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RESEARCH ARTICLE

Differential impacts of ocean acidification and warming on winter and summer progeny of a coastal squid (*Loligo vulgaris*)

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ABSTRACT

Little is known about the capacity of early life stages to undergo hypercapnic and thermal acclimation under the future scenarios of ocean acidification and warming. Here, we investigated a comprehensive set of biological responses to these climate change-related variables (2°C above winter and summer average spawning temperatures and ΔpH=0.5 units) during the early ontogeny of the squid *Loligo vulgaris*. Embryo survival rates ranged from 92% to 96% under present-day temperature (13–17°C) and pH (8.0) scenarios. Yet, ocean acidification (pH 7.5) and summer warming (19°C) led to a significant drop in the survival rates of summer embryos (47%, $P<0.05$). The embryonic period was shortened by increasing temperature in both pH treatments ($P<0.05$). Embryo growth rates increased significantly with temperature under present-day scenarios, but there was a significant trend reversal under future summer warming conditions ($P<0.05$). Besides pronounced premature hatching, a higher percentage of abnormalities was found in summer embryos exposed to future warming and lower pH ($P<0.05$). Under the hypercapnic scenario, oxygen consumption rates decreased significantly in late embryos and newly hatched paralarvae, especially in the summer period ($P<0.05$). Concomitantly, there was a significant enhancement of the heat shock response (HSP70/HSC70) with warming in both pH treatments and developmental stages. Upper thermal tolerance limits were positively influenced by acclimation temperature, and such thresholds were significantly higher in late embryos than in hatchlings under present-day conditions ($P<0.05$). In contrast, the upper thermal tolerance limits under hypercapnia were higher in hatchlings than in embryos. Thus, we show that the stressful abiotic conditions inside the embryo's capsules will be exacerbated under near-future ocean acidification and summer warming scenarios. The occurrence of prolonged embryogenesis along with lowered thermal tolerance limits under such conditions is expected to negatively affect the survival success of squid early life stages during the summer spawning period, but not winter spawning.

KEY WORDS: Ocean acidification, Global warming, Thermal tolerance limits, Early life stages, Squid

INTRODUCTION

The atmospheric concentration of CO_2 has increased from preindustrial levels of 280 ppm to present-day levels of 390 ppm, and it is expected to rise to 730–1020 ppm by 2100 (Meehl et al., 2007). Concomitantly, the continuous CO_2 uptake by the oceans is changing the seawater chemistry and is estimated to lead to a drop of 0.4–0.5 units in seawater pH (Meehl et al., 2007). The future changes in the ocean's chemistry are expected to pose particular problems for key calcifying organisms (Fabry et al., 2008; Orr et al., 2005). Yet, elevated CO_2 has also been shown to elicit negative effects on the survival, growth, calcification and physiology of cephalopods (Gutowska et al., 2008; Hu et al., 2011; Kaplan et al., 2013; Rosa and Seibel, 2008; Rosa et al., 2013c). Concomitantly, average global sea surface temperatures are expected to increase by up to 3°C by 2100 (Meehl et al., 2007), a scenario that will likely drive profound impacts on the ecophysiology (Donelson et al., 2011; Munday et al., 2009; Pörtner and Knust, 2007; Rosa and Seibel, 2008) and, consequently, the biogeography of marine biota (Perry et al., 2005; Somero, 2005). Coastal marine ecosystems are warming at a higher rate than most other ecosystems (MacKenzie and Schiedek, 2007). As several coastal organisms already live close to their thermal tolerance limits (Helmuth et al., 2006; Stillman and Somero, 2000), ocean warming is expected to negatively impact their performance and survival. The metabolism of marine ectotherms is constrained by oxygen supply at high (and low) temperatures with a progressive transition to an anaerobic mode of energy production [the 'oxygen and capacity limitation of thermal tolerance' concept (Pörtner and Knust, 2007)]. The reduction in aerobic scope is not caused by lower levels of ambient oxygen but rather through the limited capacity of oxygen supply mechanisms (ventilatory and circulatory systems) to meet an animal's temperature-dependent oxygen demand. These patterns may be influenced by rising CO_2 levels. In fact, hypercapnia is known to augment heart rates, thus eliciting the narrowing of thermal windows (Metzger et al., 2007; Walther et al., 2009). In this context, it is of paramount importance to predict how acclimation to increased future CO_2 levels and ocean temperatures may affect behavior, growth and reproduction, and possibly shape the long-term fate of marine species. Yet, it is worth noting that most studies were conducted under short-term (acute) scenarios and on adult stages. Early life stages (e.g. embryos, larvae) of marine organisms are expected to be the most vulnerable to such climatic shifts (Oyarzun and Strathmann, 2011; Pechenik, 1999; Przeslawski, 2004; Strathmann, 1985). Ultimately, this vulnerability may become a serious bottleneck for species survival (Byrne, 2011).

Squids play an important ecological role in marine trophic webs, both as prey and predators (Rosa et al., 2008; Rosa et al., 2012a). These organisms are commonly defined as keystone species due to their strong influence on ecosystem dynamics (Rosa et al., 2013a; Rosa et al., 2013b). The life history of these cephalopods is characterized by high mortality and strong selection pressures, with early life stages being influenced not only by the external environment but also by the intrinsic features of the embryo

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inherited from parental organisms (Strathmann, 1985). For instance, the European squid, *Loligo vulgaris*, has an extended spawning season with two peaks – a major one in February and a minor one in June/July (Moreno et al., 2009), which implies that squid hatched in these distinct periods experience extremely different thermal conditions. Yet, until now, there has been little information on the ability of squid early life stages to undergo acclimation as a means of coping with projected ocean acidification and warming trends (Kaplan et al., 2013). It is worth noting that the western coastal area of Portugal is located in the Western Iberian Upwelling Ecosystem (WIUE), which comprises the northern limit of the Canary Current Upwelling System (one of the four major eastern boundary currents of the world). The main feature of the region is the occurrence of coastal upwelling during spring and summer in response to the intensification of northerly winds (Rosa et al., 2010). Therefore, species inhabiting this region are exposed to seasonal high P_{CO_2} events (400–460 ppm), due to the emergence of deep hypercapnic water masses (Álvarez-Salgado et al., 1997; Borges and Frankignoulle, 2002; Pérez et al., 1999). In more northern Atlantic latitudes, some upwelling-influenced benthic habitats may reach values >3000 ppm in summer months (Thomsen et al., 2010). Therefore, in all these Atlantic regions the future P_{CO_2} levels are expected to exceed the forecasted 1000–1200 ppm (ΔpH=0.4–0.5) for 2100 (Meehl et al., 2007).

