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Lower hypoxia thresholds of cuttlefish early life stages living in a warm acidified ocean

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The combined effects of future ocean acidification and global warming on the hypoxia thresholds of marine biota are, to date, poorly known. Here, we show that the future warming and acidification scenario led to shorter embryonic periods, lower survival rates and the enhancement of premature hatching in the cuttlefish *Sepia officinalis*. Routine metabolic rates increased during the embryonic period, but environmental hypercapnia significantly depressed pre-hatchling's energy expenditures rates (independently of temperature). During embryogenesis, there was also a significant rise in the carbon dioxide partial pressure in the perivitelline fluid (PVF), bicarbonate levels, as well as a drop in pH and oxygen partial pressure (pO_2). The critical partial pressure (i.e. hypoxic threshold) of the pre-hatchlings was significantly higher than the PVF oxygen partial pressure at the warmer and hypercapnic condition. Thus, the record of oxygen tensions below critical pO_2 in such climate scenario indicates that the already harsh conditions inside the egg capsules are expected to be magnified in the years to come, especially in populations at the border of their thermal envelope. Such a scenario promotes untimely hatching and smaller post-hatching body sizes, thus challenging the survival and fitness of early life stages.

1. Introduction

Global temperature is rising at a rate unprecedented in the experience of modern human society and expected to increase between 3°C and 6°C by 2100 [1], which is predicted to dictate deleterious temperature-mediated physiological responses at organism level [2–4]. At community level, profound impacts on phenology, diversity and biogeography are also likely to occur [5–7]. In coastal areas, many organisms already live close to their thermal tolerance limits [8,9] and ocean warming will negatively impact their performance and survival. Moreover, since the industrial revolution, $[CO_2]_{atm}$ has increased from 280 ppm to levels now exceeding 380 ppm [10] and is expected to rise to 730–1020 ppm by the year 2100 [1]. Carbon dioxide reacts with seawater resulting in a net increase in the concentrations of H^+ (lowered pH), H_2CO_3 and HCO_3^- while decreasing CO_3^{2-}. This process, termed ocean acidification, is projected to decrease the pH of surface waters between 0.14 and 0.5 units, depending on emission scenario, by the end of the twenty-first century [1]. These future changes in the ocean's chemistry are expected to pose particular problems for key calcifying organisms [11,12]. Yet, elevated CO_2 has also been shown to have detrimental effects on the survival, growth and respiratory

physiology of marine animals more broadly [2,13,14] (but also see [15]). Concomitantly, in the past few decades, marine hypoxia has also become one of the major ecological concerns in the world [16], because of the: (i) increase of excessive anthropogenic input of nutrients and organic matter into coastal ecosystems, especially in estuaries and semi-enclosed seas, and (ii) climate-related expansion of oceanic-oxygen-minimum zones that can cause upwelling-driven shelf hypoxia [17,18]. Low oxygen episodes lead to major losses in local biodiversity, with extreme hypoxia causing the so-called dead-zones devoid of higher marine life. Non-migrating organisms surviving in hypoxia usually experience sublethal physiological stresses followed by reduced growth and reproductive potential [16,19]. Moreover, early life stages are assumed to be more sensitive to oxygen stress than older life stages [20], but while most research has been conducted on the latter stages, the former are expected to be more vulnerable to these new climate-change-related conditions. Ultimately, oxygen stress promoted by shifting climate conditions may constitute a major bottleneck for species survival.

The combined effects of temperature and elevated CO_2 on hypoxic thresholds of marine biota are, to date, poorly known. Here, we report the oxygen thresholds for hypoxia (critical pO_2) in the early stages (developing/intermediate embryos and pre-hatchlings) of the common cuttlefish (*Sepia officinalis*) acclimated to the environmental hypercapnia (0.16% CO_2, approx. 1600 ppmv; $\Delta pH = 0.5$) and warming (+4°C) as expected for the end of this century. Besides the quantification of routine metabolic rates (RMRs) and thermal sensitivity (Q_{10} values), we also evaluated the abiotic conditions (pH, pCO_2, pO_2, $[HCO_3^-]$) inside the egg capsules (i.e. in the perivitelline fluid, PVF).

