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Metabolomic and phenotypic effects of ocean acidification on cuttlefish differ across early life stages

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ABSTRACT

Ocean acidification (OA) affects the physiology and behaviour of some marine organisms, impacting their development and metabolism during vulnerable early-life stages. Among them, the embryo of the cuttlefish develops for about two months in encapsulated eggs with harsh perivitelline conditions of hypoxia and hypercapnia, potentially worsened by OA. In this study, common cuttlefish *Sepia officinalis* embryos and juveniles, were exposed to five pH conditions (pH_T 8.08 to 7.43). Growth, development and metabolite profiles were explored during the embryonic development period up to 10 days-post-hatching. Our results show delayed embryonic development and decreased hatching success at pH 7.43, but no effect on juvenile weight upon hatching. The ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy revealed that decreasing pH affected metabolites profiles in embryos until a metabolic suppression was observed at pH 7.43. The O_2 consumption in 10d-old juveniles did not change with pH whereas metabolites indicated a switch to anaerobic metabolism under low pH. Overall, our results suggest that the transition from the encapsulated embryonic stage to the free juvenile life shapes a metabolomic reprogramming more drastically than ocean acidification.

1. Introduction

Atmospheric carbon dioxide (CO_2) emissions are continuously increasing because of energy production, transport, manufacturing, and other human activities, causing a rise in the sea-surface partial pressure of CO_2 (pCO_2). Reaction of CO_2 with seawater leads to an ocean pH decrease known as ocean acidification (OA) (Gattuso and Hansson, 2011). The surface ocean pH is expected to decrease from 8.07 to 8.00 or 7.66 by 2100 according to the shared socioeconomic pathways (SSP) 1–2.6 or SSP5-8.5 scenarios, respectively (Cooley et al., 2022). OA is directly affecting the carbonate-silicate cycle of the ocean, resulting in a change in the calcification rate of organisms such as coralline algae (Manning et al., 2019), coccolithophores (Krumhardt et al., 2019), foraminifera (Guamán-Guevara et al., 2019), corals (Chou et al., 2020) and molluscs, in particular cephalopods (Gutowska et al., 2010a; Dorey et al., 2013a; Zhao et al., 2020). Changes in seawater pH also cause behavioural and physiological alterations in some marine organisms, such as reduced growth rates and development (Sigwart et al., 2016), altered metabolic rates (Cattano et al., 2018), acid-base imbalance (Pörtner and Farrell, 2008), lowered osmoregulatory capacity (Ramaglia et al., 2018), and altered neurosensory responses (Nilsson et al., 2012). The consequences of OA are gaining interest as an emerging threat to ecosystems, and evidence suggests that early-life stages are particularly vulnerable to acidification (Pörtner and Farrell, 2008; Dupont and Thorndyke, 2009; Zippay and Hofmann, 2010; Oliveira et al., 2020).

Among marine organisms, cuttlefish have a particularly long embryonic development time of about two months (i.e., 10% of the entire life span) in the perivitelline fluid (PVF) of encapsulated eggs. This fluid constitutes a micro-environment wherein the embryo develops, surrounded by three envelopes: the chorion, the envelope secreted by the gland of the oviduct, and the envelope secreted by the nidamental glands impregnated of ink (Boletzky, 1986). During embryonic development, the egg swells resulting in increased surface area and reduced egg wall thickness, which promotes eggshell permeability. This facilitates exchange with external media for oxygen or essential elements (De

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Wachter et al., 1988; Cronin and Seymour, 2000) but also allows contaminants to pass through the eggshell (Lacoue-Labarthe et al., 2009). Gutowska and Melzner (2008) demonstrated that common cuttlefish embryos are surrounded by tenfold higher pCO_2 values than those of ambient sea water (~2300 vs. 400 μatm, respectively) at the end of their embryonic developmental period, as well as low PVF pO_2 ~ 25% air saturation. Thus, cuttlefish embryos develop naturally in highly hypercarbic and hypoxic challenging conditions, until juveniles can hatch. Moreover, when exposed to lowered seawater pH, the PVF pH decreases as a mechanism to maintain the CO_2 gradient between the PVF and seawater to optimise exchange with external media (Dorey et al., 2013a; Sigwart et al., 2016).

Cuttlefish early life stages are therefore critical phase where environmental stressors could compromise young survival and recruitment. For instance, the duration of egg development, the embryo catabolic activities and growth are intrinsically linked to seawater temperature as a consequence of temperature effect on metabolic demands (Manning et al., 2019; Dorey et al., 2013b; Rosa et al., 2013). Elevated seawater pCO_2 may also trigger reducing oxygen consumption rate in cuttlefish pre-hatching until reaching metabolic depression, an adaptive response to the extreme hypercarbic challenge faced in the PVF (Rosa et al., 2013). Moreover, response to high pCO_2 leads to enhanced acid-base and ion-regulatory processes, leading to 1) hyper-calcification of the hatchlings cuttlebone (Dorey et al., 2013a; Sigwart et al., 2016), but also 2) energy reallocation leading to reduced embryo growth and a delayed hatching time (Hu et al., 2011; Sigwart et al., 2016). When they hatch, juveniles experience less restrictive abiotic conditions which would take them out of their "window of vulnerability". Unfortunately, information remains scarce on how pCO_2 challenges the early life stages of the cuttlefish from a metabolic perspective, especially around the critical period of transition from an encapsulated embryo to the free juvenile in seawater.

Untargeted metabolomics allows simultaneous measurement of numerous molecular endpoints facilitating the detection of perturbations to specific biological molecules and linking them to relevant biochemical processes, pathways, or mechanisms. For example, ^{1}H Nuclear Magnetic Resonance (NMR) revealed that high CO_2 exposure caused disturbances in energy metabolism and osmotic regulation in adult oysters (Wei et al., 2015) as well as suppressed amino acid metabolism and ATP (adenosine triphosphate) synthesis in D-shape larvae (Liu et al., 2020). In cephalopods, disturbances of energy and amino acid metabolism, and disruption of the balance of neurotransmitters and osmoregulatory molecules were revealed in *Sepia pharaonis,* pharaoh juveniles cuttlefish exposed to stressful hypoxia conditions (Jiang et al., 2020).

The aim of this study was threefold: (1) to determine early-life-stage responses to a pH/pCO_2 gradient, assessing egg development success, embryo growth, and oxygen consumption of 10-day-old juveniles; (2) to use ^{1}H NMR metabolomics as an innovative approach to obtain a comprehensive metabolic profile of cuttlefish across three successive early life stages (pre-hatching embryo, newly hatched juvenile, and 10-day-old juvenile); and (3) to explicitly investigate the metabolic implications of exposure to low pH/high pCO_2 at these critical ontogenic transitions. While previous studies have examined the physiological impacts of ocean acidification on cephalopods, none have focused on their metabolomic reprogramming, especially at early life stages. This study provides novel insights into the differential metabolic changes employed by embryos and juveniles in response to ocean acidification, filling a key knowledge gap in how early developmental stages adjust to environmental stressors. By integrating metabolomic profiles with physiological assessments, we offer a unique perspective on the metabolic changes occurring across key developmental stage, contributing to a broader understanding of species-specific resilience and vulnerability to ocean acidification.