Here, we provide a comprehensive and integrated view of biological (namely morphological, developmental, biochemical and physiological) responses of squid embryos and hatchlings (paralarvae) to realistic scenarios of these climate change-related variables. Recently spawned *L. vulgaris* Lamarck 1798 egg masses were collected in cooler (winter) and warmer (summer) periods, and reared until hatching at present-day (between 13 and 17°C) and future temperatures expected for the western Iberian coast in 2100 [+2°C (Santos et al., 2002)], and at present (~430 ppm, pH ~8.0) and future P_{CO_2} levels (0.16% CO_2, ~1650 ppm, ΔpH=0.5). More specifically, we examined how the different climate scenarios affect: (i) survival and development time, (ii) embryonic growth, (iii) hatching (incidence of malformations and premature paralarvae), (iv) metabolic rates and thermal sensitivity (Q_{10} values), (v) thermal tolerance limits (LT_{50} and LT_{100}) and (vi) heat shock response (HSP70/HSC70 levels).

RESULTS

Survival and development time

Survival rates ranged between 92% and 96% under present-day temperatures (i.e. between 13 and 17°C) and pH (8.0) for both winter and summer embryos. However, the projected near-future ocean warming had a significant negative effect on summer embryos, with survival decreasing to 71% (Fig. 1A, three-way nested ANOVA, $P<0.05$; for more statistical details, see supplementary material Table S1). Moreover, with future hypercapnic conditions and ocean warming during the summer (i.e. pH 7.5 and 19°C), survival decreased significantly to 47% (Fig. 1A, three-way nested ANOVA, $P<0.05$). Consequently, a significant difference between seasons was observed ($P<0.05$), i.e. while the combined effect of warming and hypercapnia was striking on summer embryo mortality, it was not evident in the winter embryos.

The duration of the embryonic period in *L. vulgaris* was significantly shortened with increasing temperature in both pH treatments (Fig. 1B, three-way nested ANOVA, $P<0.05$). At present-day pH, embryogenesis lasted 27±1 days at 13°C; warming to 19°C caused summer embryos to hatch after 14±2 days. However, a significant increase in development time occurred with the lower pH

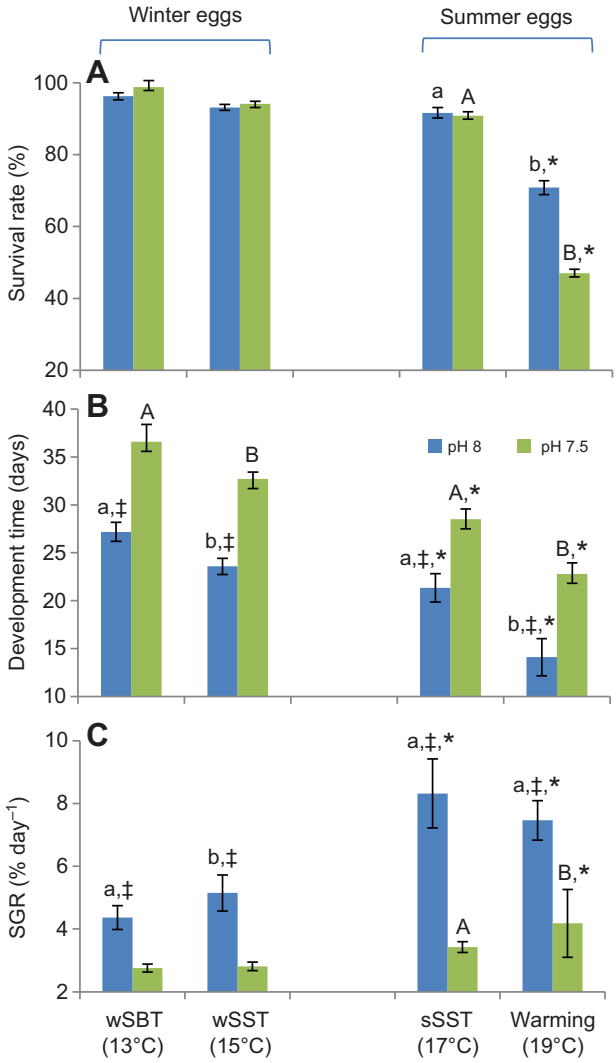

Fig. 1. Impact of ocean acidification (from pH 8.0 to 7.5) and warming (from 13 to 19°C) on survival (A), development time (B) and specific growth rate (SGR) of squid (*Loligo vulgaris*) embryos. Values are means ± s.d. In each season (i.e. winter and summer embryos), different lower and upper case letters represent significant differences within normocapnic and hypercapnic treatments, respectively; double daggers represent significant differences between pH treatments in each temperature condition ($P<0.05$); asterisks represent significant differences between seasons ($P<0.05$). wSBT (13°C), mean sea bottom temperature in winter; wSST (15°C), the expected mean sea surface winter temperature in 2100; sSST (17°C), mean sea surface summer temperature (sSST); and Warming (19°C), the future sSST warming scenario for the western coast of Portugal in 2100.

condition (Fig. 1B, three-way nested ANOVA, $P<0.05$). In fact, embryogenesis under hypercapnia and winter conditions lasted 36±2 days, while under the future warming (19°C) and hypercapnic scenario, embryonic development was completed in 23±1 days. It is also noteworthy that there were significant differences between winter and summer eggs under all experimental conditions ($P<0.05$; see also supplementary material Table S1).