2. Material and methods

(a) Egg collection and incubation

Recently spawned, stage I [21], egg masses of common cuttlefish (*S. officinalis*) were collected, between August and October 2012, in Caldeira de Tróia, a shallow water habitat near the mouth of the Sado estuary (38°29′18.42″ N; 8°53′15.12″ O), in the west coast off Portugal. After collection, eggs were immediately transferred to the aquaculture facilities in Laboratório Marítimo da Guia, Cascais. It is worth noting that the Portuguese western coast is situated in the Western Iberian Upwelling Ecosystem (and the northern limit of the Canary Current Upwelling System), one of the four major eastern boundary currents of the world, and pCO_2 levels may reach up to approximately 500 ppm [22,23]. Consequently, in these regions, the future pCO_2 levels are expected to exceed the forecasted 1000–1200 ppm ($\Delta pH = 0.4–0.5$) for 2100 [1]. To understand the physiological mechanisms by which early ontogenetic stages may (or may not) be able to withstand future ocean changes, the eggs were acclimated until pre-hatching at: (i) rising pCO_2 ($\Delta pH = 5.0$; 0.16% CO_2, approx. 1600 ppmv), and (ii) ocean warming expected in 2100 [1], 4°C above the average temperature of the main period of the spawning season (18°C) of the common cuttlefish, *S. officinalis*, in the western coast of Portugal.

Egg masses were placed in 12 life support systems (100 egg capsules per system; 400 l each) that were replenished daily with 100 l of fresh seawater to maintain total alkalinity and dissolved inorganic carbon speciation owing to bacterial activity (i.e. nitrifiers, denitrifiers) and acidification of the treatments (two of four). The semi-closed systems were filled with 1-µm and UV-irradiated filtered seawater, with the tanks being illuminated from above with white fluorescent lamps under a photoperiod of 14 L : 10 D cycle. Water quality was ensured using wet–dry filters (bioballs), protein skimmers (Schuran, Jülich, Germany) and 30 W UV-sterilizers (TMC, Chorleywood, UK). Ammonia and nitrite were monitored regularly and kept below detectable levels. pH was adjusted automatically, via solenoid valves, with the Profilux controlling system (Kaiserslautern, Germany) connected to individual pH probes. pH values were monitored every 2 s and lowered by injection of a certified CO_2 gas mixture (Air Liquid, Portugal) via air stones or upregulated by aerating the tanks with CO_2 filtered (using soda lime, Sigma-Aldrich) air. Additionally, pH values were manually controlled (daily) showing average values in the range of 8.1 ± 0.1 and 7.5 ± 0.1, respectively. Salinity throughout the experiment was 35.0 ± 1.0, and temperatures (18.0 ± 0.2°C and 22.0 ± 0.2°C) were regulated via Hailea chillers (Guangdong, China).

Seawater carbonate system speciation was calculated weekly from total alkalinity according to Sarazin *et al*. [24] (spectrophotometrically at 595 nm) and pH measurements. pH was quantified via a Metrohm pH meter (826 pH mobile, Metrohm, Filderstadt, Germany) connected to a glass electrode (Schott IoLine, SI analytics, ±0.001) and calibrated against the seawater buffers Tris–HCl (Tris) and 2-aminopyridine–HCl (Mare, Liège, Belgium) according to Dickson *et al*. [25]. Measurements were performed under temperature-controlled conditions using a water bath (Lauda, Germany, ±0.1°C). Bicarbonate and pCO_2 values were calculated using the CO2SYS software [26], with dissociation constants from Mehrbach *et al*. [27] as refitted by Dickson & Millero [28].

For each temperature and pH, 65 embryos were screened for survival rates, development time and premature hatchling. Regarding the latter, it was defined as newborn juveniles that hatched with the yolk sac still attached or detached inside egg capsule.

(b) pH, bicarbonate, pCO_2 and pO_2 levels

To assess the conditions (pH, bicarbonate, pCO_2 and pO_2) prevailing in the intermediate embryonic (ranging from 0.011 to 0.023 g; equivalent to stages XI–XV in [21]) and pre-hatching stages (ranging from 0.039 to 0.092 g; equivalent to stages XVIII–XIX in [21]) of the common cuttlefish, PVF was withdrawn using a gas-tight Hamilton syringe (500 µl, Switzerland) and disposable needles. pH of the PVF was quantified at ambient temperatures (18°C or 22°C, respectively; water bath, Lauda, Germany, ±0.1°C) using a microelectrode (WTW Mic-D) connected to a WTW pHi 340 pH meter (precision ± 0.01 units) that was calibrated with radiometer precision buffers 7 and 10 (S11M44, S11M007). Total dissolved inorganic carbon was determined in duplicates (50 µl each) via a Corning 965 carbon dioxide analyser (precision ± 0.1 mmol l^{-1}; Olympic Analytical Service, UK) that was calibrated by generating a sodium bicarbonate (Fluka, Germany) standard curve. Carbonate system speciation (pCO_2 and bicarbonate) was calculated from pH and total dissolved inorganic carbon measurements using CO2SYS software [26], with dissociation constants from Mehrbach *et al*. [27] as refitted by Dickson & Millero [28]. The seawater carbonate chemistry data for the different climate change scenarios are shown in the electronic supplementary material, table S1.

