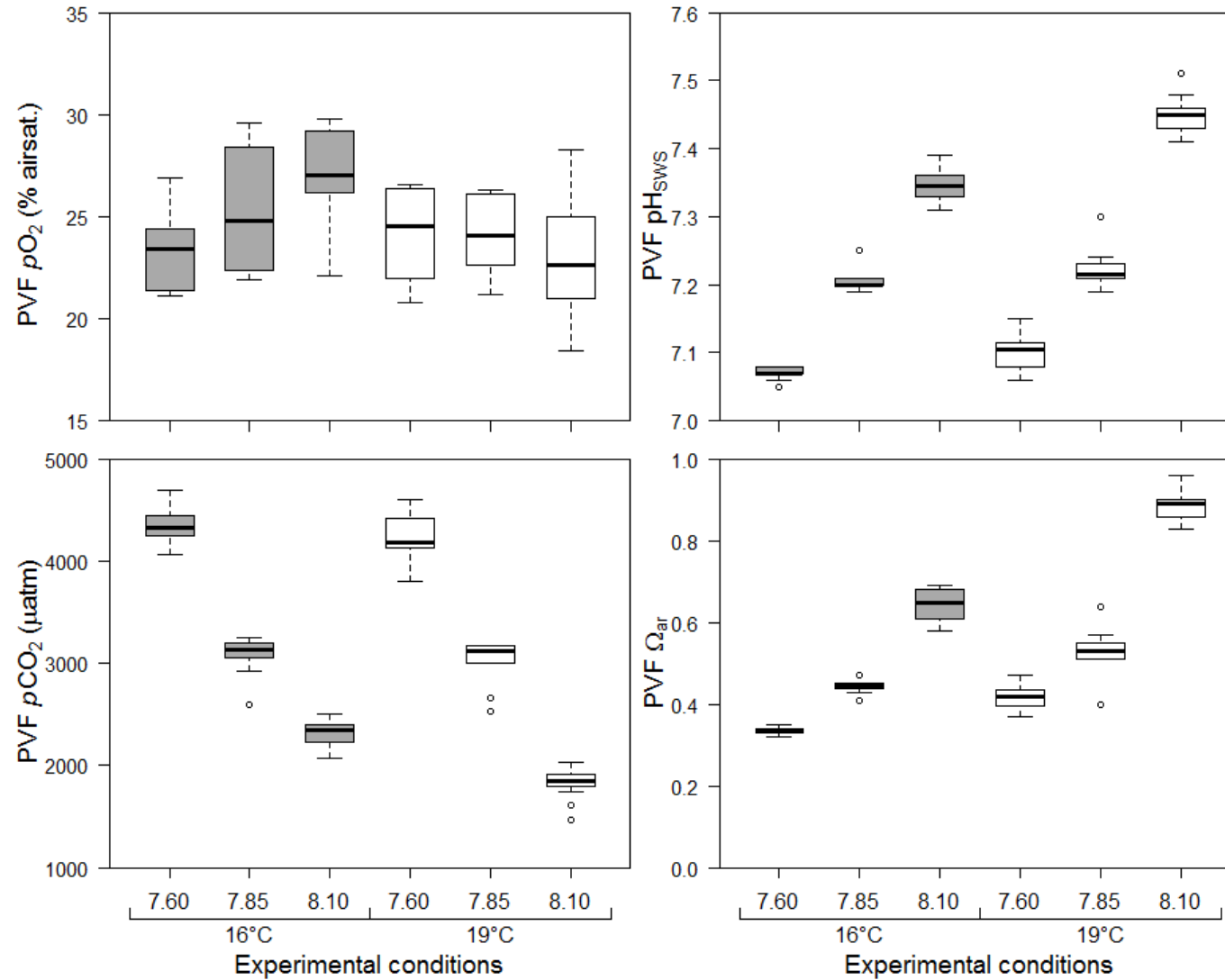


Fig. 2 Abiotic conditions in the perivitelline fluid (PVF) of *S. officinalis* eggs during *Experiment 2* (stage 30; $n = 8$ to 12) including pO_2 (% air saturation), pH (SWS scale), pCO_2 (μatm) and Ω_{ar} in the different experimental conditions ($pH = 7.60, 7.85$ and 8.10 ; temperature = $16^\circ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