Growth, premature hatching and abnormalities

Under normocapnia, the future warming scenario (19°C) significantly affected early ontogenetic growth (Fig. 1C, three-way nested ANOVA, $P<0.05$). In fact, embryo specific growth rate (SGR; % day^{-1}) increased significantly during warming within the

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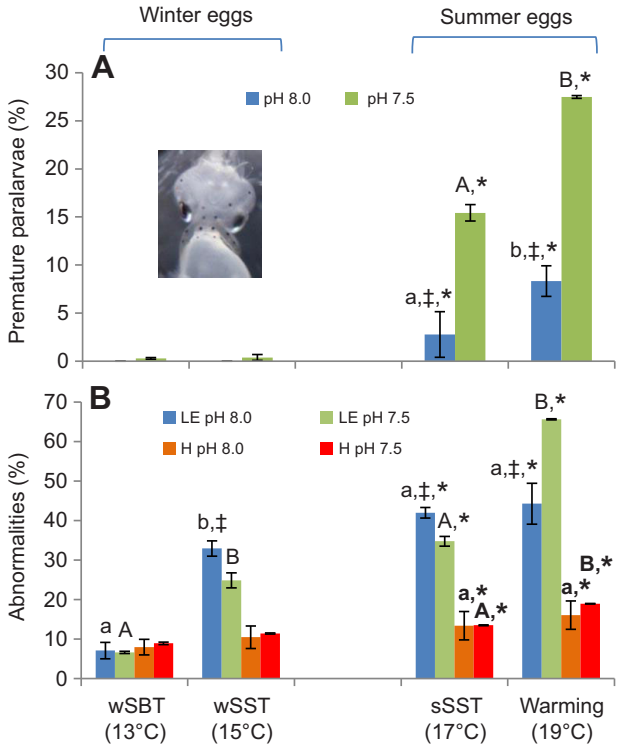

Fig. 2. Impact of ocean acidification (from pH 8.0 to 7.5) and warming (from 13 to 19°C) on the occurrence of (A) premature hatching and (B) abnormalities during the early development of *L. vulgaris*. LE, late embryos; H, hatchlings/paralarvae. Values are means ± s.d. In A, and in each season (i.e. winter and summer embryos), different lower and upper case letters represent significant differences within normocapnic and hypercapnic treatments, respectively; double daggers represent significant differences between pH treatments in each temperature condition (P<0.05); asterisks represent significant differences between seasons (P<0.05). In B, the letters and symbols have the same meaning, but those in bold are associated with the paralarval stage (P<0.05). For temperature abbreviations, see Fig. 1; more statistical details are given in supplementary material Tables S1 and S2.

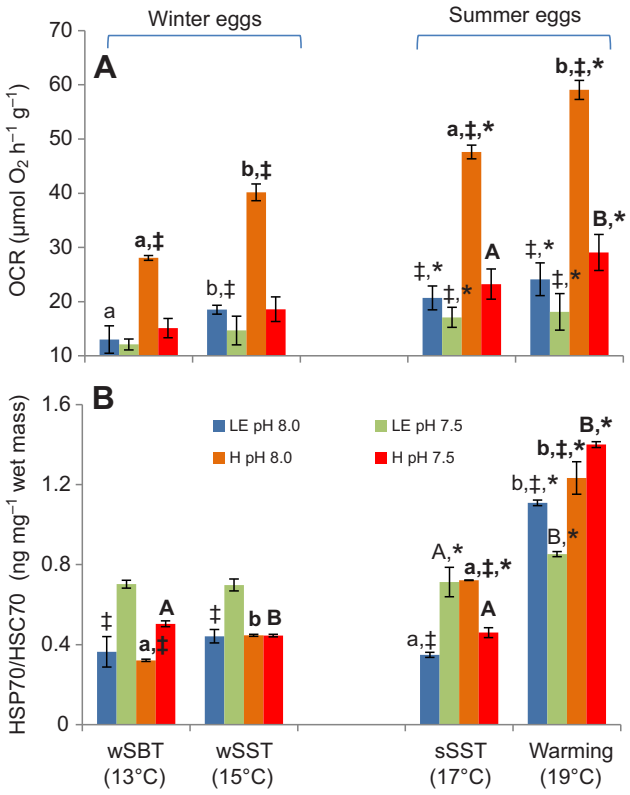

Fig. 3. Impact of ocean acidification (from pH 8.0 to 7.5) and warming (from 13 to 19°C) on (A) oxygen consumption rate and (B) heat shock protein levels during the early life stages of *L. vulgaris*. LE, late embryos; H, hatchlings/paralarvae; OCR, oxygen consumption rate. For temperature abbreviations, see Fig. 1; more statistical details are given in supplementary material Table S2.

range of present-day temperatures, but there was a significant trend reversal under future summer conditions. Moreover, when exposed to environmental hypercapnia, embryo daily growth rates were reduced and became independent of temperature (Fig. 1C, three-way nested ANOVA, P>0.05). A significant distinct growth response to warming and hypercapnia between winter and summer eggs was observed (P<0.05).

One of the most striking impacts of future warming on squid early ontogeny was premature hatching (Fig. 2A). The percentage of paralarvae that hatched with the yolk sac still attached was significantly greater at 19°C, which was related to the fact that late embryos (pre-hatchlings) still had a significant amount of yolk before hatching. Future CO_2 conditions significantly enhanced premature hatching, especially in summer eggs (Fig. 2A, three-way nested ANOVA, P<0.05). The incidence of abnormalities also increased significantly with temperature and varied between stages (Fig. 2B, four-way nested ANOVA, P<0.05; see also supplementary material Table S2). A high percentage of abnormalities was found in late embryos exposed to future summer warming and lower pH (Fig. 2B, P<0.05). Hatchlings had the lowest degree of abnormalities in all thermal and pH scenarios. It is worth noting that late embryos mostly exhibited elongated bodies and mantle deformities, while hatchlings revealed a greater incidence of mantle detachment, mantle deformity and complete body deformities.