Oxygen tension was measured by injecting a 75 ml sample of PVF into a micro-respirometry chamber (Strathkelvin MC100 Microcell) connected to a Clarke-type O_2 electrode (Strathkelvin SI130 microcathode oxygen electrode) maintained at the experimental temperature and calibrated to air- and nitrogen-saturated seawater [29]. The oxygen sensor was connected to a multi-channel oxygen interface (Strathkelvin, 929, six-channel oxygen system).

(c) Routine metabolic rates and thermal sensitivity

Oxygen consumption measurements (RMRs) were determined according to Rosa *et al*. [3,30]. Intermediate embryonic stages

and pre-hatchings were incubated in sealed water-jacketed respirometry chambers (RC300 respiration cell, Strathkelvin, North Lanarkshire, UK) containing filtered seawater mixed with antibiotics (50 mg l^{-1} streptomycin) to avoid bacterial respiration. Water volumes were adjusted in relation to animal mass (up to 4 ml) in order to minimize locomotion and stress. Concomitantly, bacterial controls were conducted to correct for possible bacterial respiratory activity. Respiration chambers were placed in water baths (Lauda, Lauda-Königshofen, Germany) to control temperature. Oxygen concentrations were recorded with Clarke-type O_2 electrodes connected to a multi-channel oxygen interface (Strathkelvin). The duration of respiratory runs varied from 6 to 12 h.

Thermal sensitivity (Q_{10}) was determined using the standard equation:

$$Q_{10} = \left[\frac{R(T_2)}{R(T_1)}\right] \times \frac{10}{(T_2 - T_1)}, \quad (2.1)$$

where $R(T_2)$ and $R(T_1)$ represent the oxygen consumption rates at temperatures T_2 and T_1, respectively.

(d) Critical oxygen partial pressures

The external critical oxygen partial pressures ($P_{c,ext}$; the point at which the rate of oxygen consumption was no longer maintained independent of ambient oxygen partial pressure) were determined by plotting specific rates of oxygen consumption against oxygen partial pressure. Regressions were calculated for the two distinct sections of the curve, the regulated (higher pO_2) segment and the very sloped (low pO_2) segment. P_c was defined as the point where the two regressions intersected [31].

The internal critical oxygen partial pressures ($P_{c,in}$) were estimated based on the diffusion model proposed by Cronin & Seymour [32] for the giant cuttlefish *Sepia apama*. Briefly, it is known that the oxygen diffusion is determined by a partial-pressure gradient between the external ($pO_{2,ext}$) and internal ($pO_{2,in}$) environments of the egg, and it is driven by oxygen consumption (here as RMR) and the conductance of the capsule (GO_2). Thus, according to these authors, the embryo's oxygen uptake may be described by the Fick equation: $RMR = GO_2 \times (pO_{2,ext} - pO_{2,in})$.

GO_2 depends on the effective surface area (ESA) of the capsule, its thickness (X) and Krogh's oxygen-diffusion coefficient in water—the product of diffusitivity (1/7500 of that in air) and capacitance (1/30 of that in air). To determine ESA (the mean of the inner and outer radii of each capsule, see equation 3 in [32], p. 864) and X, the embryo capsules (exposed to 18°C pH 8.0, and 22°C pH 8.0) were measured under a microscope. Unfortunately, we could not undertake this task in the four treatments, namely in the high CO_2 ones, and therefore, in our $P_{c,in}$ estimations we assumed that both ESA and X-values did not change from normocapnia (ESA: 1.2 ± 0.8 cm^2 at intermediate stages and 3.5 ± 1.3 cm^2 at hatchling stage; X-values: 0.6 ± 0.1 mm at intermediate stages and 0.1 ± 0.0 mm at pre-hatching stage) to hypercapnia.

(e) Statistical analyses

Two-way ANOVAs were conducted to detect significant differences in RMRs, oxygen thresholds for hypoxia (i.e. critical pO_2, P_c), pH, pCO_2, pO_2 and HCO_3^- between the different climate change scenarios and development stages (intermediate embryonic stages and pre-hatchings) of common cuttlefish *S. officinalis*. Subsequently, Tukey post-hoc tests were performed. All values are expressed as means ± s.d. All statistical analyses were performed for a significance level of 0.05, using STATISTICA v. 10.0 software (StatSoft Inc., Tulsa, USA).