2. Material and methods

2.1. Cuttlefish collection and exposure

Freshly spawned eggs (<24h) of common cuttlefish *Sepia officinalis* were caught in the Bay of Biscay, Port des Minimes, France (46°08′44.7″N 1°10′16.4″W) during summer 2019. The eggs were separated, randomized and acclimated for one week in 5 open-circuit glass aquaria (n = 90 per 20 L aquarium; water flux: 60 L h^{-1}; salinity: 38; temperature: 17.4 ± 0.1 °C; pH: 8.0 ± 0.1; 12 h light:12 h dark cycle) at the International Atomic Energy Agency, Marine Environment Laboratories (IAEA-MEL). Embryos, and later juveniles, were exposed to five conditions of seawater pH/pCO_2 (i.e., control; pH = 8.08 equivalent to 395 μatm and four acidified conditions; pH = 7.82; 7.65; 7.54; 7.43 equivalent to 792, 1220, 1603, 2097 μatm, respectively, i.e., without "pH" tank replication). The water renewal was kept constant (water flux: 60 L h^{-1}) throughout the experiment duration to maintain as constant as possible the seawater parameters including the total alkalinity. The pCO_2 was regulated by an IKS system from Aquastar© with a flow-through bubbling CO_2 and pH measurements every 20 min and a weekly calibration of the IKS probes with NIST (National Institute of Standards and Technology) standard pH solution (pH 4.0 and pH 7.0). The registered pH values expressed in the NIST scale were then weekly corrected and reported on the total scale (pH_T) using a glass electrode (Metrohm©, ecotrode plus) and using TRIS buffer solutions (salinity 35, provided by A. Dickson, Scripps Institution of Oceanography, USA). Total alkalinity was measured every two days in duplicate potentiometrically using a Metrohm© titrator (Titrando 888). The pCO_2 values and carbonate chemistry parameters were calculated from pH_T and the alkalinity with the seacarb package from R (Gattuso et al., 2021). Experimental conditions are outlined in Table S1.

After hatching, cuttlefish were placed in a new aquarium (same conditions as eggs) and individualized with circular baskets (60 mm diameter). Juveniles were fed with live ditch shrimp *Palaemon varians* twice daily, for 10 days. The exposure conditions and endpoints are summarised in Fig. 1. The experiments were referenced and authorized by the French Ministry of Higher Education and Research for the use of animals for scientific purposes (APAFIS # 20520–2019050614554709).

2.2. Hatching and development

The embryonic stages (according to Boletzky et al., 2016) were regularly determined by removing the eggshell to check the embryo development and roughly predict the hatching event. Eggs used for the latter purpose were then definitively removed from the aquarium. Few hours before hatching, the eggs were laid horizontally, photographed and their frontal surface was measured with ImageJ software (version 1.53, 2021) and weighted (g) just before hatching. Once eggs started to hatch, hatchlings were counted twice a day. Metrics from the day when 50% of the embryos had hatched were used for statistical analysis. Both newly born cuttlefish (<24h) and 10d-old cuttlefish were measured (i.e., dorsal mantle length) and weighed as previously described.

2.3. Respirometry

2.3.1. Experimental set up

Two oxymeters (Microx, PreSens, Germany) and height cylindrical respirometers (61 mL; 3.75 cm diameter) were used to assess metabolic performance of 10 d-old juvenile cuttlefish. Each oxymeter was connected to four submerged respirometers in a 20 L buffer tank, and filled with temperature-controlled (i.e., 17 °C) and oxygenated mixed water as in the rearing system. Mass specific oxygen consumption (MO_2 in mg O_2 kg^{-1} h^{-1}), associated with the metabolic activity of cuttlefish, was measured by intermittent-flow respirometry (Steffensen, 1989). Water supply in each respirometer was provided by flush pumps, which controlled water flow from the buffer tank to the respirometer.

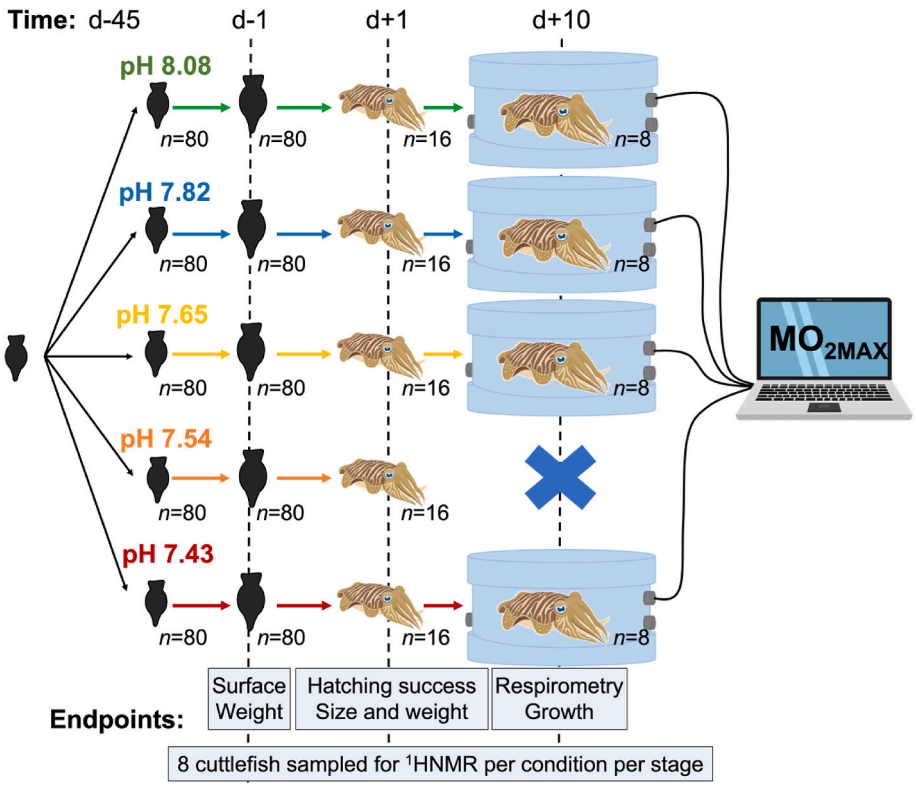

Fig. 1. Schematic summary of eggs and juvenile cuttlefish *Sepia officinalis* exposed to 5 pHs (8.08, 7.82, 7.65, 7.54, 7.43). Endpoints: surface and weight of the eggs at last embryonic stage (stage 30), hatching success and size and weight of the newly hatched, respirometry and growth of the 10 days-old cuttlefish, ^{1}H nuclear magnetic resonance of cuttlefish from the 3 development stages previously cited.

