Regional Studies in Marine Science 102 (2026) 105382

Contents lists available at ScienceDirect

Regional Studies in Marine Science

journal homepage: www.elsevier.com/locate/rsma

Controlled incubation supports baseline developmental and molecular comparability in cuttlefish (*Sepia officinalis*) embryos

Giulia Chemello [a], Chiara Fini [a], Matteo Zarantoniello [b], Erica Trotta [a], Francesca Perretta [c], Giorgia Gioacchini [a,*]

[a] Department of Life and Environmental Science, Polytechnic University of Marche, Via Brecce Bianche, Ancona 60131, Italy
[b] Department of Agricultural, Food and Environmental Science, Polytechnic University of Marche, Via Brecce Bianche, Ancona 60131, Italy
[c] Agency for Innovation in the agrifood and fisheries sectors, (AN), Via Thomas A. Edison 2, Osimo 60027, Italy

ARTICLE INFO

Keywords:
Embryo development
Cephalopods
Biological sentinels
Physiological patterns
Growth performance
Incubation system

ABSTRACT

Early life stages are increasingly used as biological sentinels in marine environmental research, yet the ecological valence of controlled systems is still poorly understood. Cephalopod embryos are commonly used to study developmental, physiological, and molecular responses to environmental stressors, but it remains unclear whether embryos incubated under controlled conditions exhibit developmental and molecular patterns comparable to those observed under field conditions. This study aimed to evaluate biometric parameters and gene expression patterns in common cuttlefish (*Sepia officinalis*) embryos incubated under natural environmental and controlled tank conditions to assess their baseline comparability within the investigated developmental window. Eggs from a single sampling area were collected and randomly divided into two groups: one was maintained in a tank-based incubation system, and the other was returned to the sampling area. Embryos from both groups were sampled 7 (T1) and 14 (T2) days after collection. Embryos' weight and mantle length were recorded at each sampling time. PCR analysis evaluated the expression of genes associated with neural and visual system development, stress response, and oxygen transport. Results showed a stage- and time-specific difference in embryo weight was detected, whereas the other endpoints showed no consistent tank-specific alterations, providing preliminary support for the evaluation of tank-based incubation systems across different experimental contexts and developmental stages, including beyond hatching. These findings should nevertheless be interpreted cautiously and not as evidence of formal biological equivalence between controlled and field conditions.

1. Introduction

The Northern Adriatic Sea is a highly dynamic coastal system where multiple anthropogenic pressures, combined with climate-related drivers and riverine inputs, generate substantial environmental variability (Solidoro et al., 2009; Giani et al., 2012; Cossarini et al., 2021). Long-term observations indicate that this region is experiencing progressive sea warming, deoxygenation, and changes in carbonate equilibrium, with increasing frequency of extreme and short-term fluctuation events, particularly in shallow coastal areas (Grilli et al., 2013; Kapsenberg et al., 2017; Ricci et al., 2024). Such dynamics make the Northern Adriatic a natural laboratory for studying the responses of marine organisms to environmental stressors.

Embryonic and juvenile life stages are widely recognized as critical windows of vulnerability, as environmental conditions strongly shape physiological and developmental patterns and may have carry-over effects on subsequent life stages (Pechenik, 2006; Leung and McAfee, 2020; Marshall and Connallon, 2022). For this reason, early life stages are increasingly employed as biological sentinels in marine environmental research, where subtle physiological and molecular responses can provide early warning signals of environmental change.

Among different key species inhabiting the shallow continental shelf of the Northern Adriatic, cephalopods are a class of mollusks characterized by high responsiveness to external drivers, particularly during their embryonic and juvenile stages (de la Chesnais et al., 2019).

In cephalopods, embryogenesis takes place within eggs that are attached to benthic substrates or artificial structures for prolonged periods during which they are exposed to local environmental fluctuations. As a consequence, embryos can be affected by both short-term variability and chronic changes in those key abiotic factors that are typically

* Corresponding author.
E-mail address: giorgia.gioacchini@univpm.it (G. Gioacchini).

https://doi.org/10.1016/j.rsma.2026.105382
Received 23 February 2026; Received in revised form 10 August 2026; Accepted 21 August 2026
Available online 29 August 2026
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subjected to rapid shifts in many coastal ecosystems (Gutowska et al., 2010; Rosa et al., 2012; Bloor et al., 2013; Peng et al., 2017; Márquez et al., 2021; Jiang et al., 2022a, 2023; Court et al., 2024; Bouchaud, 1991; O'Brien et al., 2017; Choi et al., 2024). A growing body of literature has demonstrated that cephalopod embryos respond to a wide range of potential stressors, including fluctuations in marine abiotic parameters that may induce hypoxia and acidification, as well as exposure to contaminants. These stressors often result in sub-lethal effects that may not be apparent during later stages (Bouchaud, 1991; O'Brien et al., 2017; Jiang et al., 2023; Choi et al., 2024; García-Flores et al., 2025; Martins et al., 2026). One of the cephalopod species with a high ecological and economic impact within the Adriatic region is the common cuttlefish (*Sepia officinalis*), which is increasingly regarded as an effective sentinel for environmental research.

In particular, several studies have shown that cuttlefish eggs may be exposed to a range of anthropogenic contaminants, highlighting their relevance as integrative biological matrices for ecotoxicological assessments in coastal environments (García-Flores et al., 2025; Martins et al., 2026; Lacoue-Labarthe et al., 2016; Chemello et al., 2023; Cocci et al., 2023; Gouveneaux et al., 2023). Considering their early-life exposure to contaminants and their potential for high bioaccumulation, this species has attracted considerable attention. This interest is further driven by its commercial importance in the Adriatic region (FAO, 2025) and the high level of human consumption, which may represent a potential pathway for the transfer of contaminants to humans. Consequently, new information regarding this species can provide valuable insights not only from a scientific perspective but also concerning its economic significance.