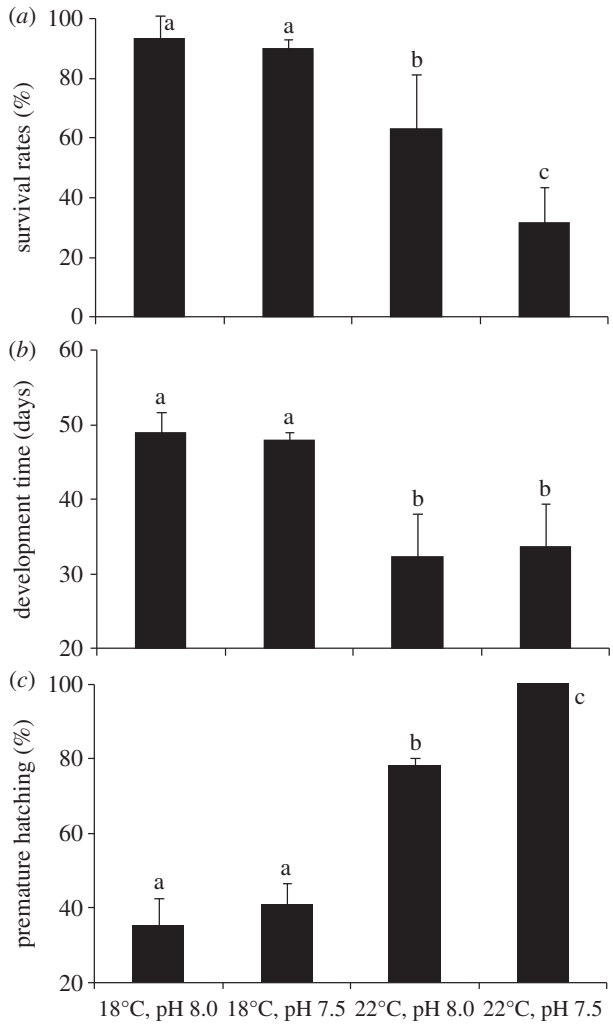

Figure 1. Effect of hypercapnia ($\Delta pH = 0.5$) and warming (+4°C) on the: (*a*) survival rates (%); (*b*) embryonic development time (days); and (*c*) premature hatching (%) in the common cuttlefish *Sepia officinalis*. Values represent the mean ± s.d. Different letters represent significant differences between treatments ($p < 0.05$).

3. Results

Embryonic survival rates of the common cuttlefish (*S. officinalis*) were significantly affected by temperature and pH ($p < 0.05$; figure 1*a*). Although at 18°C there was no differences between the normocapnic and hypercapnic treatments (93.7% and 90.3%, respectively; $p > 0.05$), the future warming and acidification scenarios led to significantly lower survival ($p < 0.05$, 31.8%). Regarding development time, and as expected, temperature significantly decreased the embryonic period, from 48–49 days at 18°C to 32–34 days at 22°C ($p < 0.05$). Yet, pH did not elicit any significant change ($p > 0.05$). Premature hatching increased significantly from present-day conditions (35.2% at pH 8.0 and 41.0% at pH 7.5) to the future warming and acidification scenario (22°C pH 7.5; $p < 0.05$; figure 1*c*), reaching 100%, i.e. all newborn juveniles still had unconsumed yolk inside the egg capsule.

The RMRs also rose significantly during the embryogenesis ($p < 0.05$, figures 2*a*,*b* and 3). At the intermediate embryonic stages, RMR ranged between 1.3 (18°C, pH 7.5) and 2.2 μmol g^{-1} h^{-1} (22°C, pH 8.0), and at pre-hatchling stage ranged between 2.2 (22°C, pH 7.5) and 5.6 μmol g^{-1} h^{-1} (18°C, pH 8.0). It is noteworthy that high CO_2