Dissolved oxygen in the water was measured every 5 seconds by oxygen probes (minisensor, PreSens, Germany) connected to an oxymeter. The probes were calibrated once at the beginning of the test using 0% and 100% air saturation for a controlled temperature of 17 °C.

2.3.2. Maximal oxygen uptake measurement (MO_{2Max})

The cuttlefish were starved 24 h before respirometry assays as previous research showed that, it avoid any excretions and bias in the experimental systems, cuttlefish are adaptable to short-term hunger which has no significant effect on their respiration rate and oxygen consumption (Grigoriou and Richardson, 2009). The MO_{2Max} was established by using an exercise challenge immediately before oxygen uptake measurement. To achieve MO_{2Max}, cuttlefish were chased in a circular tank for 3 min (Brennan et al., 2016), then held in a dip net and periodically lifted in and out of the water (10:10 s) for 1 min immediately prior to introduction to the respirometry chamber (Norin and Clark, 2016; Reemeyer and Rees, 2020). Then MO_{2Max} were measured during 20 min followed by a phase of oxygen renewal of 10 min constituting a first cycle of 20:10 min. Two more cycle of 30:10min were repeated to verify the first cycle MO_{2Max} consumption. The total experimental trial lasted 2 h for each cuttlefish. The total experiment lasted approximately 10 h. The running order of the juveniles and their respective conditions were randomized. The O_2 concentration did not fall below 70% air saturation during measurement periods in any of the chambers.

2.3.3. Calculations MO_{2Max}

Mass specific oxygen consumption MO_{2Max} is expressed in mg O_2 kg^{-1} h^{-1} and calculated using the following equation:

$$MO_{2Max} = S.V_{resp}.M_{cut}^{-1}$$

where S is the slope (in milligrams of O_2 per litre per second) of O_2 consumption function of time, V_{resp} is the volume of the respirometer (chamber and tubing) minus the volume of the cuttlefish (water displacement, in litres) and M_{cut} is the mass of the cuttlefish (in kilograms). Only the steepest slope (S) of the 3 cycles were considered. The R^2 of slopes were always above 0.95.

2.4. Nuclear magnetic resonance spectroscopy

The polar metabolites of embryos and juveniles reared at different pH were analysed by ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy.

2.4.1. Sample description

The following samples (n = 112) were collected and analysed to facilitate comparing the effects of varying pH conditions on metabolite profiles at three developmental stages.

- 40 stage 30 embryos sampled at the end of the development period (n = 8 per pH; 5 pH: 8.08, 7.82, 7.65, 7.54, 7.43).
- 40 newly hatchlings collected immediately post-hatching (n = 8 per pH; 5 pH: 8.08, 7.82, 7.65, 7.54, 7.43).
- 32 samples of 10d-old juveniles (n = 8 per pH; 4 pH: 8.08, 7.82, 7.65, 7.43). The 7.54 condition was stopped due to lab space limitation. We chose to keep the ambient pH and the two more realistic pH (8.08, 7.82, 7.65 respectively) in an OA scenario as well as the lowest pH (7.43), as an extreme condition.

2.4.2. Measurement

The cuttlefish were flash-frozen via submersion in liquid nitrogen, freeze-dried, and shipped on dry-ice to Griffith University, Australia, where they were stored at −80 °C until extraction of metabolites and analysis using NMR spectroscopy. Prior to analysis, individual samples were transferred to glass test tubes and again placed in a freeze-dryer overnight to ensure complete removal of residual water (e.g.,

atmospheric absorption or uptake during cold storage), after which they were weighed (mg) for normalisation of metabolite relative abundance per unit mass. Samples were manually ground then homogenised with an Ultra-Turrax® T10 tissue homogenizer (IKA®, Selangor, Malaysi), after which ice cold methanol was added (800 μl) and they were incubated overnight at −20 °C. The following morning 1600 μL chloroform and 400 μL deionised water were added, the samples were vortexed and then centrifuged (10 min, 15,000 rpm at 4 °C). After centrifugation, the polar fraction (top layer) was transferred to glass amber vials and dried in a centrifugal evaporator (GeneVac). The dried samples were resuspended in deuterium oxide (D_2O) containing 0.05% sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) as an internal standard. Samples were transferred to 3 mm NMR tubes using glass pipettes.

NMR spectra were acquired with an 800 MHz Bruker® Avance III HDX spectrometer, fitted with a Triple (TCI) Resonance 5 mm Cryoprobe with Z-gradient and automatic tuning and matching. A SampleJet automatic sample changer controlled via the software IconNMR™ (Bruker Pty Ltd., Victoria, Australia) was used to load the samples. Spectra were acquired at 298 K, with D_2O for field locking and TSP (^{1}H δ 0.00) as a reference. ^{1}H spectra were acquired using the zg30 pulse program with 64 scans, 0.8 s relaxation delay, 5.25 μs pulse width and 16 kHz spectral width (^{1}H δ −3.75–16.28). An edited ^{1}H-^{13}C HSQC spectra was acquired for a representative sample with 200 scans, 0.8 s relaxation delay, 5.25 μs pulse width and spectral widths of 12.8 kHz (^{1}H δ −3.23–12.79) and 33.1 kHz (^{13}C δ −9.40–155.2). Due to high salt content in the samples, there was considerable suppression of the ^{13}C signal, meaning several metabolites could only be identified using ^{1}H spectra. Nevertheless, the samples were highly concentrated, and identification was achieved with a high degree of confidence for most of the spectral features (i.e., metabolites).

2.4.3. Peak assignments and processing of ^{1}H NMR spectra

MestReNova v8.1.4 (Mestrelab Research S.L., Spain) was used for post processing of NMR spectra. ^{1}H NMR free induction decay (FID) data were Fourier transformed with line broadening of 0.3 Hz and HSQC spectra were processed with default Bruker post-processing parameters. All spectra were manually phase corrected, automatically baseline adjusted (ablative), and referenced and normalised to TSP (^{1}H δ 0.00, ^{13}C δ 0.0). Once processed, individual features (resonances) were manually integrated and normalised to the mass of each sample to provide a comparative measure of the relative abundance of metabolites.