Different studies evaluating the effects of environmental stressors adopted controlled incubation systems to investigate cephalopod embryogenesis under defined, reproducible conditions. Thanks to advances in egg collection, handling, and incubation protocols, laboratory approaches allow precise manipulation of abiotic variables and are commonly used to study physiological responses, stress tolerance, and developmental plasticity in cephalopod early life stages (Jiang et al., 2022a; Domingues et al., 2004; Sykes et al., 2012; Huang et al., 2024). However, since husbandry conditions can themselves impact developmental pathways, their influence should be considered when interpreting environmentally oriented results. Controlled incubation approaches have been tested mainly in the context of commercial farming, evaluating egg lipid composition, hatching rate, or gonadal development (Sykes et al., 2009; Peng et al., 2019; Zeng et al., 2024). However, it remains unclear whether embryos incubated under such conditions follow developmental and physiological patterns representative of those in the natural environment. Artificial incubation may introduce confounding stress responses or unknown biological signals that differ from those generated under natural environmental variability (García-Flores et al., 2025; Villanueva et al., 2026).

This represents a critical methodological gap, particularly for studies that aim to use molecular and physiological responses of embryos as indicators of environmental quality. Without demonstrating that controlled incubation preserves biologically meaningful developmental processes, there is a risk that experimental outcomes may reflect incubation artifacts rather than environmentally driven responses.

Considering this context, the present study utilized *Sepia officinalis* embryos maintained under natural conditions and in a tank system to assess whether stable controlled conditions are associated with developmental and physiological patterns comparable to those observed in the field. Focusing on molecular markers associated with neural and visual system development, stress response, and oxygen transport, this study provided an integrative evaluation of embryonic responses under controlled and natural conditions. The results provide baseline information on developmental and molecular responses under controlled and natural conditions within the investigated developmental window, to support further evaluation of controlled incubation systems in studies using cuttlefish embryos as sentinels.

2. Materials & methods

2.1. Experimental design and sampling

Sepia officinalis eggs were collected in June 2023, during the spawning season, in collaboration with local small-scale fishermen of Numana (AN), Italy.

Eggs were collected by fixing ropes (6 ropes of ~60 cm each) inside traditional fishing gears (3 fishing gears, 2 ropes per gear), commonly used for cuttlefish capture, located at a 7–10 m depth close to the coast (43°34.5678'N, 13°35.0548'E). Ropes served as substrates for egg deposition by cuttlefish females once trapped inside the gear. Fishing gears were monitored during routine fishing operations for a week, the time required to obtain a sufficient number of eggs (>500) to perform 3 replicate trials. Since egg deposition did not occur synchronously across the 6 fishing gears, 3 replicate trials were initiated on 3 different days. For each trial, 2 egg-covered ropes from 2 randomly selected fishing gears were cut in half (each half carrying approximately 100 eggs). One half of each rope was randomly assigned to a tank system (group = tank) at the fish facility of the Polytechnic University of Marche (Italy), while the other half remained in the fishing gear (group = wild) at the egg collection site. Eggs were kept attached to the rope to avoid mechanical damage until embryos were sampled.

The experimental system consisted of three 100 L conical tanks (one tank per replicate trial) operating as independent closed-recirculating systems with a constant water flow. Water conditions were maintained under average natural environmental conditions (23 °C, 31 psu, pH 8.0, and a 15 L:9D photoperiod), corresponding to the mean values recorded at the sampling site during June 2023. Sea surface temperature, salinity, and pH data were retrieved from the Copernicus Marine Service Marine Data Store using the SST_MED_SST_L4_REP_OBSERVATIONS_010_021 (Mediterranean Sea High Resolution L4 Sea Surface Temperature Reprocessed), MEDSEA_MULTIYEAR_PHY_006_013 (Mediterranean Sea Multi-Year Physical Reanalysis), and MEDSEA_MULTIYEAR_BGC_006_008 (Mediterranean Sea Multi-Year Biogeochemical Reanalysis) datasets.

Each recirculating system was equipped with mechanical, biological, and UV filtration, ensuring complete water turnover every 3 h. Dissolved oxygen concentration was monitored twice weekly using a ProODO dissolved oxygen meter (YSI Inc., Yellow Springs, OH, USA) and was consistently maintained above 5 mg L^{-1} . Water quality was further monitored twice weekly by measuring total ammonia (NH_3/NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) concentrations using JBL ProAquaTest NH_4, NO_2, and NO_3 kits (JBL GmbH & Co. KG, Neuhofen, Germany). Total ammonia and nitrite remained undetectable throughout the experiment, while nitrate concentrations were consistently maintained below 20 mg L^{-1} . Concerning the 3 fishing gears, their surfaces were closed to prevent predation, while allowing continuous exposure to natural environmental conditions, and were maintained in situ for the whole duration of the experiment.

Embryos from both wild and tank groups were sampled after 7 (T1) and 14 days (T2) from the beginning of each trial.

At each sampling time, 60 embryos were collected from each group (30 from each of the 2 half-ropes), sampled on ice to anesthetize the animals, singularly weighted, and photographed at 10x magnification using a camera (Invenio 10SCIII, DeltaPix, Denmark) installed on a stereomicroscope (Leica, Wetzlar, Germany) to measure mantle length. Each embryo was staged following the criteria described by Boletzky et al (Boletzky et al., 2016). based on morphological traits such as eye color and position, arm structure, distribution of chromatophores, and mantle length. Only embryos from stages 27–30 were selected and stored at −80°C to perform molecular analysis.

Table 1
List of primers and their efficiency used for Real-Time PCR.