Metabolic rates, thermal sensitivity, heat shock response and thermal tolerance limits

Oxygen consumption rates (OCRs) were significantly affected by temperature, pH and developmental stage, and were also significantly different between winter and summer eggs under all experimental conditions (Fig. 3A, four-way nested ANOVA, P<0.05). Under normocapnia, late embryos displayed OCRs ranging from 13.0 µmol O_2 h^{-1} g^{-1} (winter temperature; 13°C) to 24.1 µmol O_2 h^{-1} g^{-1} (future summer warming – 19°C). At pH 7.5, the embryo's OCR ranged from 12.1 to 18.2 µmol O_2 h^{-1} g^{-1} at 13 and 19°C, respectively. Squid paralarvae displayed significantly higher OCRs at normal pH, ranging between 28.1 µmol O_2 h^{-1} g^{-1} (winter temperature, 13°C) and 59.1 µmol O_2 h^{-1} g^{-1} (19°C). At lower pH, paralarval OCR decreased significantly, ranging from 15.1 to 29.0 µmol O_2 h^{-1} g^{-1} at 13 and 19°C, respectively (Fig. 3A, four-way nested ANOVA, P<0.05; see also supplementary material Table S2). Consequently, embryonic Q_{10} values ranged between 1 and 1.5 across all temperatures, and were around 1.5 in hatchlings acclimated to low pH (Fig. 4). At normal operating temperatures, metabolic demand for oxygen should increase with temperature following a Q_{10} of around 2–3. This 'expected' trend was only observed in: (i) embryos exposed to normocapnia below 15°C and (ii) hatchlings exposed to normocapnia in all thermal scenarios.

Concomitantly, there was a significant progressive increase of the heat shock response (HSP70/HSC70) with warming in both developmental stages and in both pH treatments (Fig. 3B, four-way nested ANOVA, P<0.05; see also supplementary material Table S2).

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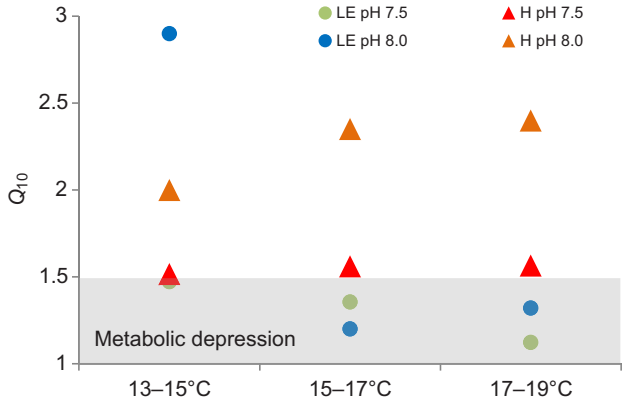

Fig. 4. Thermal sensitivity (Q_{10}) of late embryonic stages and hatchlings of *L. vulgaris* in the different pH and temperature treatments. Q_{10} values between 2 and 3 indicate active metabolic regulation; Q_{10} values lower than 1.5 suggest active metabolic suppression below expected rates.

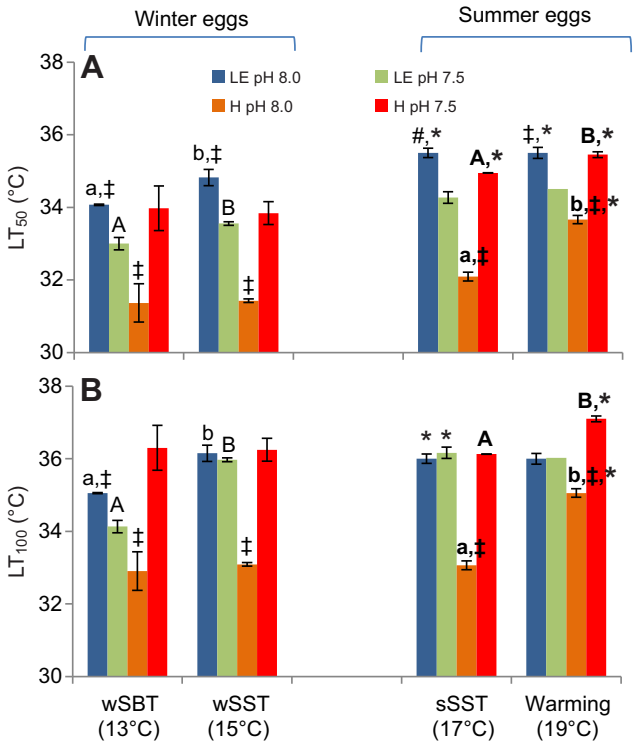

Fig. 5. Impact of ocean acidification (from pH 8.0 to 7.5) and warming (from 13 to 19°C) on thermal tolerance limits during the early life stages of *L. vulgaris*. (A) LT_{50} and (B) LT_{100}. LE, late embryos; H, hatchling/paralarvae. Values are means ± s.d. For temperature abbreviations, see Fig. 1; symbol definitions are given in Fig. 2; more statistical details are given in supplementary material Table S2.

The response was significantly greater in summer eggs (*t*-test, $P<0.05$) and in the hatchling stage (above 1.2 ng mg^{-1}).

Lastly, the thermal tolerance experiments revealed that the upper thermal tolerance limits were significantly affected by temperature, pH and developmental stage (Fig. 5, four-way nested ANOVA, $P<0.05$; see also supplementary material Table S2). Both LT_{50} and LT_{100} values were positively influenced by the acclimation temperature, and in normocapnic conditions, such thresholds were significantly higher in late embryos than in hatchlings. In contrast, lower pH caused higher upper thermal tolerance limits among hatchlings than in embryos.