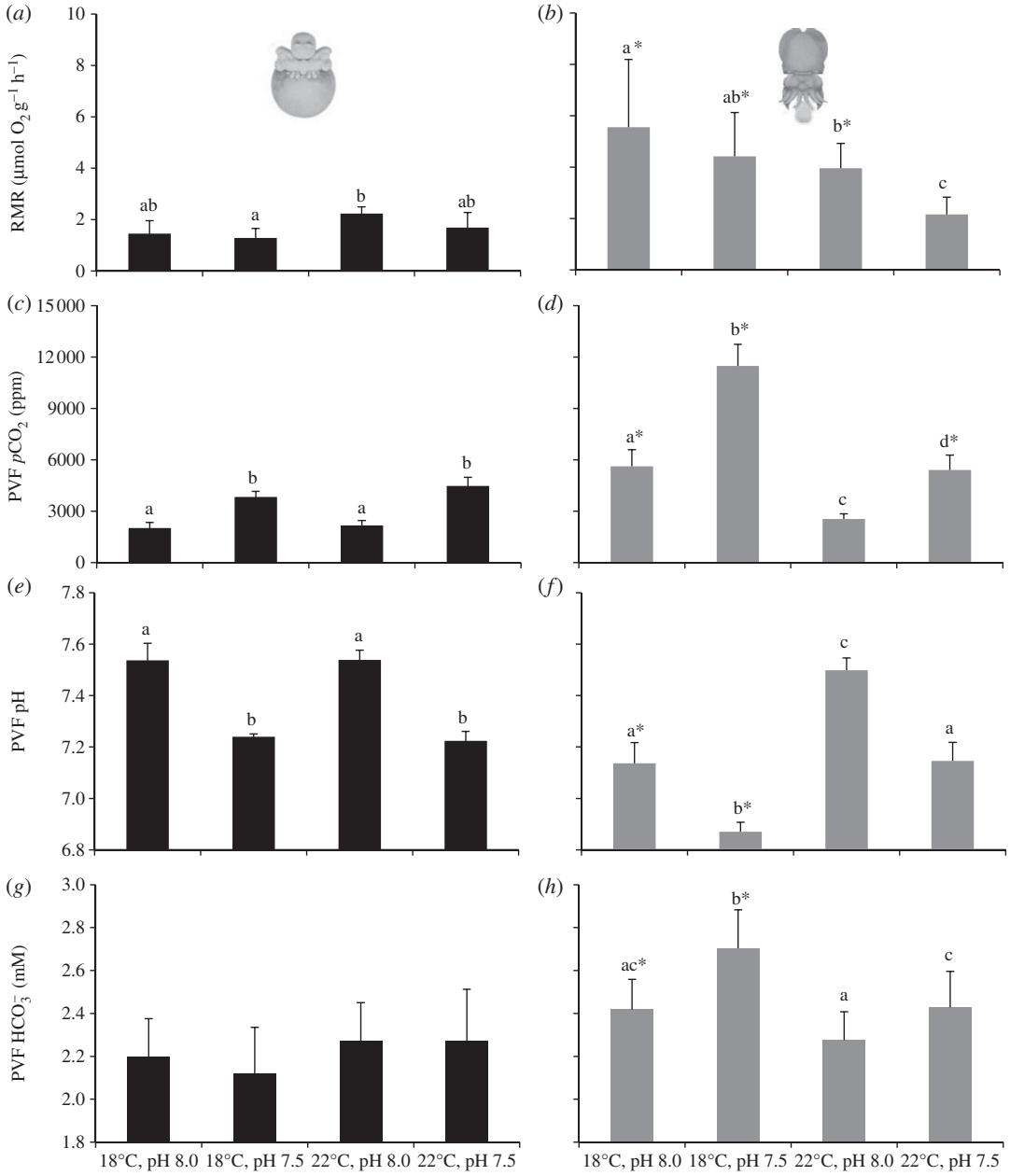

Figure 2. Effect of hypercapnia (ΔpH = 0.5) and warming (+4°C) on the: (*a*,*b*) routine metabolic rates (RMR, μmol O_2 h^{-1} g^{-1} wet weight; $n = 20$); (*c*,*d*) pCO_2 in the PVF (ppm; $n = 20$); (*e*,*f*) pH ($n = 20$); and (*g*,*h*) HCO_3^- (mM; $n = 20$) and in the PVF of intermediate embryonic (*a*,*c*,*e*,*g*) and pre-hatching (*b*,*d*,*f*,*h*) stages of common cuttlefish *Sepia officinalis*. Values represent the mean ± s.d. Different letters represent significant differences between treatments ($p < 0.05$). Asterisks represent significant differences between life stages ($p < 0.05$).

significantly lower pre-hatchings RMR independently of temperature ($p < 0.05$; figure 2*b*). Regarding the thermal sensitivity data, the embryos' Q_{10} values ranged around 2 and 3 at the intermediate stage and below 1 at the pre-hatching stage, which was indicative of metabolic depression (figure 4). With the increase in energy expenditure rates throughout embryogenesis, there was a significant rise in PVF pCO_2 ($p < 0.05$, figure 2*c*,*d*) and HCO_3^- ($p < 0.05$, figure 2*g*,*h*), as well as a drop in pH ($p < 0.05$, figure 2*e*,*f*), especially at 18°C. More specifically, at the intermediate embryonic stages, PVF pCO_2 ranged between 2020.5 (18°C, pH 8.0) and 4478.6 ppm (22°C, pH 7.5), and at pre-hatching stage ranged between 2546.5 (22°C, pH 8.0) and 11 476.8 ppm (18°C, pH 8). Regarding PVF HCO_3^-, the values ranged between 2.2 (18°C, pH 8.0) and 2.3 mM (22°C, pH 7.5; $p > 0.05$) in intermediate stages, and between 2.3 (22°C, pH 8.0) and 2.7 mM (18°C, pH 7.5; $p < 0.05$) in pre-hatchings. The drop in pH was more noticeable at the pre-hatching stage, ranging from 6.9 (18°C, pH 7.5; $p < 0.05$) and 7.5 (22°C, pH 8.0; $p < 0.05$).