Gene	Forward Primer (5'-3')	Efficiency (%)	Reference
HOX1	ACGAGACGCACACTGATCTTT TTGCCATGTCCACCGTTT	73.2	(Cocci et al., 2023)
ARX	CGTCCACTGGCAACGGAGAATATCA CCTTCTCCAGTTCCTCCAACT	81.9	(Cocci et al., 2023)
VSX	CCGGACACAGTACTCCGTTT CTTTGTCATCAACGCCCTCT	80.5	(Cocci et al., 2023)
r-opsin1	GAGTCCCTATGCTGTCGTGG ACGGAGTAGATGAGTGGATTGTG	91.4	(Bonadè et al., 2020)
reti1	CAGTCACTTGGCGGGTCATA AGTGCGGGCAGTAGCAATAA	92.7	(Bonadè et al., 2020)
reti2	TAGGTTGCTGTGTCTATGTCAGT CAGCGATACAGGCCAAAAGA	100.8	(Bonadè et al., 2020)
Cu/Zn SOD	GAGACTTTCGTTAGGACGGATA AGCCATTCCCCTTATTTCAC	91.2	(Jiang et al., 2022b)
HSP70	GAGTTCAAGCGAAAGCACA AGCGACCTACAAGGACAAT	100.2	(Jiang et al., 2022b)
SofHc1	CTTTTCGAGTTTACCAGCTCTTGTT CCTGCAACGTCAATATATGAGTGAT	92.4	(Strobel et al., 2012)
SofHc2	TCTCCCGTTTTTGGTAACTGAAC TGTCAGCAACATCAATATAACCATGA	100	(Strobel et al., 2012)
SofHc3	TGCTCCGTCAACCAATGTCC TCCAGGGCATCTCGTTTTCAC	101.9	(Strobel et al., 2012)
TUBB1	GGTGCTTGTCAAGGTCCCTA GTCAACAGCAACCAGTTTCCT	93.0	(Maradonna et al., 2024)
EF1α	TGCCAGGTGACAATGTTGGT CAATGTGTGCAGTGTGGCAA	98.7	(Bonadè et al., 2020)

2.2. Molecular analysis

2.2.1. Total RNA extraction and cDNA synthesis

Total RNA was extracted from 4 pools of 8 embryos per tank/gear (each pool consisted of 2 embryos at 27, 28, 29, and 30 stage/tank) for a total of 12 pools per group (4 pools x 3tanks/gears). RNA extraction was performed using TRIzol TM reagent (Invitrogen, Waltham, MA, USA). The extracted RNA was resuspended in 30 μL of RNase-free water (Qiagen). Final RNA concentration was determined using a NanoPhotometer P Class spectrophotometer (Implen, München, Germany), and RNA integrity was verified by GelRedTM staining by highlighting 28S and 18S ribosomal RNA bands on 1% agarose gel. After RNA extraction, samples were treated with the DNase I kit (Sigma-Aldrich, Milan) to digest and remove double- and single-stranded DNA from RNA-extracted samples. RNA was stored at −80 °C until use. cDNA synthesis was performed using the iScript TM cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) using 1 μg of total RNA.

2.2.2. Real-time PCR

Real-time PCR (RT-qPCR) analyses were performed using SYBR Green chemistry on an iQ5 iCycler Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Each reaction (10 μL final volume) contained 1 μL of cDNA (1:10 dilution), 5 μL of 2 × iQ™ SYBR Green Supermix (Bio-Rad), 0.3 μM of each forward and reverse primer, and nuclease-free water. Thermal cycling consisted of an initial denaturation at 95 °C for 3 min, followed by 45 cycles of 95 °C for 20 s, primer-specific annealing temperature for 20 s, and 72 °C for 20 s. Fluorescence was measured at the end of each amplification cycle. Amplification specificity was verified by melting curve analysis, which yielded a single peak for each primer pair. No amplification was detected in no-template controls, and no primer-dimer formation was observed.

Relative transcript abundance was determined for homeobox genes (*HOX1*, *ARX* and *VSX*), visual system genes (*r-opsin1*, *reti1* and *reti2*), oxidative stress-related genes (*Cu/ZnSOD* and *HSP70*), and oxygen transport genes (*SofHc1*, *SofHc2* and *SofHc3*). Primer sequences, amplification efficiencies, and the corresponding references for the primer sequences are reported in Table 1.

To identify the most appropriate reference genes for the experimental conditions investigated, three candidate housekeeping genes (*18S* rRNA, β-tubulin (*TUBB1*) and elongation factor 1 alpha (*EF1α*)) were experimentally evaluated. Gene expression stability was assessed using geNorm, NormFinder, BestKeeper, and the comparative ΔCt method. Based on the overall stability ranking, *EF1α* and *TUBB1* were identified as the two most stable reference genes and were therefore selected for normalization. Relative transcript abundance of all target genes was normalized using the geometric mean of the amplification efficiency-corrected relative quantities of *EF1α* and *TUBB1*, following the normalization strategy proposed by Vandesompele (Vandesompele et al., 2002).

Relative quantification was performed using the amplification efficiency-corrected mathematical model implemented in the GeneEx Macro iQ5 workflow, according to the method described by Pfaffl (2001) (Pfaffl, 2001). Quantification data were processed using iQ5 Optical System Software version 2.0 (Bio-Rad), followed by GeneEx Macro iQ5 Conversion and GeneEx Macro iQ5. All samples were analyzed in technical duplicate.