2.5. Statistical analysis

All statistical analysis were performed using Rstudio (Version 4.0.5, http://www.r-project.org), and the threshold of statistical significance was fixed at 0.05. As many experiments revealed a large variability in response depending on the pH considered and especially demonstrated "tipping point" curve responses in other organisms (e.g. Dorey et al., 2013b; Lutier et al., 2021), our approach was to investigate this potential tipping point in cuttlefish, with respect to the endpoints reported in this study. Consequently, we tested fived controlled pH conditions considering the pH (and the subsequent pCO_2) as a continuous variable (Havenhand et al., 2010), without tank replications (i.e. pseudo-replications). The size and surface of the eggs and juveniles as well as the MO_{2Max} have been tested considering the measured pH_T as a continuous variable and were computed using regression models (see Table S2). Metabolites multivariate analysis was performed on each of the 3 groups of samples independently using the MetaboAnalyst 5.0 platform. Spectra were log transformed and auto-scaled and were first examined by Principal Components Analysis (PCA) to explore differences in metabolite profiles among the treatments. Partial Least-Squares Discriminant Analysis (PLS-DA) was subsequently applied to further characterize the observed separation among treatment groups. Permutation analysis (n = 2000 permutations) was applied to explore whether PLS-DA separation distance was statistically significant. Significance Analysis of Metabolites (SAM) and ANOVAs with False Discovery Rate (FDR) adjusted *p*-values were used to identify specific metabolites driving the observed differences among treatments and life-stage respectively. Important Features analysis was also performed – this test highlights the most important metabolites contributing towards separation in the multivariate (PLS-DA) analysis irrespective of statistical significance. ANOVA-Simultaneous Component Analysis (ASCA) was used to split the data into sub-sets that describe the variation between treatment, life-stage, and their interaction, and to identify specific metabolites contributing the most to the variation in each of these data sub-sets (Smilde et al., 2005).

3. Results

3.1. Egg development and hatching success

Monitoring of egg hatching revealed a minor hatching delay corresponding to the lowering pH treatments, with embryonic development lengthening from 61.7 ± 3.5 days at pH 8.08 to 61.7 ± 4.0, 62.3 ± 4.6, 62.7 ± 4.2, 63.9 ± 5.4 days at pH 7.82, 7.65, 7.54, 7.43, respectively (Fig. 2). Moreover, hatching success was significantly reduced (*p*-value = 0.0078, see Table S2) at the lowest pH condition, from 84.4% at pH 8.08–72.5% at pH 7.43 (Table 1). No mortality was observed in juveniles.

3.2. Eggs size

Egg weight (g) and surface area (cm^2) showed a significant increase with decreasing pH (*p*-value <0.001, see Table S2). Specifically, mean weight increased from 4.05 ± 0.29 g at pH 8.08 to 4.39 ± 0.41 g at pH 7.43 (Fig. 3A) and mean surface increased from 3.45 ± 0.19 cm^2 at pH 8.08 to 3.77 ± 0.25 cm^2 at pH 7.43 (Fig. 3B).

3.3. Oxygen consumption rate in 10-d-old juvenile under lower pH

Oxygen uptake measurement demonstrated an apparent effect of pH on the maximum consumption, but this was non-significant (*p*-value = 0.270). MO_{2Max} dropped from 668 ± 254 mg kg^{-1} h^{-1} at pH 8.08 to 574 ± 236 and 340 ± 229 mg kg^{-1} h^{-1} at pH 7.82 and 7.65, respectively, and rose back to 594 ± 191 mg kg^{-1} h^{-1} at pH 7.43 (Fig. 4).

3.4. Metabolomic changes during the successive early life stages at ambient pH

Metabolites profiles were first compared among the three developmental stages at ambient pH to highlight the differing metabolic demands that occur during the progression from embryonic to juvenile life. The PLS-DA performed on metabolite relative abundances of late developed embryo, newly hatched, and 10d-old juveniles (Fig. 5A) identified clear separation between encapsulated individuals and older juveniles. The multivariate plots of newly hatched cuttlefish partly overlap with those of the other developmental stages, suggesting a transitional metabolic state. ANOVAs with False Discovery Rate adjusted *p*-values of $p = 0.05$ identified 8 spectral features that decreased significantly in relative abundance from the embryo to the juvenile phase (Fig. 5B). These features corresponded to the metabolites: alanine, proline, glutamate, asparagine, trimethylamine, acetoacetate, formate, and a region with overlapping signals for proline/glutamate.

3.5. Metabolomic responses to lowered pH

Analyses of metabolomic profiles among pH treatments revealed a differing magnitude of response to pH according to the three life stages.

Clear separation among pH treatments was confirmed in late embryonic stage samples via PCA, PLS-DA (Fig. 6A), and permutation

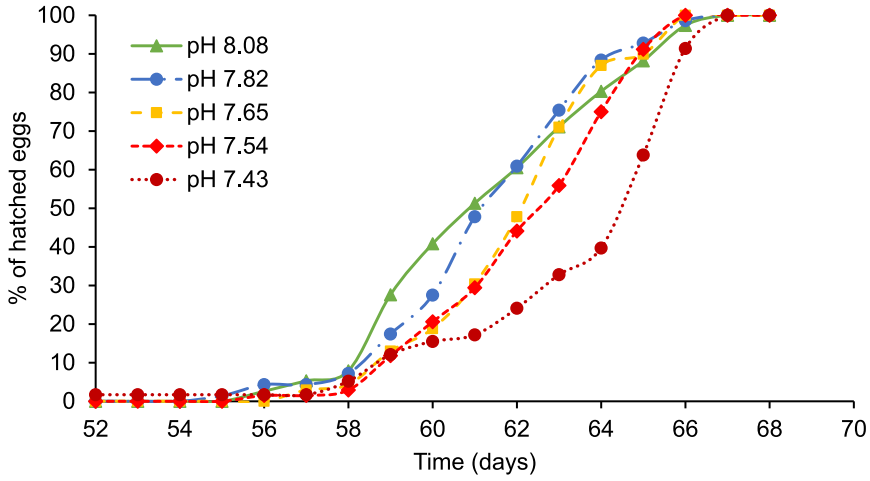

Fig. 2. Percentage of the total hatched eggs of common cuttlefish *Sepia officinalis* exposed to five different pH (8.08, 7.82, 7.65, 7.54, 7.43) over time.