Additionally, pO_2 decreased significantly throughout the embryonic development ($p < 0.05$; figure 5*a*,*b*). While pO_2 ranged between 2.2 (22°C, pH 8.0) and 6.1 kPa (18°C, pH 8.0; $p < 0.05$) in intermediate stages, it ranged between 1.8 (22°C, pH 8.0) and 3.4 kPa in the more advanced ontogenetic stage (18°C, pH 8.0; $p < 0.05$). The external P_c ($P_{c,ext}$) of the intermediate embryos was fairly similar between all the treatments, varying non-significantly between 3.2 and 3.7 kPa ($p > 0.05$; figure 5*c*). On the other hand, the pre-hatchlings' $P_{c,ext}$ were significantly lower than those observed for the intermediate embryos (except for the high CO_2 and warming scenario; figure 5*d*), and increased significantly from 1.6 (at 18°C, pH 8.0) to 3.7 kPa (22°C, pH 7.5; $p < 0.05$). The

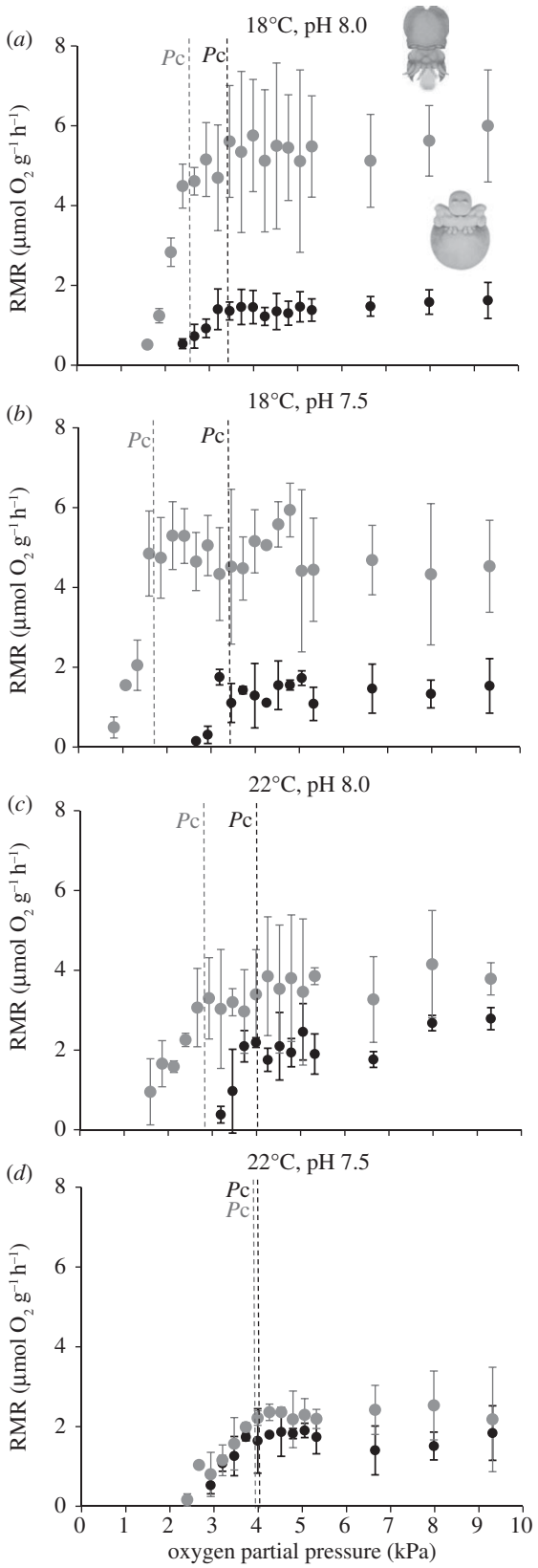

Figure 3. (*a–d*) Effect of hypercapnia (ΔpH = 0.5) and warming (+4°C) on the mass-specific routine metabolic rates (RMR, µmol O_2 h^{-1} g^{-1} wet weight) averaged over 2-min intervals as a function of available oxygen (mmHg) in intermediate embryonic stages (black circles) and pre-hatchlings (grey circles) of common cuttlefish *Sepia officinalis*. Values represent the mean ± s.d. of the 20 individual runs.

estimated internal P_c ($P_{c,in}$) followed the same trend, i.e. it did not vary between treatments in intermediate stages (ranging between 2.6 and 3.0 kPa; $p > 0.05$; figure 5*e*), whereas the pre-hatchlings' $P_{c,in}$ were significantly lower than those observed