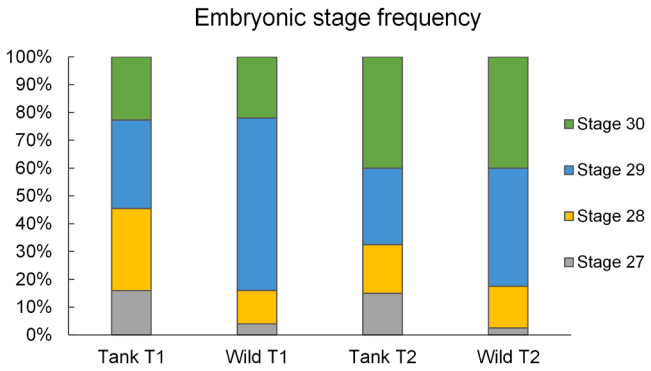


Fig. 1. *S. officinalis* embryonic frequency (%) at T1 and T2 from tank and wild conditions (n = 60).

2.3. Statistical analysis

Statistical analyses were performed with R software (version 4.6.1; R Foundation for Statistical Computing, Vienna, Austria). Data were analyzed using a linear mixed-effects model (LMM) fitted with the lmerTest package. The model included Group, Time, and their interaction as fixed effects, while Unit_ID (corresponding to the individual tanks for the tank group and the individual gears for the wild group, representing the biological replicates) was included as a random intercept to account for the non-independence of biological replicates. Type III analysis of variance with Satterthwaite's approximation was used to assess the significance of fixed effects. When a significant interaction was detected, post hoc pairwise comparisons were performed on the estimated marginal means (EMMs) using the emmeans package, with Holm's adjustment applied to correct for multiple comparisons. Comparisons were conducted between groups within each time point and between time points within each group. Gene expression analyses were considered exploratory, and no global multiple-comparison correction was applied across target genes. Results were considered statistically significant at $p < 0.05$. EMMs for all linear mixed-effects models are provided in the Supplementary Material (Tables S1–S17).

3. Results

3.1. Embryonic development and biometric parameters

The frequency of embryonic developmental stages was assessed at each sampling time (T1 and T2) for both experimental conditions (wild and tank). All stages observed were present at each sampling time for each condition (Fig. 1).

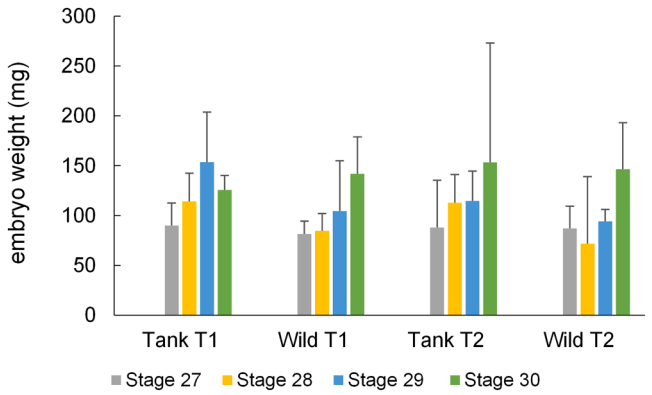


Fig. 2. Average embryonic weight (mg) of *S. officinalis* at stages 27, 28, 29, and 30 across sampling times (T1 and T2) from tank and wild conditions (n = 60).

Table 2
LMM results for embryo weight at stage 28, 29, and 30. The effects of Group, Time, and Group*Time interaction are reported as degrees of freedom (df), F-values, and *p*-values.

Embryonic stage	Effect	df	F	*p*
28	Group	1, 4.80	3.020	0.145
	Time	1, 24.33	0.7699	0.389
	Group * Time	1, 24.33	0.7090	0.408
29	Group	1, 4.39	13.51	**0.018**
	Time	1, 64.99	7.925	**0.006**
	Group * Time	1, 64.99	4.191	**0.045**
30	Group	1, 5.11	0.1332	0.730
	Time	1, 38.70	0.08435	0.773
	Group * Time	1, 38.70	0.8172	0.372

Embryonic weight increased progressively with developmental stage (Fig. 2). Mean total weight values showed limited variability between embryos under tank and wild conditions across sampling times (T1 and T2).

The LMM analysis showed no significant effects of Group (tank and wild), Time (T1 and T2), or the Group*Time interaction on embryo weight at stages 28 and 30 (Table 2) (Fig. 3A and C).

In contrast, the LMM analysis revealed a significant effect of Group ($p = 0.018$), Time ($p = 0.006$), and Group*Time interaction ($p = 0.045$) concerning weight values of embryos at stage 29 (Table 2). The post hoc pairwise comparisons of the estimated marginal means showed that at T1, embryo weight was significantly higher in the Tank group than in the Wild group ($p = 0.002$) (Table 3) (Fig. 3B), whereas no significant difference between groups was detected at T2. Within the Tank group, embryo weight differed significantly between T1 and T2 (p = 0.003), while no significant temporal difference was observed in the Wild group.

Similarly, the LMM analysis showed no significant effects of Group (tank and wild), Time (T1 and T2), or the Group*Time interaction on embryo length at all embryonic stages evaluated (Table 4). At all stages, embryo length did not differ significantly between sampling times or incubation conditions (Fig. 4A, B, and C).

3.2. Gene expression analyses

3.2.1. Homeobox and visual system-related genes

The LMM analysis showed no statistically significant effects of Group, Time, or the Group*Time interaction on the expression of the

Table 3
Post hoc pairwise comparisons of estimated marginal means (EMMs) for embryo body weight obtained from LMM. *P*-values were adjusted using Holm's method. Estimate, estimated difference between the estimated marginal means; SE, standard error; df, degrees of freedom; t-ratio, t statistic.

Group	*Contrast*	*Estimate*	*SE*	*df*	*t-ratio*	*p-value*
Tank	T1 – T2	39.01	12.80	64.9	3.038	**0.003**
Wild	T1 – T2	6.16	9.62	65.2	0.640	0.524
Time						
T1	Tank – Wild	52.7	11.8	8.76	4.481	**0.002**
T2	Tank – Wild	19.8	13.6	14.97	1.458	0.166

Table 4
LMM results for embryo length at stage 28, 29, and 30. The effects of Group, Time, and Group*Time interaction are reported as degrees of freedom (df), F-values, and *p*-values.