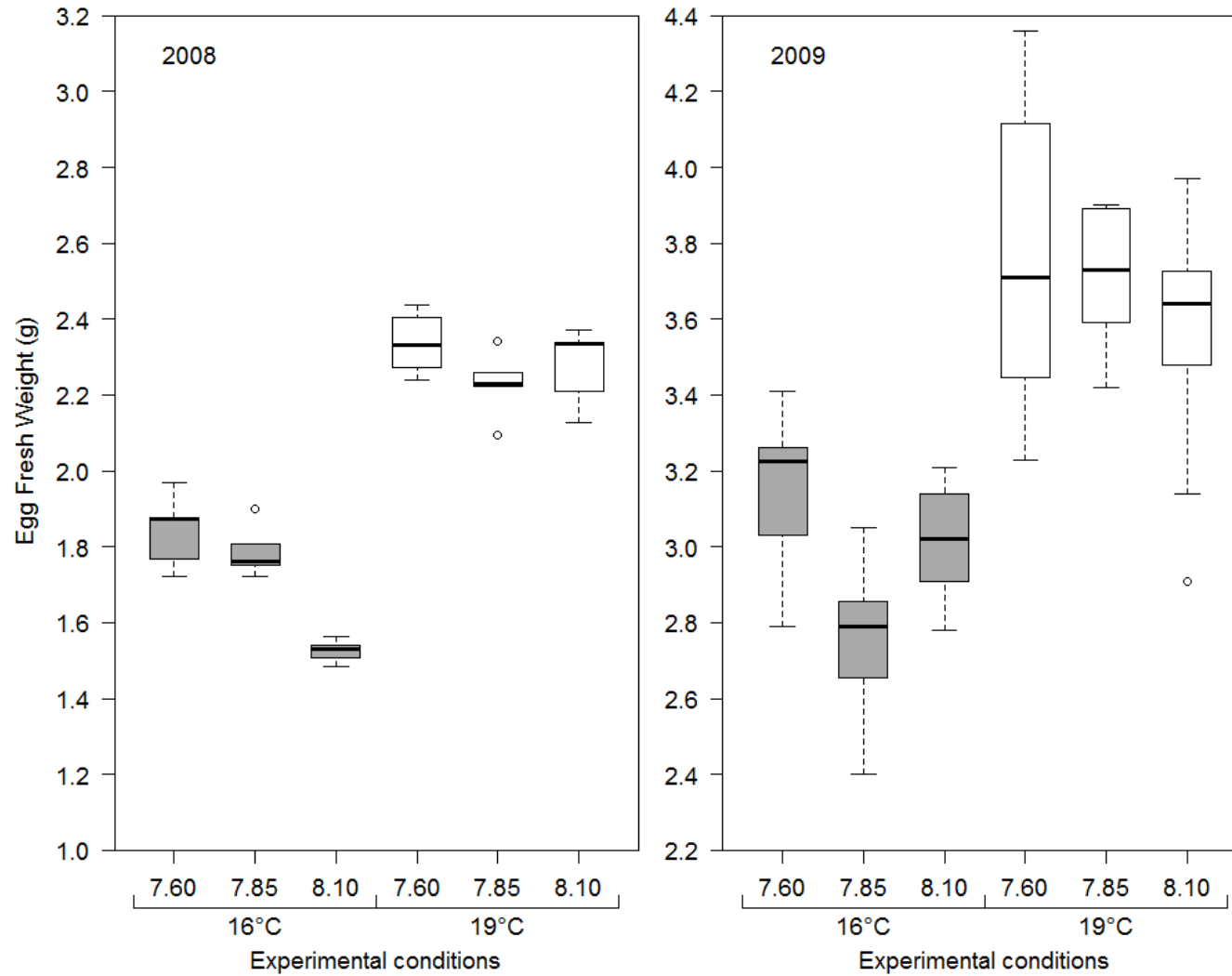


Fig. 3 Weights of *S. officinalis* nearly hatched eggs (g; stage 30; n = 10) in the different experimental conditions (pH = 7.60, 7.85 and 8.10; temperature = 16°C and 19°C). Data are issued from two experiments carried out in 2008 (*Experiment 1*; left) and 2009 (*Experiment 2*; right; note the different 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 ($\Delta pCO_2 = PVF pCO_2 - \text{seawater } pCO_2$) 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.