Table 1
Hatching success of common cuttlefish eggs *Sepia officinalis* exposed to 5 pHs (8.08, 7.82, 7.65, 7.54, 7.43) n = 90, *significant effect of pH treatment on hatching success (binomial exact test, *p*-value < 0.05).

pH	pCO_2 (µatm)	Hatching success (%)	*p*-value
8.08	400	84.4	/
7.82	800	86.2	0.7587
7.65	1200	86.2	0.7587
7.54	1600	85.0	0.9986
7.43	2100	72.5*	**0.0078**

analysis (*p*-value <0.05). SAM indicated that this was driven by differences in 29 metabolites among pH treatments: 3-methoxytyramine, acetylcholine, AMP (adenosine monophosphate), ADP (adenosine diphosphate), alanine, arginine, asparagine, betaine, choline, creatinine, cytosine, fumarate, galactarate, glucose, glutamate, glycine, hydroxykynurenine, isoleucine, lactic acid, leucine, NAD+ (nicotinamide adenine dinucleotide), phenylalanine, proline, taurine, trimethylamine, tryptophan, uracil, uridine, valine, plus 4 notable spectral features that could not be conclusively identified (Fig. S1). The characteristic pattern of response for all metabolites was a general decrease in relative abundance in pH 7.43 as compared to the other treatments.

In newly hatched juvenile, the PLS-DA displayed very little visible separation of metabolite profiles with respect to pH (Fig. 6B). Interestingly, considerable variance existed within each pH treatment during the juvenile stage apart from individuals at pH 7.43. Only one metabolite, AMP, was identified as significantly different among treatments in hatchlings (Fig. S2). However, this observation should be interpreted with caution, since other integrals corresponding to AMP were not identified as significantly different based on the SAM.

The 10 d-old juvenile cuttlefish exhibited significant differences in 13 metabolites: acetylcholine, ATP, alanine, asparagine, creatinine, cytosine, fumarate, glutamate, GMP (guanosine monophosphate), lactic acid, proline, trimethylamine, uracil, plus 2 notable spectral features that were not identified (Fig. 6C). All but 2 of these metabolites (i.e., GMP and trimethylamine), plus one unknown feature, were also significantly altered in the stage 30 embryos. However, an additional 9 metabolites were significantly altered by pH in embryos that were not identified as significantly different among treatments in 10 d-old juvenile cuttlefish group. As with hatchlings, the 10 d-old juveniles generally exhibited lower metabolite relative abundance (overall) in pH 8.08 compared to the other treatment groups.

Finally, ASCA computed on all metabolomic data in individuals sampled at the three development stages and the pHs 8.08, 7.82, 7.65 and 7.43 confirmed metabolite differences related to ontogeny and pH exposure conditions. The iPCA (Fig. 7A) clearly showed a separation of ontogenic groups (especially according to the first axe that accounts for 84% of the variance) and pH conditions to a lesser extent. Nevertheless,

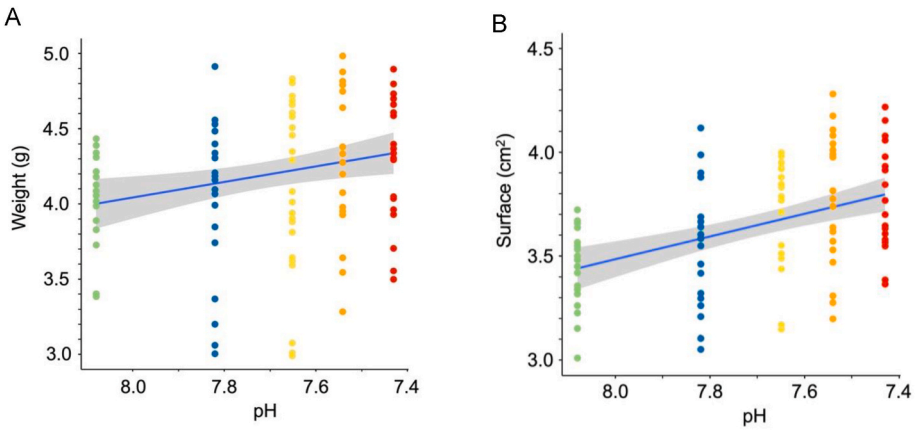

Fig. 3. A) Weight (g) and B) surface (cm^2) of common cuttlefish eggs *Sepia officinalis* before hatching (stage 30) exposed to 5 different pHs (8.08, 7.82, 7.65, 7.54, 7.43). Significant effect of pH on eggs weight and surface (regression model *p*-value < 0.05, see Table S2). The graphical blue bar represents the linear regression. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

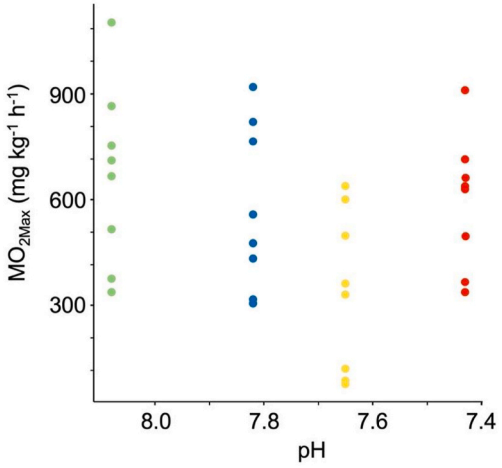

Fig. 4. Maximal oxygen uptake (MO_{2Max} in mg kg^{-1} h^{-1}) of common cuttlefish *Sepia officinalis* 10 days-old exposed to 4 different pHs (8.08, 7.82, 7.65, 7.43). No significant effect of pH on juveniles MO_{2Max} (regression model *p*-value > 0.05, see Table S2).

a permutation test indicates that the model may be overfit for all but ontogeny (*p*-value <0.05) suggesting that ontogeny is the most important factor in this dataset in terms of causing effects on the metabolome. The heatmap (Fig. 7B) clearly segregated the individuals belonging to the embryos and the 10d-old juveniles at pH 8.08, 7.82 and 7.65, whereas metabolite abundance changes appeared less drastic at pH 7.43 between both groups. Newly hatched individuals showed contrasting profiles at all pH conditions; probably linked to the acute and drastic metabolic transition that occurs with hatching.

4. Discussion

4.1. Effect of decreasing pH on egg and embryo development

The embryonic development period was observed to lengthen with decreasing extreme pH along with a significant decrease in hatching success. Extended embryonic development as a consequence of OA has been previously reported by Sigwart et al. (2016), with 50% of *S. officinalis* hatching after 65–69 d of incubation in ambient conditions and a delay to 67–70 d at 16 °C under pH 7.76. However, Moura et al. (2019) found the development duration at 18 °C of *S. officinalis* embryos did not differ (59 ± 9 days) between ambient and acidified pH (i.e., 7.7). Likewise, they found that neither hatching success (73.33 ± 1.80% and 70.20 ± 1.36% at pH 8.1 and 7.7, respectively), nor survival rate 10 days after hatching (66.86 ± 1.10% and 69.30 ± 1.99% at pH 8.1 and 7.7, respectively) were significantly affected by low pH/high CO_2 treatment (Moura et al., 2019). The discrepancies with our results are most likely due to the widest range of pH treatments we tested (i.e., down to pH 7,43) for a given temperature.