Embryonic stage	Effect	df	F	*p*
28	Group	1, 5.73	3.174	0.127
	Time	1, 25.05	0.8637	0.362
	Group * Time	1, 25.05	0.06557	0.800
29	Group	1, 5.39	2.969	0.141
	Time	1, 65.59	2.361	0.129
	Group * Time	1, 65.59	0.3450	0.559
30	Group	1, 4.03	0.05038	0.833
	Time	1, 40.03	0.3405	0.563
	Group * Time	1, 40.03	1.607	0.212

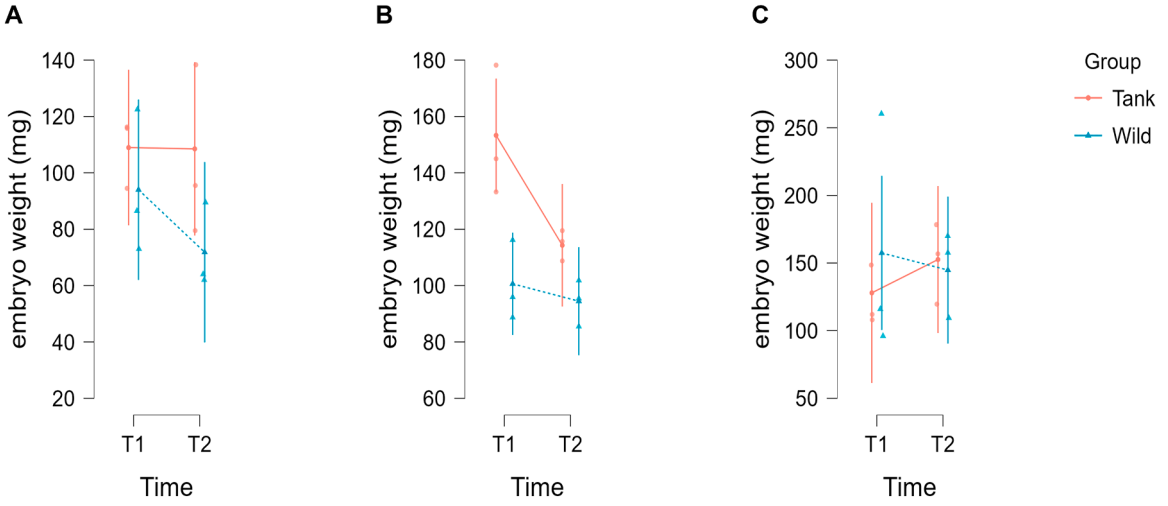


Fig. 3. Embryo weight of *Sepia officinalis* at developmental stages 28 (A), 29 (B), and 30 (C) under tank and wild incubation conditions at T1 and T2. Symbols represent individual observations, while connected points and error bars indicate estimated marginal means and model-based 95% confidence intervals, respectively.

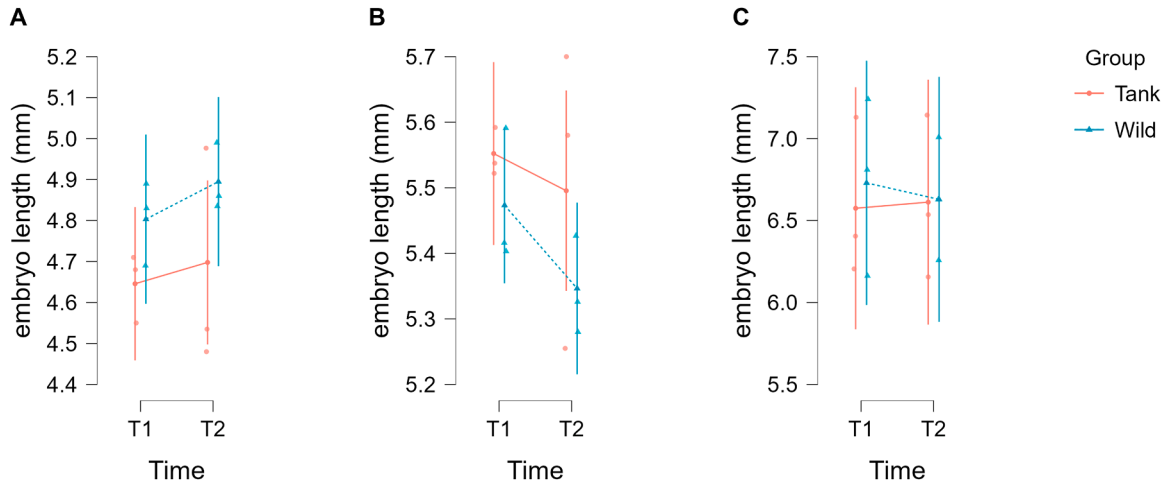


Fig. 4. Embryo length of *Sepia officinalis* at developmental stages 28 (A), 29 (B), and 30 (C) under tank and wild incubation conditions at T1 and T2. Symbols represent individual observations, while connected points and error bars indicate estimated marginal means and model-based 95% confidence intervals, respectively.

Table 5
LMM results for HOX1, ARX, and VSX relative expression. The effects of Group, Time, and Group*Time interaction are reported as degrees of freedom (df), F-values, and *p*-values.