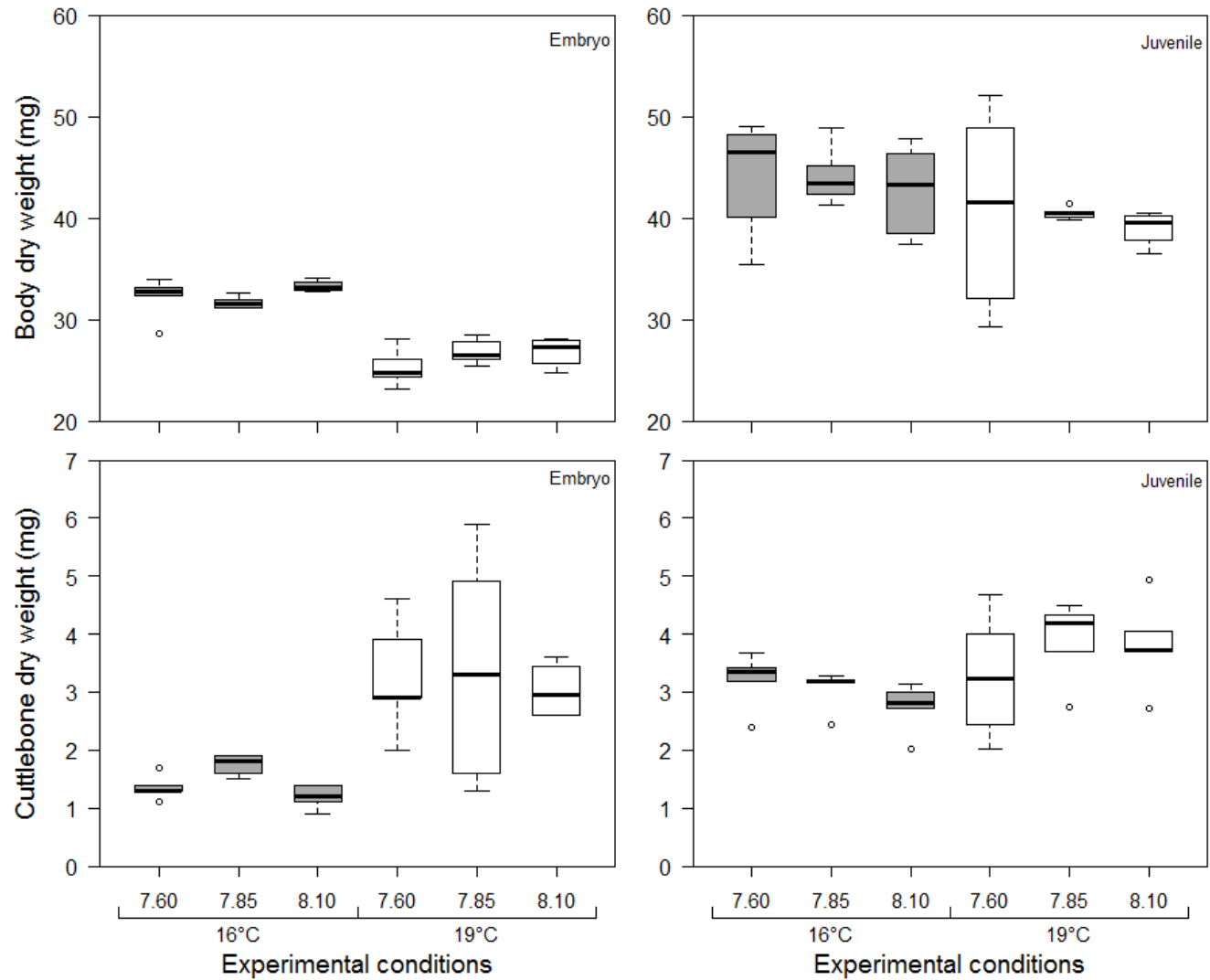


Fig. 4 Body (g; upper figures) and cuttlebone (mg; bottom figures) dry weight of *S. officinalis* embryos (left figures; stage 30; n = 5) and juveniles (right figures; 19-days; n = 5) in the different experimental conditions (pH = 7.60, 7.85 and 8.10; temperature = 16°C and 19°C). Results of the statistical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