A significant increase in egg size (including greater weight and surface area) was observed at low pH (i.e., 7.65, 7.54, and 7.43), being likely explained by an enhanced swelling of the egg (Dorey et al., 2013a) with increasing pCO_2. Indeed, during the last third of the development period, the egg swells due to a massive water intake in the PVF (Lacoue-Labarthe et al., 2009). In addition, because of gas exchanges properties between the seawater and the PVF through the eggshell, cuttlefish embryos are naturally exposed in their PVF to low pO_2 <6 kPa, and high pCO_2 >2000 µatm (Gutowska and Melzner, 2008). Subsequently, the pCO_2 in extracellular fluids of marine animals such as cephalopods are about 2000 µatm above those of the ambient seawater (Johansen et al., 1982; Portner et al., 1991), meaning that embryos deal with extreme extracellular acidosis. When exposed to ambient pH (8.08, pCO_2 ~ 400 µatm), the egg's PVF is about 7.4 (pCO_2 ~ 2000 µatm) while at low pH (7.43, pCO_2 ~ 2100 µatm) the PVF pH decreases to about 7.0 (pCO_2 ~ 4200 µatm), as a mechanism to maintain the CO_2 gradient between the PVF and seawater, and optimise exchanges with external media (Dorey et al., 2013a; Sigwart et al., 2016). Under hypercarbic conditions, enhanced egg swelling appeared as a biological response allowing an increased egg surface area, a reduced thickness contributing to facilitate gas exchanges (Cronin and Seymour, 2000) and limiting hypercapnia aggravation in the PVF.

Thus, both extended egg development duration and increased egg swelling are in line with previous findings of the ocean acidification

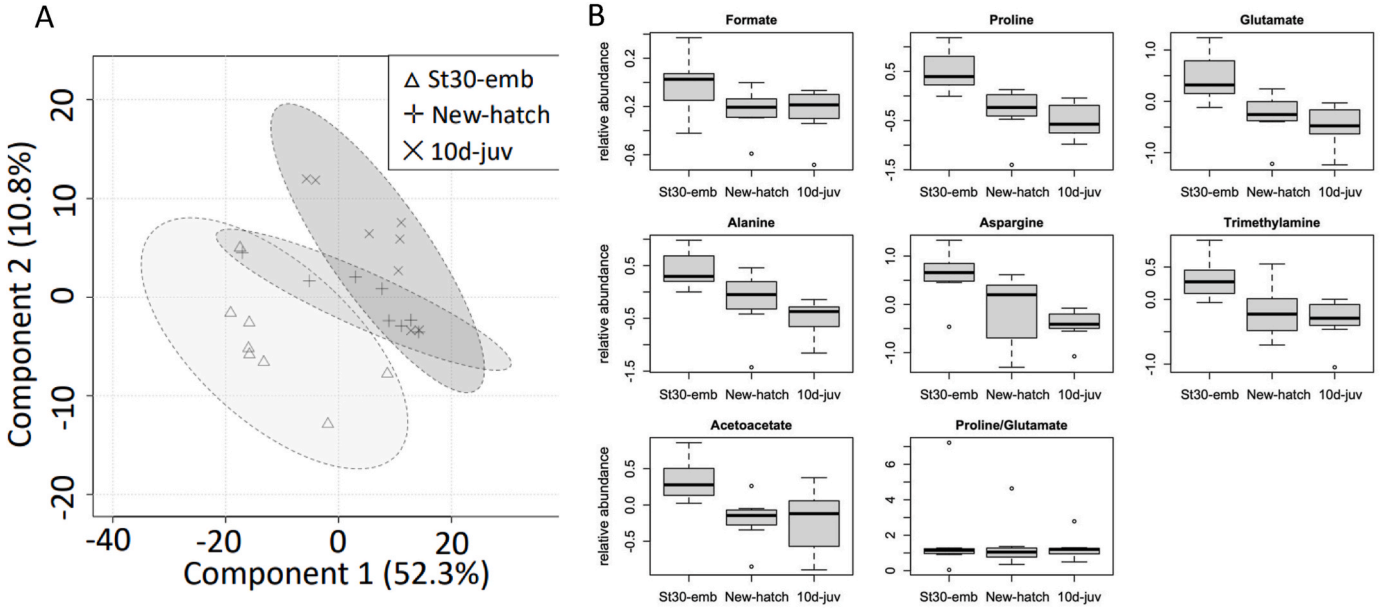

Fig. 5. A) Scores plot from Partial Least-Squares Discriminant Analysis (PLS-DA) of metabolites extracted in stage 30-embryo (St30-emb) n = 8, newly hatched juvenile (New-hatch) n = 8 and 10-d old juvenile (10d-juv) n = 8 raised under ambient pH condition and B) relative abundances of the eight metabolites that differed significantly between the three cuttlefish age groups, based on ANOVA with FDR *p*value = 0.05. The graphical black middle bars represent median and the error bars represent interquartile range.

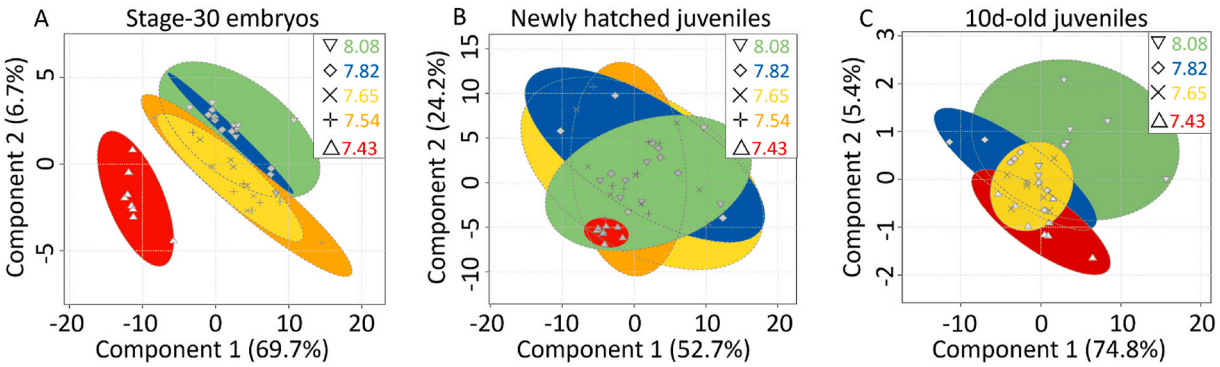

Fig. 6. Partial Least-Squares Discriminant Analysis (PLS-DA) of metabolite profiles common cuttlefish *Sepia officinalis* between pH treatment A) end of the embryonic development period cuttlefish B) immediately post-hatching cuttlefish C) 10d-old cuttlefish.