Gene	Effect	df	F	*p*
HOX1	Group	1, 4.05	1.741	0.257
	Time	1, 36.95	1.536	0.223
	Group * Time	1, 36.95	0.2066	0.652
ARX	Group	1, 4.17	0.08617	0.783
	Time	1, 37.10	1.352	0.252
	Group * Time	1, 37.10	0.08073	0.778
VSX	Group	1, 4.01	5.651×10^{-4}	0.982
	Time	1, 37.00	0.005872	0.939
	Group * Time	1, 37.00	0.3042	0.585

developmental genes *HOX1*, *ARX*, and *VSX* (Table 5; Fig. 5A, B, and C). Similarly, no statistically significant effects were detected for *r-opsin*, *reti1*, and *reti2* expression (Table 6), as indicated also by Fig. 6A, B, and C.

3.2.2. Stress-response and oxygen-transport genes

The expression of stress response-related genes (*HSP70* and *CuZn-SOD*) and oxygen-transport genes (*SofHc1*, *SofHc2*, and *SofHc3*) was not significantly influenced by Group, Time, or their interaction, as indicated by the LMM analysis (Table 7) (Table 8). Estimated marginal means of both stress response-related genes (Fig. 7A and B) and oxygen-transport genes (Fig. 8A, B, and C) did not differ significantly between incubation groups (Tank and Wild) or between sampling times (T1 and T2).

4. Discussion

Controlled incubation systems are increasingly employed in

Table 6
LMM results for r-opsin, reti1, and reti2 relative expression. The effects of Group, Time, and Group*Time interaction are reported as degrees of freedom (df), F-values, and *p*-values.

Gene	Effect	df	F	*p*
r-opsin	Group	1, 4.03	3.833×10^{-30}	1.000
	Time	1, 37.01	1.952×10^{-31}	1.000
	Group * Time	1, 37.01	3.097×10^{-31}	1.000
reti1	Group	1, 4.05	0.04160	0.848
	Time	1, 37.02	0.02920	0.865
	Group * Time	1, 37.02	0.08745	0.769
reti2	Group	1, 3.92	0.2977	0.615
	Time	1, 36.89	0.01610	0.900
	Group * Time	1, 36.89	0.2417	0.626

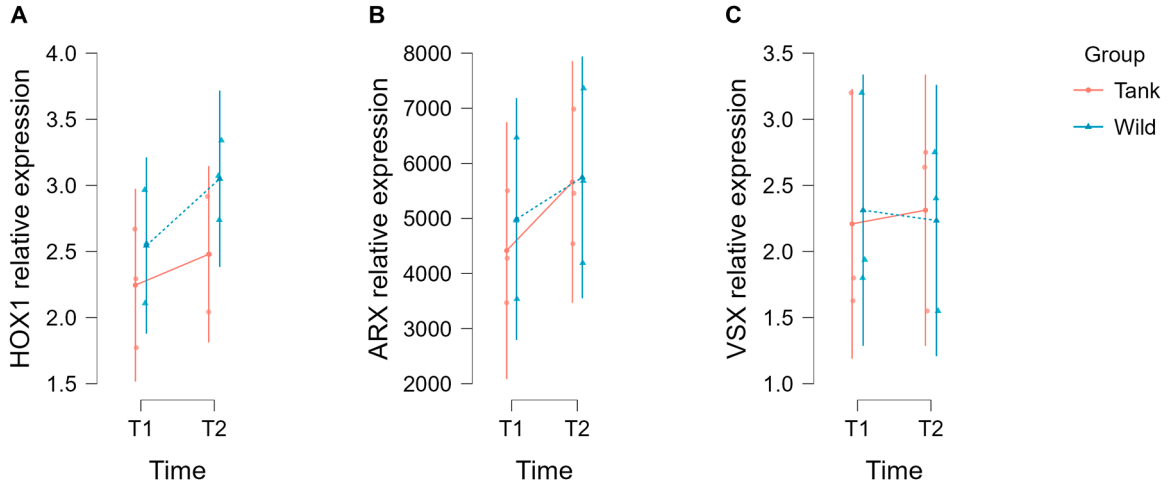


Fig. 5. Relative gene expression of *HOX1* (A), *ARX* (B), and *VSX* (C) under tank and wild incubation conditions at T1 and T2. Symbols represent individual observations, while connected points and error bars indicate estimated marginal means and model-based 95% confidence intervals, respectively.

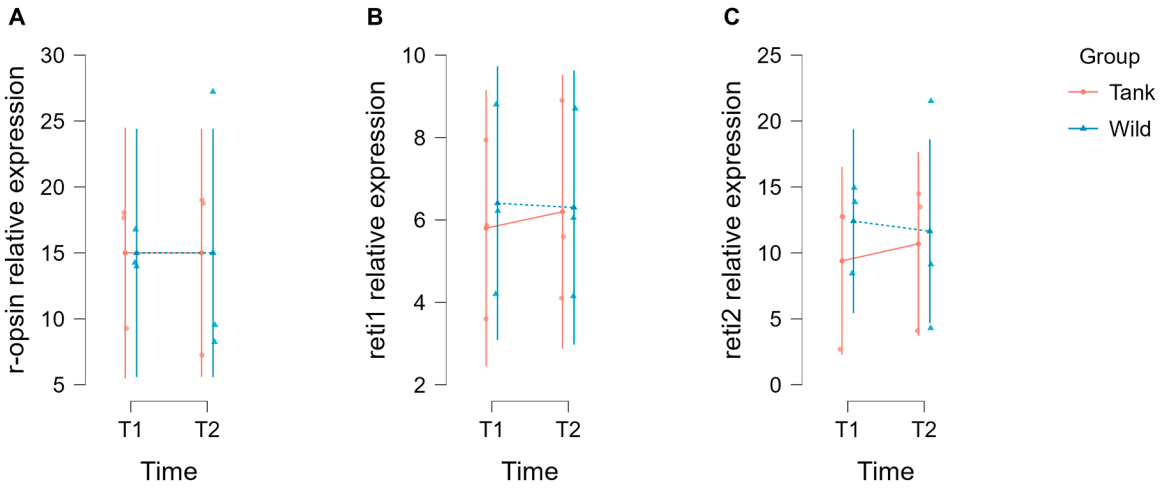


Fig. 6. Relative gene expression of *r-opsin* (A), *reti1* (B), and *reti2* (C) under tank and wild incubation conditions at T1 and T2. Symbols represent individual observations, while connected points and error bars indicate estimated marginal means and model-based 95% confidence intervals, respectively.