DISCUSSION

The present study showed that projected conditions of ocean acidification and warming for the summer spawning period are already outside the range of tolerance of the squid *L. vulgaris* early life stages as they elicited: (i) about 50% mortality, (ii) a reduction in the embryonic growth potential and (iii) an increase in the occurrence of developmental abnormalities. Moreover, late stage embryos displayed a greater amount of yolk before hatching (data not shown) and a higher percentage of paralarvae with their yolk sacs still attached to their body. As in the present study, the recent work of Kaplan et al. (Kaplan et al., 2013) also found that embryos of the squid *Doryteuthis pealeii* raised under elevated P_{CO_2} had an increased time to hatching (and shorter mantle lengths).

The premature hatching observed here may be a response to hypoxia (Kamler, 2008), as a decrease in the oxygen flux (by means of diffusion) leads to egg swelling and, consequently, greater surface area and reduced egg wall thickness (Cronin and Seymour, 2000; Lacoue-Labarthe et al., 2009; Wolf et al., 1985). Swelling does not prevent P_{O_2} from consistently falling (to critical levels) and P_{CO_2} from rising within cephalopod eggs (Gutowska and Melzner, 2009; Rosa et al., 2013c). The rise in OCR throughout embryogenesis may have accelerated hypoxia within egg capsules, eliciting a metabolic depression response (low Q_{10} values). This finding may be linked to a reduced capacity to extract oxygen under hypoxic and hypercapnic conditions within developing embryos. As squid early life stages are known to display incomplete development and functionality of ion regulatory epithelia (Hu et al., 2011), the physiological challenges imposed by the joint increase of perivitelline fluid P_{CO_2} and environmental hypercapnia is expect to be greater (Rosa et al., 2013c). Moreover, it is worth noting that the transition from an encapsulated embryo to a planktonic life form is accompanied by a drastic change of surrounding conditions, which may also elicit significant stress responses (e.g. increased HSP expression).

The planktonic paralarvae of squid rely predominantly on a pulsed jet for locomotion (Bartol et al., 2009) and, therefore, the energy losses due to swimming activity are expected to increase with warming. Yet, here we show that the metabolic increment typically observed during the transition from an encapsulated embryo to a jet propelled planktonic stage under normocapnia (Pimentel et al., 2012; Rosa et al., 2012b) no longer occurred under hypercapnia (Fig. 3A). While it is predicted that ocean warming may enhance the food requirements of squid hatchlings, environmental hypercapnia may lead to a hypometabolic state, accompanied by a reduction in protein synthesis and, consequently, growth (Hochachka and Somero, 2002; Storey and Storey, 2004). Therefore, such a strategy will not be beneficial for organisms living under chronic conditions of high CO_2 in tomorrow's oceans.

Marine early life stages are known to display narrow thermal windows due to developmental constraints and insufficient capacity of central organs (Pörtner and Farrell, 2008; Pörtner et al., 2004). In the present study, there were significant differences in thermal tolerance limits between winter and summer embryos, with the latter displaying higher heat limits. It is noteworthy that parental effects may have played a key role in such trends, as winter spawners tend to show higher fecundity and smaller oocytes, a feature that can favor higher offspring numbers surviving natural mortality (Boavida-Portugal et al., 2010). Our findings highlight that the extent to which these season-related spawning cohorts are adapted to the prevailing temperatures may have implications for population

survival and genetic composition under a rapidly changing thermal regime. In fact, transgenerational acclimation may be a key mechanism for coping with this rapid climate change (Donelson et al., 2012).

The few available ontogenetic studies on LT_{50} suggest that the thermal tolerance range is narrower in embryos than in larvae; once larvae hatch, the temperature over which they can endure increases significantly (Jordaan and Kling, 2003). In the present study, however, thermal tolerance data revealed three distinct trends, namely: (i) embryos were more heat tolerant (independent of the thermal environment) than hatchlings under normocapnia, (ii) LT_{50} and LT_{100} increased steadily with temperature in both life stages (late stage embryos and hatchlings) regardless of P_{CO_2} treatment, and (iii) hypercapnia caused heat tolerance limits to be higher in hatchlings. First, we propose that the loss of physical protection (egg capsules) during hatching may contribute to the lower thermal tolerance of the hatchlings under normocapnia. Nonetheless, the lower thermal tolerance limits exhibited by squid hatchlings may be related to the higher metabolic rates they display due to their planktonic existence. Their greater sensitivity to environmental stress may be reflected in physiological mechanisms that respond to such reduced negative stress effects. In fact, the HSC70/HSP70 concentrations were higher in hatchlings under the near-future warming scenario (for both P_{CO_2} treatments). Their increased oxygen demands in the pelagic realm may be associated with radical oxygen species (ROS) formation, and HSPs are among the molecules that are expressed in response to ROS formation (de Oliveira et al., 2005). Yet, the HSP levels were not indicative of the LT_{50} or LT_{100} values observed for the two life stages surveyed. The values recorded instead reflected their sublethal limits characterized by the 'oxygen- and capacity-limited thermal tolerance' hypothesis (Pörtner and Knust, 2007; Pörtner et al., 2004).

As thermal tolerance windows are directly related to oxygen supply and energy demand (Pörtner and Knust, 2007; Pörtner et al., 2004), one of the major limitations that the hatchlings might face under a future warming scenario (19°C) is the ability to extract enough oxygen from the water to match the demand dictated by biochemical processes. Yet, thermal tolerance limits of both embryos and paralarvae increased with thermal acclimation. Through acclimation, organisms may compensate and reduce the increase in oxygen demand and thereby shift thermal tolerance limits. Early life stages may have balanced their baseline costs, by adjusting mitochondrial densities and functional properties (e.g. reducing membrane proton leakage) in order to shift their thermal tolerance windows to higher temperatures (Pörtner, 2002). This capacity is influenced by the level of ambient hypercapnia. The fact that embryos were less heat tolerant than hatchlings under hypercapnia might be related to the more stressful abiotic conditions inside egg capsules (higher P_{CO_2} and lower P_{O_2}). In fact, the greater degree of metabolic suppression in embryos under hypercapnia implies greater deleterious effects on their survival and growth over longer time scales.