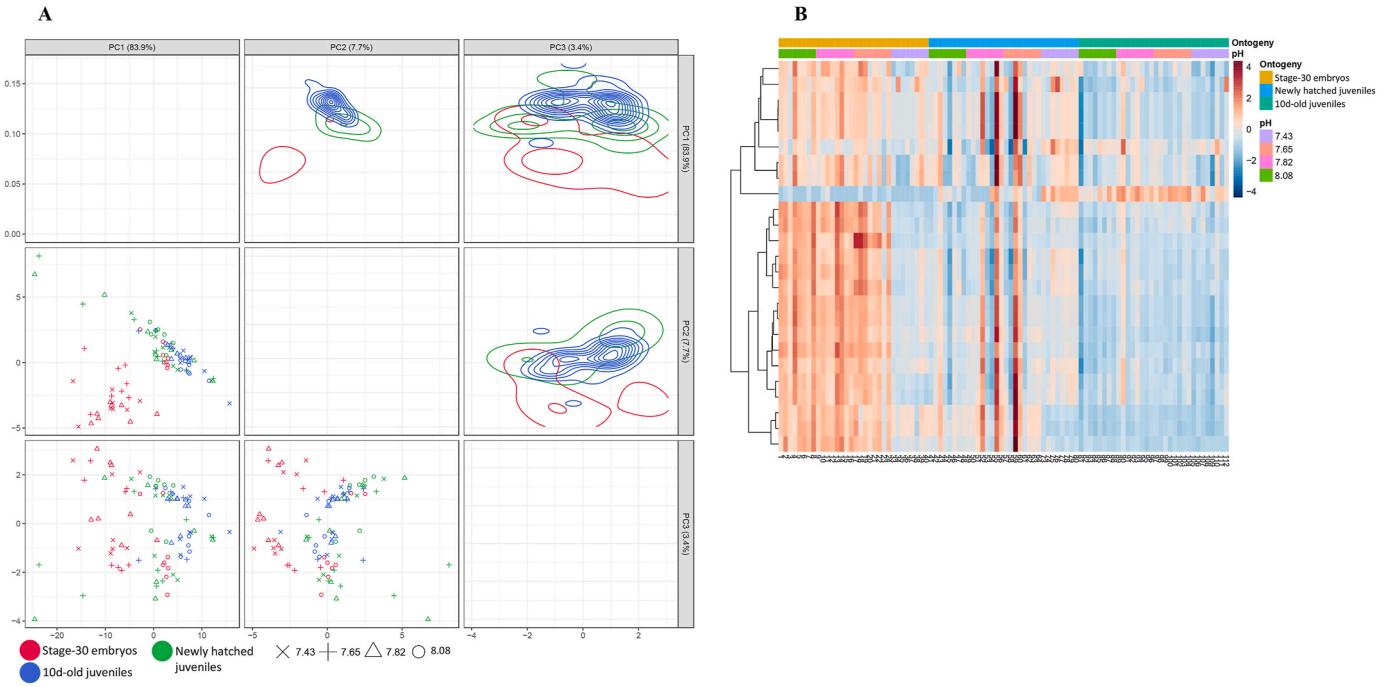

Fig. 7. A) interactive PCA representation and B) heatmap produced from ANOVA-simultaneous component analysis on metabolites extracted in individuals sampled at the three development stages and the pHs 8.08, 7.82, 7.65 and 7.43.

effects on cuttlefish embryonic development (see above). These results contribute to validate the experimental conditions of exposure to lowered pH, strengthening the significance of the metabolomic responses discussed below.

4.2. *Metabolic profiles according to ontogenic stages and pH conditions*

When comparing the metabolome of the three studied developmental stages, changing levels of several metabolites are consistent with the transition from embryos in harsh hypoxic and hypercarbic perivitelline conditions to juveniles breathing seawater. For example, the high relative abundances of asparagine and glutamate in late stage embryo are consistent with a metabolism under low oxygen and high CO_2, since these are well known as metabolic fuels for anaerobic pathways (Storey, 2005). Similarly, the high relative abundance of alanine (fermentation products of glucose and aspartate) is consistent with metabolism under hypoxic conditions. Specifically, elevated alanine is a key survival response associated with the buffering of H^+ ions, the regulation of intracellular osmotic pressure, and serves as a substrate for alanopine production during hypoxic stress (Fujimori and Abe, 2002; Garrett and Grisham, 2010; Müller et al., 2012). Venter et al. (2018) suggested that increased proline in the tissues of South African abalone *Haliotis midae* exposed to environmental hypoxia could be the result of reduced proline catabolism due to redox imbalance. The overall higher relative abundances of these and other metabolites in embryos compared to juveniles are therefore largely consistent with the fact that hypoxic and hypercarbic conditions lead to an embryonic metabolism dominated by anaerobic pathways, which represents an adaptive mechanism to ensure energy conservation and growth during development.

Our metabolomic analysis revealed that stage 30 embryos appear to be rather resilient to decreasing pH, with relatively stable metabolite profiles except at the lowest pH of 7.43. Among the metabolites significantly impacted by the pH conditions, increased relative abundances of ketogenic amino acids (i.e. leucine, isoleucine, phenylalanine) with decreasing pH until 7.54 may be consistent with a reduced conversion of these compounds in the citric acid cycle, as a clue for a switch from aerobic to anaerobic metabolism (Venter et al., 2018). Indeed, oxygen affinity of cuttlefish haemocyanins decreases with decreasing pH (Bridges, 1995), thus reducing their metabolic scope. Concomitantly,

lactate, a known product of anaerobic metabolism, also increased before reaching its lowest value at pH 7.43. Indeed, under these conditions, the strong decrease in the relative abundance of all the metabolites but dihydrouracil suggests an overall metabolic depression in embryos at this extreme value (Rosa et al., 2013). As mentioned before, the partial pressure gradient conservation between the PVF and seawater implies that seawater pH of 7.54 and 7.43 (i.e., $pCO_2 = 2097$ µatm) would lead to PVF pH values of ~7.1 and 7.0 (Dorey et al., 2013a; Sigwart et al., 2016). These values are consistent with pH responses of various morphological and physiological traits in the pacific oyster *Crassostrea gigas* which showed an identified tipping point at pH 7.3-6.9, coinciding with a major reshuffling in membrane lipids and transcriptome (Lutier et al., 2021). Similarly, sea urchin larvae appeared plastic to decreasing seawater pH but showed a physiological tipping point around pH 7.0 (Dorey et al., 2013b). Our results suggest therefore that a reduction in broad metabolic processes in cuttlefish also occurs around pH 7.0, but encapsulation allows the embryo to experience this extreme condition in coastal areas where seawater pH can be as low as 7.50 (Petton et al., 2024).