Table 7
LMM results for HSP70 and Cu/ZnSOD relative expression. The effects of Group, Time, and Group*Time interaction are reported as degrees of freedom (df), F-values, and *p*-values.

Gene	Effect	df	F	*p*
HSP70	Group	1, 4.31	0.4165	0.551
	Time	1, 37.23	1.819	0.186
	Group * Time	1, 37.23	0.1214	0.729
CuZnSOD	Group	1, 39.00	0.01576	0.901
	Time	1, 39.00	2.078	0.157
	Group * Time	1, 39.00	0.7462	0.393

Table 8
LMM results for SofHc1, SofHc2, and SofHc3 relative expression. The effects of Group, Time, and Group*Time interaction are reported as degrees of freedom (df), F-values, and *p*-values.

Gene	Effect	df	F	*p*
SofHc1	Group	1, 39	2.105	0.155
	Time	1, 39	5.296×10^{-4}	0.982
	Group * Time	1, 39	0.6111	0.439
SofHc2	Group	1, 4.38	1.907	0.234
	Time	1, 37.28	2.411	0.129
	Group * Time	1, 37.28	0.07733	0.782
SofHc3	Group	1, 3.83	0.08437	0.787
	Time	1, 36.76	3.803	0.059
	Group * Time	1, 36.76	0.5673	0.456

environmental physiology, ecotoxicology, and developmental studies involving cephalopods (García-Flores et al., 2025; Zeng et al., 2024). However, their capacity to preserve biologically meaningful developmental and molecular patterns under environmentally relevant conditions has rarely been examined. This study addressed a central methodological question in marine environmental research, specifically whether controlled incubation can be used to study early life stages without generating misleading effects that obscure biologically meaningful signals driven by environmental conditions.

The present study suggests that, within the developmental window and endpoints investigated, no major differences in developmental and molecular parameters were detected between embryos maintained under controlled and field conditions. However, these findings should not be interpreted as formal evidence of biological equivalence, particularly considering the limited number of independent experimental units, which may have reduced the sensitivity of the analyses to detect

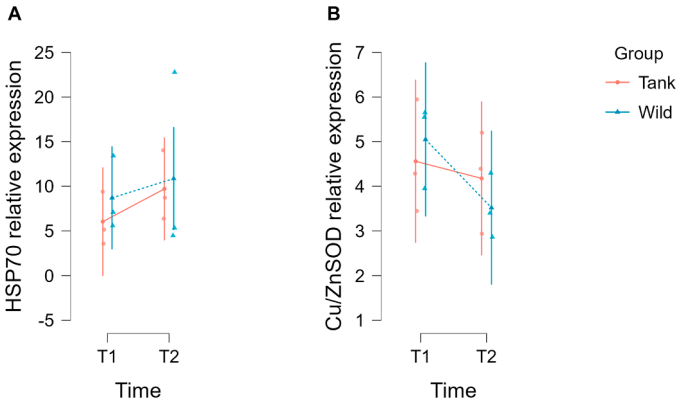


Fig. 7. Relative gene expression of *HSP70* (A) and *Cu/ZnSOD* (B) under tank and wild incubation conditions at T1 and T2. Symbols represent individual observations, while connected points and error bars indicate estimated marginal means and model-based 95% confidence intervals, respectively.

small or moderate condition-related effects. The absence of statistically significant effects on growth performance and on the expression of core developmental genes suggests that no detectable alterations in these parameters were observed under the tested conditions. Indeed, the difference in embryo weight detected at developmental stage 29 was no longer evident at stage 30. Moreover, these results are not reflected when considering core developmental pathways. Genes analyzed in the present study (*HOX1*, *ARX*, and *VSX*), which are associated with neural and visual system development, did not show consistent differences between embryos incubated under natural and controlled conditions. These pathways are among the most tightly regulated during cephalopod embryogenesis and support the development of complex neural networks and sensory systems that are critical for post-hatching performance (Jiang et al., 2022b; Strobel et al., 2012). In *Sepia officinalis*, neurogenesis and visual system differentiation occur predominantly during late embryogenesis and involve highly conserved molecular processes (Boletzky et al., 2016; Strobel et al., 2012; Maradonna et al., 2024; Lee et al., 2003).

The absence of statistically significant effects on these genes suggests that no detectable alterations in their expression were observed under the controlled incubation conditions tested. Importantly, the absence of major divergence in neural- and vision-related gene expression should not be interpreted as a lack of sensitivity of these pathways to environmental conditions. Rather, it suggests that the controlled incubation