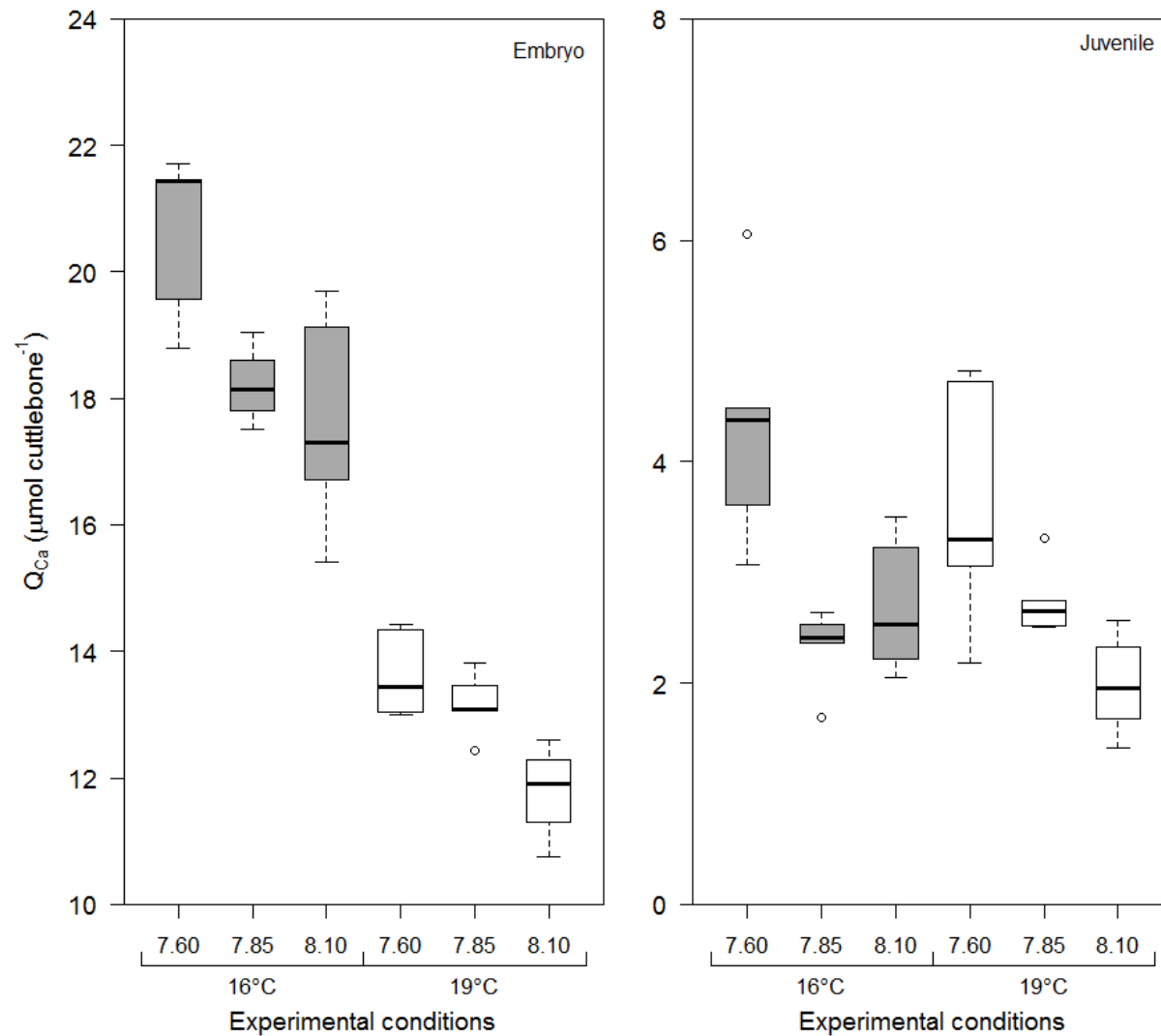


Fig. 5. Calcium-45 content (Q_{Ca} : $\mu\text{mol cuttlebone}^{-1}$; $n = 5$ to 12) in cuttlebones of *S. officinalis* embryos (left; stage 30) and juveniles (right; 19-days old) following incubation to ^{45}Ca in the different experimental con(pH = 7.60, 7.85 and 8.10; temperature = 16°C and 19°C). 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 ⁴⁵Ca 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	<i>Embryo</i>	1	269.22	131.37***	5.10 ⁻¹¹	2	4.58	2.24 ^{ns}	0.13	2	2.92	1.43 ^{ns}	0.26
	<i>Juvenile</i>	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	<i>Embryo</i>	1	23.71	25.38***	4.10 ⁻⁵	2	0.50	0.54 ^{ns}	0.59	2	0.038	0.040 ^{ns}	0.96
	<i>Juvenile</i>	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}	<i>Embryo</i>	1	249.76	224.66***	2.10 ⁻¹³	2	13.93	12.53***	2.10 ⁻⁴	2	2.29	2.06 ^{ns}	0.15
	<i>Juvenile</i>	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.