In summary, we found evidence that extended embryogenesis (linked to metabolic depression and a higher incidence of premature hatching) and lower embryonic heat tolerance limits under chronic hypercapnia and warming conditions will negatively affect the survival success of squid early stages during the summer spawning period. In contrast, during the winter spawning period, near-future changes in temperature and ocean chemistry did not seem to elicit significant stress responses in the early life stages of *L. vulgaris*. As the squid species studied here is an intermittent terminal spawner (i.e. with sequential maturation of distinct batches of oocytes over a protracted period of time), it is hard to predict whether in the future they will continue to display these two main spawning seasons, or whether they will be able to take more advantage of the less-stressful winter prevailing conditions.

MATERIALS AND METHODS

Egg collection and incubation

Recently spawned egg masses [eggs with cleavage and formation of germinal layers; stage I–V (Naef, 1928)] were collected by commercial fishing vessels off Figueira da Foz and Cascais (western coast of Portugal) between 36 and 54 m depth, in the winter (February) and summer (June) periods of 2012. After collection, eggs were transported in thermal cases and immediately transferred to the aquaculture facilities of Laboratório Marítimo da Guia (Cascais). Egg masses were suspended 5–15 cm below the water surface, to ensure good aeration, and distributed between 12 tanks of 17.5 l volume. It is worth noting that each egg mass may include embryos from multiple mothers. Winter egg masses were reared at: (i) 13°C – the mean sea bottom temperature in winter (wSBT) along the western coast of Portugal (Moreno et al., 2009), and (ii) 15°C – the expected mean winter sea surface temperature (wSST) in 2100 [+2°C (Santos et al., 2002)]. Summer egg masses were reared at: (i) 17°C – the mean sea surface summer temperature (sSST), and (ii) 19°C – the future sSST warming scenario for the western coast of Portugal in 2100 [+2°C (Santos et al., 2002)]. Ten egg masses were incubated per thermal and CO_2 treatment [4 temperatures×2 pH values=8 experimental treatments; 8 treatments×10 egg masses (replicates) per treatment=80 egg masses in total]. All thermal treatments were conducted at present (~430 ppm) and future P_{CO_2} [ΔpH=0.5; 0.16% CO_2, ~1650 ppm]. Independent life support systems were operated with 0.35 µm-filtered and UV-irradiated natural seawater, under a semi-closed water circulation regime. The selection of this circulation regime aimed to minimize bacterial activity (i.e. nitrifiers, denitrifiers) and dissolved inorganic carbon speciation in each life support system. Tank illumination was provided through overhead fluorescent lighting (Digilamp FL-40SS/36 Daylight, 6400K, 2450 lumen), under a photoperiod of 14 h light:10 h dark. Ammonia and nitrite levels were monitored regularly using colorimetric tests and maintained below detectable levels. pH was adjusted automatically, via solenoid valves, with the Profilux controlling system (Profilux 3, Kaiserslautern, Germany) being connected to individual pH probes (Blueline 25 pH, SCHOTT Instruments, Mainz, Germany). pH (NBS) values were monitored every 2 s and lowered by injection of a certified CO_2 gas mixture (Air Liquide, Miraflores, Algés, Portugal) via air stones or upregulated by aerating the tanks with CO_2-filtered (using soda lime, Sigma-Aldrich, St Louis, MO, USA) air. Additionally, pH, temperature and salinity values were manually surveyed on a daily basis (see supplementary material Table S3) throughout the experimental period. Salinity was maintained by adding 1 µm charcoal-filtered freshwater, while temperatures were controlled by means of water chillers (Hailea, Guangdong, China) and 300 W heaters (Eheim, Deizisau, Germany).

Seawater carbonate system speciation was calculated weekly from total alkalinity as described previously (Sarazin et al., 1999) (spectrophotometrically at 595 nm; see supplementary material Table S3) and pH (total scale) measurements. pH was quantified via a Metrohm pH meter (826 pH mobile, Metrohm, Filderstadt, Germany) connected to a glass electrode (±0.001; Schott IoLine, SI analytics, Mainz, Germany) and calibrated against the seawater buffers Tris-HCl (Tris) and 2-aminopyridine-HCl (AMP) (Mare, Liège, Belgium) according to Dickson et al. (Dickson et al., 2007). Measurements were performed under temperature-controlled conditions using a waterbath (±0.1°C; Lauda, Lauda-Königshofen, Germany). Bicarbonate and P_{CO_2} values were calculated using CO2SYS software (Lewis and Wallace, 1998) (see supplementary material Table S3), with dissociation constants from Mehrbach et al. (Mehrbach et al., 1973) as refitted by Dickson and Millero (Dickson and Millero, 1987).

Survival, growth and abnormalities

In order to follow survival and individual growth, 45 embryos were randomly isolated at the beginning of the experiment and stocked in three

independent boxes (with 15 slots each) that were placed in the different (independent) experimental systems. Growth and survival of individual embryos were monitored every other day under a microscope at 48 h intervals until hatching. It was assumed that handling promoted no significant differences (Oosthuizen et al., 2002). Developmental stage was identified and the following measurements were performed: egg length and width, yolk sac length and width, and embryo length (from the moment that the yolk sac and embryo were well differentiated. The specific embryonic growth rate (SGR, % day^{-1}) was calculated as:

$$\{[\ln \text{embryo size } (T_2) - \ln \text{embryo size } (T_1)] / \text{number of days elapsed between } T_1 \text{ and } T_2\} \times 100\,, \quad (1)$$

where T_1 and T_2 are temperature 1 and temperature 2, respectively. From egg masses incubated at each temperature, 100 late stage embryos and 200 hatchlings were screened for abnormalities. Abnormalities were defined as anomalous body shape, such as underdeveloped mantle, mantle detached, eye dimorphism, elongated body and complete body deformity, among others (see Oosthuizen et al., 2002). For each temperature and pH, the percentage of individuals with abnormalities was determined. Premature hatching was also considered, being defined as paralarvae that hatched with the yolk sac still attached.