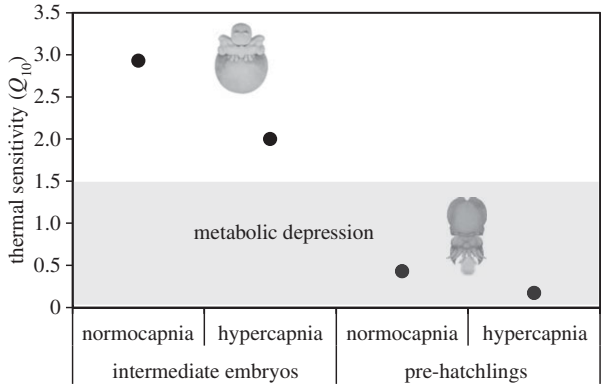

Figure 4. Effect of hypercapnia (ΔpH = 0.5) on the thermal sensitivity (Q_{10}; between 18°C and 22°C) of intermediate embryonic stages (left-hand side panels) and pre-hatchlings (right-hand side panels) of common cuttlefish *Sepia officinalis*.

for the earlier stage ($p < 0.05$), and increased significantly from 0.15 (at 18°C, pH 8.0) to 3.1 kPa (22°C, pH 7.5; $p < 0.05$; figure 5*f*). Consequently, the $P_{c,in}$ of the intermediate stages never reached higher than the partial pressure of the perivitelline space (figure 5*g*). A quite different situation occurred in the pre-hatching stage, because the $P_{c,in}$ (hypoxic threshold) was higher than the PVF pO_2 at the warmer and hypercapnic condition (negative value in figure 5*h*).

4. Discussion

As expected, the metabolic demand rose during embryogenesis, but the future warming scenario led to lower RMR at the pre-hatching stage. Temperature-independent metabolism [33] and hypoxia-related metabolic suppression [2] are well-known energy-conserving strategies in marine molluscs, but a heat induction of hypometabolism has rarely been described [34]. In this study, warming *per se* caused a notable metabolic depression in the pre-hatching stage (at pH 8.0–28.7%, $p > 0.05$; at pH 7.5–51.1%, $p < 0.05$). Based on thermal sensitivity data, we argue that the metabolic depression is a short-term method of extending the timeframe over which unfavourable conditions inside the egg can be withstood.

Cuttlefish embryos develop within egg capsules that act as physical protection and barrier to the diffusion of dissolved gases. During the present experiments, the eggs were swelling due to the entry of ambient water into the hypertonic PVF, with the eggshell becoming much thinner. Concomitantly, energy expenditure increased during development (e.g. for sustaining cellular growth, organogenesis and muscular activity) and led to a significant rise in PVF pCO_2 and HCO_3^-, as well as a drop in pH, especially at 18°C. Such trends were less evident at 22°C possibly as a consequence of the hatchling's physiological strategy. Low pH or high CO_2 are common triggers of metabolic depression [35], and themselves caused a significant metabolic drop in the pre-hatchlings (18°C, 20.4%, $p > 0.05$; 22°C, 45.4%, $p < 0.05$; figures 1*b* and 3).

Oxygen depletion within eggs was partially compensated for by egg swelling (i.e. increased surface area, reduced egg wall thickness; see a comprehensive examination of the subject in [32]) but it still did not prevent pO_2 from consistently falling to potential critical levels. Low pO_2 levels may also