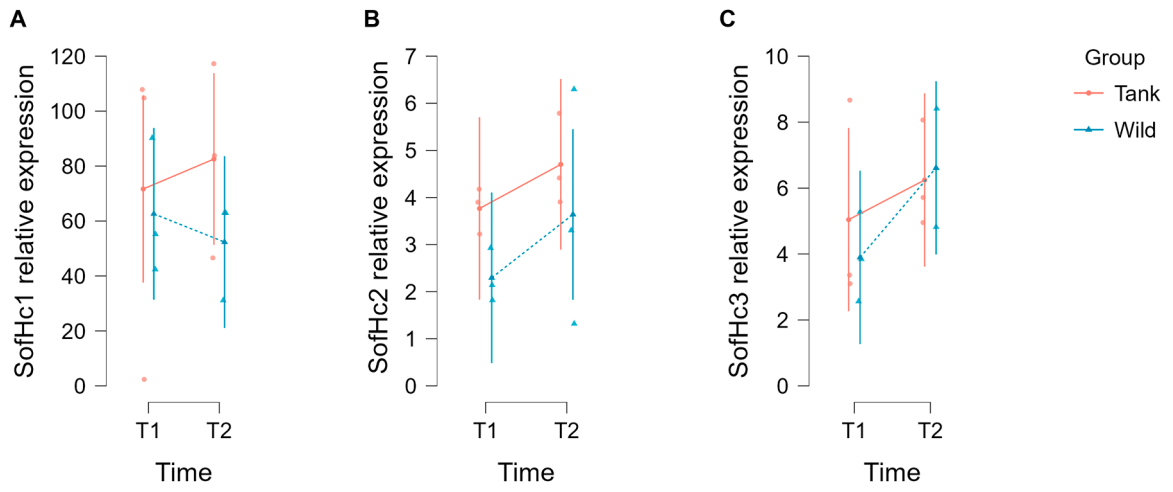


Fig. 8. Relative gene expression of *SofHc1* (A), *SofHc2* (B), and *SofHc3* (C) under tank and wild incubation conditions at T1 and T2. Symbols represent individual observations, while connected points and error bars indicate estimated marginal means and model-based 95% confidence intervals, respectively.

conditions did not induce detectable alterations in these pathways under the conditions tested.

Markers associated with stress response and oxygen transport exhibited limited and context-independent variation between natural and controlled incubation conditions.

In *S. officinalis* and other cephalopods, stress-related genes such as *HSPs* and genes encoding antioxidant enzymes (e.g., *Cu/ZnSOD*) are constitutively expressed during embryonic development, reflecting high metabolic activity, rapid cellular differentiation, and increasing energetic demands (Choi et al., 2024; García-Flores et al., 2025). However, the overproduction of heat shock proteins (HSPs) can be induced by temperature fluctuations or other environmental stressors (Focareta et al., 2014). In mollusks, several studies have demonstrated that HSP70 proteins respond to a wide range of stress factors, including variations in temperature, hypoxia, pH, salinity, and exposure to pollutants (Buresi et al., 2016).

Although no incubation influence was detected for stress-related markers, the available design and the absence of direct environmental logging do not exclude moderate environmentally driven modulation in the field group. Therefore, the observed profiles cannot be attributed exclusively to intrinsic developmental processes. However, it should be considered that early life stages often operate close to energetic and physiological limits, making the presence of stress-related molecular signals a normal feature of development rather than an unequivocal indicator of environmental impairment (Koenig et al., 2016).

The expression patterns observed for *HSP70* across sampling times in embryos maintained under tank conditions may similarly reflect intrinsic developmental processes, as no stress-inducing events were applied to this group. The absence of a clear modulation associated with either incubation conditions or sampling time further supports the interpretation that *HSP70* expression primarily reflects normal embryonic development rather than an environmentally induced stress response. Similarly, genes involved in oxygen transport and metabolism (*SofHc1*, *SofHc2*, and *SofHc3*) are dynamically regulated during late embryonic stages, when oxygen demand increases as embryos approach hatching. Oxygen-transport gene expression did not show consistent tank-specific modulation, suggesting that oxygen availability and metabolic demands in the controlled system remained broadly consistent with those observed in field-incubated embryos. Taken together, the present findings reinforce the value of cuttlefish embryos as sentinel organisms in marine environmental research. This study provides preliminary baseline support for the use of tank-based systems as promising platforms for investigating environmentally driven responses in early life stages. This outcome is particularly urgent given the increasing reliance on embryos and larvae to detect sub-lethal effects of climate-related stressors and anthropogenic pressures in coastal ecosystems (Bouchaud, 1991; Chemello et al., 2023).

5. Conclusion

In conclusion, the present study suggests that, within the experimental conditions and endpoints investigated, the analyses did not identify consistent or systematic tank-specific alterations in *Sepia officinalis* embryonic development, based on biometric parameters and molecular markers related to developmental processes, oxygen transport, and stress response. Nevertheless, the experimental design, together with the absence of post-hatching assessments and experimental stress exposure, reduced the precision of some estimates and may have limited the detection of small or moderate condition-related effects. Future studies including additional spawning seasons, broader environmental characterization, and the evaluation of post-hatching performance under both baseline and stress conditions would provide a more comprehensive understanding of the effects of incubation environment on development. Overall, these findings support the use of controlled incubation systems as a potential approach for tank-based experimental studies on cuttlefish embryos investigating biological responses to environmental variability, while acknowledging that the absence of statistically significant differences does not constitute formal evidence of equivalence.

CRediT authorship contribution statement

Giorgia Gioacchini: Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration. **Giulia Chemello:** Writing – original draft, Validation, Investigation, Formal analysis, Conceptualization. **Chiara Fini:** Investigation, Formal analysis. **Matteo Zarantoniello:** Writing – review & editing. **Erica Trotta:** Writing – review & editing. **Francesca Perretta:** Writing – review & editing, Project administration.

Funding

This research was funded by Regione Marche within the “ShARed GOvernance of Sustainable fisheries and aquaculture activities as leverage to protect marine resources in the Adriatic Sea”-ARGOS INTERREG ITALIA-CROAZIA 2014–2020 program, grant ID 10255153.

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.

Acknowledgment

The authors would like to thank the local fishermen of Numana, with regard to Giorgio Manfrini, for the assistance during sample collection and the monitoring of fishing gears.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.rsma.2026.105382.

Data availability

Data will be made available on request.

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