Embryonic development is highly dependent on metabolic performance. However, it is noteworthy that the lower abundance of metabolites in embryos reared at extreme low pH values did not lead to decrease in body mass. Previous studies reported lighter cuttlefish embryos at very high pCO_2 (i.e. 3950 and 3650 µatm) but not at intermediate pCO_2 (i.e., 1580 and 1380 µatm), close to our extreme pCO_2 of 2200 µatm (Hu et al., 2011; Sigwart et al., 2016 respectively). Interestingly, lobster larvae displayed no significant changes in morphology and mineralization when exposed to high pCO_2 levels, despite intense metabolomic changes (Noisette et al., 2021). Thus, unlike other factors such as temperature, pH does not seem to strongly affect vitelline consumption and conversion to body mass, but the metabolic depression observed at pH 7.43 pH appears to have partly compromised hatching success.

Although pH 7.43 is very low and far from the IPCC predictions for 2100, it should be noted that pH varies over time (daily and seasonal) in the coastal nurseries where cuttlefish eggs develop, and that the effects observed may be dictated by the lowest value reached during these variations (Vargas et al., 2022).

It is noteworthy that the relative abundances of metabolites in hatchlings did not show significant differences among the pH treatments and only few apparent alterations to their metabolite profiles were observed. The pH treatment effect seems to be overshadowed by the extreme stress caused by hatching and the shift from a hypercarbic and hypoxic PVF to normoxic and normocarbic seawater (Dorey et al., 2013a; Sigwart et al., 2016). This stress was illustrated by considerable variability in the relative abundances of metabolites, whatever the pH treatment. Furthermore, the passage from PVF to seawater might require hatchlings to be more resilient to their environment through physiological plasticity as a mechanism to enhance survival at this stage.

Like embryos, 10-d-old juveniles displayed global metabolite profiles slightly varying with pH. Several of the significantly affected metabolites, including ATP, fumarate, and GMP are involved in energy production and showed higher relative abundances in lower pH conditions, suggesting increased energetic consumption to maintain their development. Furthermore, acidification increased the average ATP allocation to protein synthesis and ion transport in sea urchin, *Strongylocentrotus purpuratus,* for larval growth (Pan et al., 2015). However, decreased pH did not cause a consistent reduction in cuttlefish juvenile MO_{2Max}, as expected. Indeed, the interference of CO_2 with oxygen extraction is known to reduce aerobic performance, as cephalopods possess hemocyanin characterized by a strong Bohr effect (Bridges, 1995) whose oxygen affinity strongly depends on the blood pH. Thus, environmental acidosis caused by ocean acidification is expected to decrease the O_2 binding and transport capacities by the respiratory pigment, which should result in a decrease of MO_{2Max}. *De facto*, the Humbold squid *Dosidicus gigas* exposed to an acute decrease of pH applied rapidly (from 7.93 to 7.62) displayed a reduction of its MO_{2Max} (Rosa et al., 2013). However, a slower lowering of pH spread over several hours or days resulted in no modification of $MO_{2routine}$ or MO_{2Max} in the bigfin reef squid *Sepioteuthis lessoniana*, the pigmy squid *Idiosepius pygmaeus* (Spady et al., 2019), nor the longfin squid *Doryteuthis pealeii* and Humbold squid *Dosidicus gigas* (Birk et al., 2018). In addition, as observed in OA exposed stage CIV copepodites *(Calanus glacialis;* Thor et al., 2022), the increase in several amino acids (i.e., proline, glutamate, alanine, asparagine), as energy sources for the aerobic respiration at lower pH (Storey and Storey, 1983), together with the increase of lactate (or octopine), suggests a transition to anaerobic metabolism to compensate for the metabolic cost of acclimation to hypercapnia: ionic and acid-base regulation (Gutowska et al., 2010b; Hu et al., 2015). Cephalopods demonstrated physiological machinery that can nearly fully compensate environmental blood acidosis (Gutowska et al., 2010b), limiting alteration of the O_2 transport capacity of hemocyanin within environmental realistic ranges of seawater pH.

Interestingly, the higher relative abundances of the amino acids glutamate and acetylcholine suggest an alteration in neurological processes when juveniles were reared at lower pH. Published studies on fish and cephalopods suggest a dysregulation of the GABAergic system (Hamilton et al., 2014), consistent with altered behavior caused by ocean acidification (Spady et al., 2019). Further investigations are needed to explore the different neurological pathways that may be altered in the juvenile cuttlefish by increased seawater pCO_2 and consequences for behavior.

Finally, the ASCA showed that between both factors, ontogeny more drastically affected the metabolite profiles of early life stages of cuttlefish, likely due transition from encapsulated embryo to the seawater free juveniles. Encapsulation is considered as a protective barrier against predation of invertebrates (Pechenik, 1979), especially in *S. officinalis* where the egg capsule is thick and coloured by ink adapted to the long and direct development of the embryo (Boletzky, 1986). In turn, embryos experience adverse physico-chemical conditions (hypoxia et hypercapnia) that push them close to the edge of oxygen limitation (Pörtner, 2002; Rosa et al., 2013), as suggested by the metabolic suppression observed at low pH. Logically, the hatching event causes an evident metabolomic reprogramming when juveniles find ambient abiotic conditions.

5. Conclusion

Decreased pH conditions delayed embryonic development and increased egg surface area, as mechanisms to maximize gas exchange with seawater. Cuttlefish were apparently most sensitive to pH reductions and pCO_2 increases (i.e., exhibited significant change in the greatest number of metabolites) during embryonic development compared to the juvenile life-stages, with a metabolic depression observed at the lowest pH. Embryos developing in hypercarbic and hypoxic eggs already constitute harsh conditions, whereas just after hatching, juvenile cuttlefish displayed few significant alterations to their metabolite profiles due to greater variability relating to intense physiological/metabolic demands associated with hatching and the passage from a hypercarbic and hypoxic medium to seawater. Finally, 10 days after hatching, juveniles exposed to low pH exhibited higher metabolite relative abundance overall compared to control conditions which can be attributed to an effort to catch up with the embryonic development delay.

CRediT authorship contribution statement

Antoine Minet: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Steven Melvin:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Marc Metian:** Writing – review & editing, Validation, Supervision,

Methodology, Funding acquisition, Conceptualization. **Angus Taylor:** Writing – review & editing, Formal analysis. **François Oberhänsli:** Writing – review & editing, Supervision, Methodology. **Christel Lefrançois:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Peter Swarzenski:** Writing – review & editing, Validation, Supervision. **Paco Bustamante:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Conceptualization. **Thomas Lacoue-Labarthe:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2025.107013.

Data availability

Data will be made available on request.

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