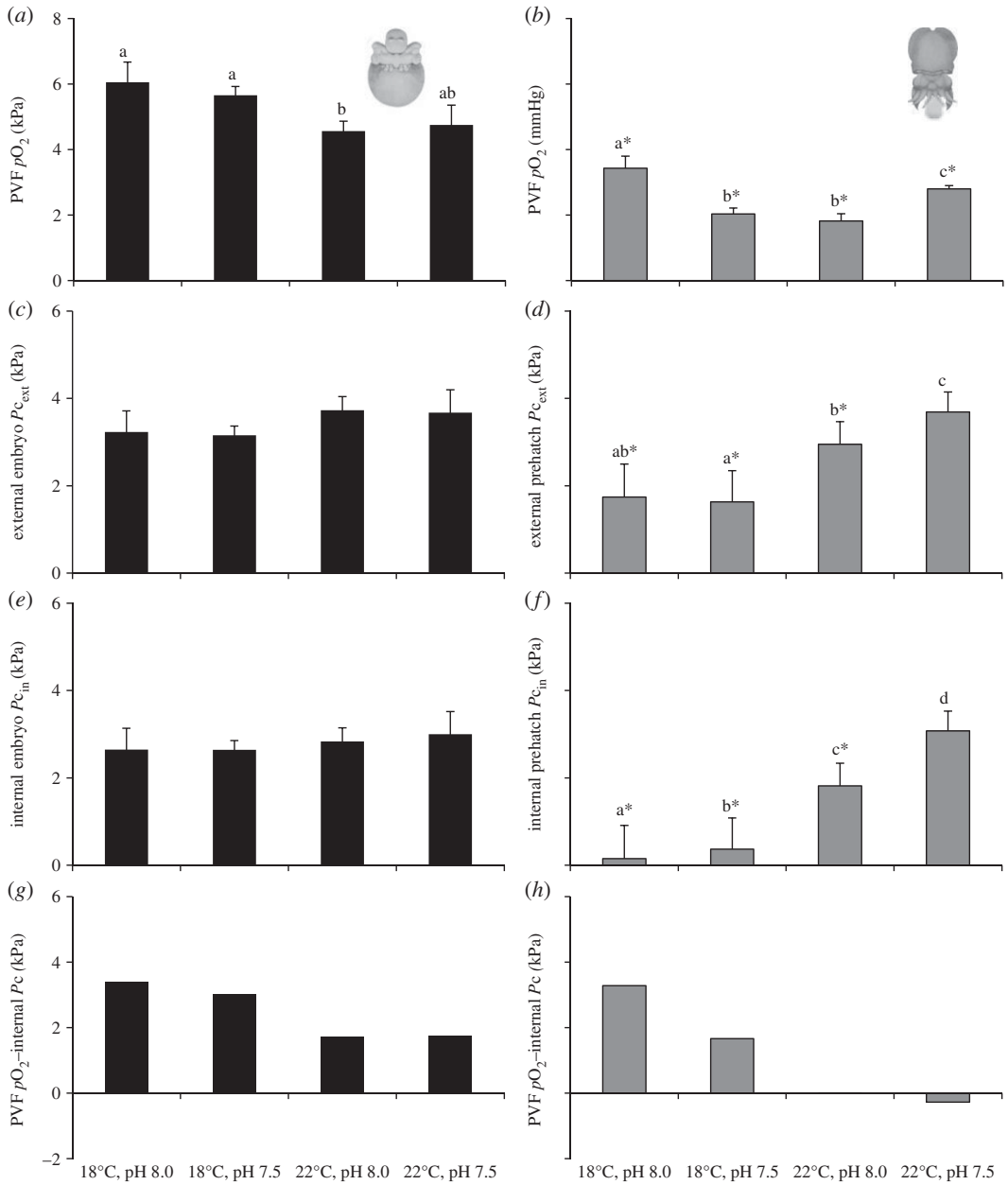

Figure 5. Effect of hypercapnia (ΔpH = 0.5) and warming ($+4^{\circ}$C) on the: (*a*,*b*) PVF pO_2 (kPa, $n = 20$), (*c*,*d*) measured external (ambient) critical partial pressure ($P_{c,ext}$, kPa; $n = 20$), (*e*,*f*) estimated internal critical partial pressure ($P_{c,in}$, kPa; $n = 20$), and (*g*,*h*) difference between mean values of PVF pO_2 and $P_{c,in}$ (kPa), of intermediate embryonic stages (*a*,*c*,*e*,*g*) and pre-hatchlings (*b*,*d*,*f*,*h*) of common cuttlefish *Sepia officinalis*. Values represent the mean $\pm$ s.d. Different letters represent significant differences between treatments ($p < 0.05$). Asterisks represent significant differences between life stages ($p < 0.05$).

have contributed to the hypometabolic state as a possible consequence of the reduced capacity to extract oxygen at hypoxic and hypercapnic conditions within egg capsules [36]. In fact, the hatchlings acclimated to the warmer scenarios were exposed to low oxygen levels, similar to (at pH 8.0) or even below (at pH 7.5; figure 5*h*) the values observed for their critical pO_2. The ability to live for limited time periods below P_c, via metabolic depression, has already been previously described for embryos [37] and older life stages [2,38], and is known to be accompanied by the reallocation of cellular energy to essential ATP demand processes as well as the transition of anaerobic metabolism [39]. Thus, with the decrease in pre-hatchlings' oxygen supply, metabolic processes could be partly shifted towards less efficient anaerobic processes [4,5].

One prevailing strategy used to achieve metabolic depression is reducing protein synthesis [40] and, consequently, growth. Although considered a sublethal reversible process, metabolic depression is only an effective adaptive strategy for the survival of short-term hypercapnia and hypoxia [40,41], but not advantageous under persistent elevations of CO_2 [12,14]. Thus, we expect that the stressful abiotic conditions inside molluscan eggs will be aggravated with ocean acidification, warming and expanding hypoxia, especially in populations at the border of their thermal envelope [4]. These stressors may act together as a main trigger for premature hatching (as shown in figure 1*c*) and smaller post-hatching body sizes [3,42] and, consequently, dictate negative effects on survival and development of posterior ontogenetic stages. These effects may constrain species survival in such areas and, thereby, cause a shift on species' biogeographic range.

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