OCRs and thermal sensitivity

Oxygen consumption measurements were determined according to previously established methods (Rosa et al., 2012b; Rosa et al., 2013c; Rosa et al., 2009). Late eggs (pre-hatching) and paralarvae (hatchlings) were incubated in sealed water-jacketed respirometry chambers (RC300 respiration cell, Strathkelvin, North Lanarkshire, UK) containing 1 µm filtered and UV-irradiated seawater from the different incubation systems (i.e. with the different thermal and CO_2 treatments). Water volumes were adjusted in relation to animal mass (up to 4 ml) in order to minimize locomotion and stress but still allow for spontaneous and routine activity rates of hatchlings. Controls (blanks) were used to correct for possible bacterial respiratory activity. Respiration chambers were immersed in waterbaths (Lauda) to control temperature. Oxygen concentrations were recorded with Clarke-type O_2 electrodes connected to a multi-channel oxygen interface (Model 928, Strathkelvin). The duration of respiratory runs varied from 12 to 24 h. Preliminary experimental runs showed that using the first 4 h of the runs, the handicaps of the closed system (increasing P_{CO_2} and hypoxia and ammonia build-up) were avoided. Thermal sensitivity (Q_{10}) was determined using the standard equation:

$$Q_{10} = [R(T_2) / R(T_1)] \times 10 / (T_2 - T_1)\,, \quad (2)$$

where $R(T_2)$ and $R(T_1)$ represent the OCRs at temperatures T_2 and T_1, respectively.

Thermal tolerance limits

The upper lethal temperature limits were determined based on Stillman and Somero (Stillman and Somero, 2000). In brief, 60 late embryos and 60 squid paralarvae were incubated in small containers with ~100 ml of 0.35 µm-filtered and UV-irradiated seawater from the different incubation systems (i.e. with the different thermal and CO_2 treatments). Each container was stocked with 20 specimens (either late embryos or paralarvae) for a total of three replicates per experimental treatment (total N=60). These glass containers were suspended in a temperature-regulated waterbath that was controlled to the nearest 0.1°C. The temperature of the waterbath was set to the acclimation temperature and maintained for 30 min. Thereafter, temperature was increased at a rate of 1°C 30 min^{-1}. Every 30 min, the water was aerated with an air stone and the temperature in each container was checked (with thermocouple probes), while swimming activity (i.e. jet propulsion) and mantle contractions (when at the bottom of the container) of each paralarvae were visually monitored. If no responsiveness was noticed, the specimen was considered to be dead. The percentage of living individuals at each temperature was calculated and then transformed by the arcsine square root function and expressed in radians. Linear regression analysis was then used to find the slope of the line, from which the temperature at which 50% of the organisms had died (0.785 rad) was calculated. This was used as the measure of upper thermal tolerance limits and referred to as the LT_{50}. The LT_{100} (temperature at which all specimens were dead) was also recorded at the end of the experiment.

Heat shock proteins

Homogenates were prepared in triplicate for each experimental treatment, by using 150 mg of frozen tissue of both developing stages (late embryos or paralarvae). To obtain enough tissue, 50 specimens (late embryos and hatchlings) were homogenized for each replicate sample. The pooled tissues were homogenized in 500 µl of the homogenization buffer, phosphate-buffered saline (PBS, pH 7.3: 0.14 mol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 8.1 mmol l^{-1} Na_2HPO_4, 1.47 mmol l^{-1} KH_2PO_4), by using a glass hand-held homogenizer. All homogenates were then centrifuged (20 min at 14,000 ***g*** at 4°C) and heat shock proteins were measured in the supernatant fraction. HSP70/HSC70 content was assessed by ELISA, adapted from a previously published protocol (Njemini et al., 2005). Briefly, 10 µl of the supernatant was diluted in 250 µl of PBS (1×), and 50 µl of the diluted sample was added to a 96 well microplate (Nunc, Roskilde, Denmark) and allowed to incubate overnight at 4°C. The next day, the microplates were washed (3×) in 0.05% PBS-Tween-20. A 100 µl sample of blocking solution (1% BSA, Sigma-Aldrich) was added to each well and left to incubate at room temperature for 2 h. Microplates were washed and 50 µl of 5 µg ml^{-1} primary antibody (anti-HSP70/HSC70; Acris, San Diego, CA, USA), detecting 72 and 73 kDa proteins corresponding to the molecular mass of inducible hsp and hsc70, was added to each well and then incubated at 37°C for 90 min. The non-linked antibodies were removed by washing the microplates again, which were then incubated for 90 min at 37°C with 50 µl of 1 µg ml^{-1} of the secondary antibody, anti-mouse IgC (Fab-specific, alkaline phosphatase conjugate, Sigma-Aldrich). After another wash, 100 µl of substrate (*FAST p*-nitrophenyl phosphate tablets, Sigma-Aldrich) was added to each well and incubated for 10–30 min at room temperature. Then, 50 µl of stop solution (3 mol l^{-1} NaOH) was added to each well and the absorbance was read at 405 nm in a 96 well microplate reader (Benchmark, Bio-Rad, Hercules, CA, USA). The amount of Hsp70/Hsc70 in the samples was calculated from a curve of absorbance based on serial dilutions of purified HSP70 active protein standard (Acris) to a range from 0 to 2000 ng ml^{-1}. The results were expressed in relation to wet mass of the sample (ng hsp70/hsc70 mg^{-1} wet mass).

Statistical analyses

A nested analysis of variance was performed to evaluate the existence of significant differences in the following dependent variables: survival, development times, SGR and percentage of premature paralarvae. Three categorical factors were considered; seawater temperature (with two levels: present-day sea surface temperature and projected sea surface temperature for the western Iberian coast in 2100), pH (with two levels: present-day and futures values predicted for 2100) and season (with two levels: winter and summer); the categorical factors seawater temperature and pH were considered to be nested within the categorical factor season. Another nested analysis of variance was also performed to evaluate any significant differences in the following dependent variables: percentage of abnormalities, OCRs, thermal tolerance limits (LT_{50} and LT_{100}) and HSP70/HSC70 levels. For this analysis, four categorical factors were considered: seawater temperature (with the same two levels described above), pH (with the same two levels described above), squid life stage (with two levels: late stage embryos and paralarvae) and season (with the same two levels described above); in this statistical analysis the categorical factors seawater temperature, pH and squid life stage were considered to be nested within the categorical factor season. Normality and homogeneity of variances were verified by Kolmogorov–Smirnov and Bartlett tests, respectively. Moreover, percentage data (% survival, % abnormalities and % premature paralarvae) were transformed by an arc sine square root function. Subsequently, *post hoc* tests (Tukey HSD and unequal N HSD) were performed. All statistical analyses were performed for a significance level of 0.05, using Statistica 10.0 software (StatSoft Inc., Tulsa, OK, USA).

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Competing interests
The authors declare no competing financial interests.

Author contributions
R.R. designed the research and provided the reagents; R.R., K.T., M.S.P., J.B.-P., F.F., M.B., G.B. and T.R. performed the research; R.R., M.S.P., R.C. and H.O.P. analyzed the data and wrote the paper.

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Table S1. Results of nested analysis of variance to evaluate the differences in the following dependent variables: survival, development times, specific growth rates (SGR) and % of premature paralarvae

	df	MS	F	p
Survival				
pH (Season)	2	232.0	5.3	0.015
T (Season)	2	1595.0	36.6	0.000
Season	1	2530.1	58.0	0.000
Development time				
pH (Season)	2	223.4	135.8	0.000
T (Season)	2	83.8	50.9	0.000
Season	1	416.9	253.4	0.000
SGR				
pH (Season)	2	30.9	62.0	0.000
T (Season)	2	0.2	0.5	0.591 (n.s.)
Season	1	25.9	52.0	0.000
% premature paralarvae				
pH (Season)	2	379.0	137.6	0.000
T (Season)	2	116.1	42.1	0.000
Season	1	1049.6	380.9	0.000

The three categorical factors were: temperature (T), pH and season. The former two factors were considered to be nested within the factor season. (n.s. – non-significant; $p>0.05$)

Table S2. Results of nested analysis of variance to evaluate the differences in the following dependent variables: oxygen consumption rates (OCR), HSP70/HSC70 levels and thermal tolerance limits (LT50 and LT100)

	df	MS	F	p
% abnormalities				
pH (Season)	2	63.6	1.4	0.239 (n.s.)
T (Season)	2	770.8	17.9	0.000
Stage (Season)	2	3117.7	72.6	0.000
Season	1	3579.6	83.3	0.000
OCR				
pH (Season)	2	1055.9	29.9	0.000
T (Season)	2	193.7	5.48	0.000
Stage (Season)	2	1526.1	43.2	0.000
Season	1	1161.6	32.9	0.000
HSP70/HSC70				
pH (Season)	2	0.1	6.5	0.003
T (Season)	2	1.0	60.0	0.000
Stage (Season)	2	0.2	9.4	0.000
Season	1	1.6	91.9	0.000
LT50				
pH (Season)	2	2.4	2.3	0.113 (n.s.)
T (Season)	2	1.3	1.2	0.309 (n.s.)
Stage (Season)	2	6.9	6.43	0.003
Season	1	18.3	17.5	0.000
LT100				
pH (Season)	2	10.8	10.6	0.000
T (Season)	2	3.3	3.21	0.045
Stage (Season)	2	2.9	2.9	0.066 (n.s.)
Season	1	6.0	5.9	0.019

The four categorical factors were: temperature (T), pH, life stages (late embryos and hatchlings) and season. The former three factors were considered to be nested within the factor season. (n.s. – non-significant; $p>0.05$).

Table S3. Seawater physiochemical parameters in all experimental (temperature and pH) setups

	13 °C		15 °C		17 °C	
	8.0	7.5	8.0	7.5	8.0	7
measured						
Temperature (°C)	13.2 ± 0.3	13.2 ± 0.3	15.1 ± 0.3	15.3 ± 0.2	17.1 ± 0.3	16.9
Salinity	35.1 ± 0.3	35.0 ± 0.4	35.3 ± 0.4	34.9 ± 0.5	35.0 ± 0.3	35.3
pH_T	8.03 ± 0.02	7.51 ± 0.03	8.03 ± 0.01	7.53 ± 0.02	8.04 ± 0.01	7.53
A_T (µmol kg^{-1} SW)	2403.4 ± 11.3	2490.0 ± 13.8	2417.0 ± 11.9	2498.5 ± 10.9	2416.4 ± 19.0	2501.9
calculated						
pCO_2 (ppm)	433.2 ± 24.1	1681.9 ± 131.7	439.8 ± 11.7	1641.4 ± 80.2	429.5 ± 13.5	1627.3
C_T (µmol kg^{-1} SW)	2196.8 ± 11.9	2502.6 ± 20.3	2198.3 ± 7.4	2474.2 ± 13.4	2176.1 ± 13.1	2467.5
Ω calcite	3.6 ± 0.2	1.3 ± 0.1	3.9 ± 0.1	1.4 ± 0.1	4.2 ± 0.1	1.5

Salinity and temperature were measured daily and averaged per replicate aquarium over the whole experimental period. The combination of total alkalinity (AT) and pHT (pH total scale) was used to calculate carbonate system parameters pCO_2, CT (total carbon) and ΩCa (calcite). Values are represented as means ± standard deviation.