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# Shark critical life stage vulnerability to monthly temperature variations under climate change

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#### ABSTRACT

In a 10-month experimental study, we assessed the combined impact of warming and acidification on critical life stages of small-spotted catshark (*Scyliorhinus canicula*). Using recently developed frameworks, we disentangled individual and group responses to two climate scenarios projected for 2100 (SSP2-4.5: Middle of the road and SSP5-8.5: Fossil-fueled Development). Seasonal temperature fluctuations revealed the acute vulnerability of embryos to summer temperatures, with hatching success ranging from 82% for the control and SSP2-4.5 treatments to only 11% for the SSP5-8.5 treatment. The death of embryos was preceded by distinct individual growth trajectories between the treatments, and also revealed inter-individual variations within treatments. Embryos with the lowest hatching success had lower yolk consumption rates, and growth rates associated with a lower energy assimilation, and almost all of them failed to transition to internal gills. Within 6 months after hatching, no additional mortality was observed due to cooler temperatures.

#### 1. Introduction

Climate change is predicted to have unprecedented impacts at all levels of marine fish biological organisation (Pörtner and Peck, 2010). These impacts starts with the alteration of gene expression, such as protons pumps (Bunse et al., 2016) or heat shock proteins, and catalysts for lipid synthesis (Bernal et al., 2020). Additionally, whole-organism responses, including growth and fecundity are also affected (Neuheimer et al., 2011; Crozier and Hutchings, 2014; Butzin and Pörtner, 2016; Foo and Byrne, 2017; Huang et al., 2021), ultimately impacting population (Neer et al., 2007) and ecosystem (Morell et al., 2023) dynamics. While temperature has a direct thermodynamic effect on biochemical reaction rates (Alfonso et al., 2021; Little et al., 2020), ocean acidification can lead to acid-base disturbance affecting multiple physiological systems including mineralization process and neurological functions (Munday et al., 2009; Heuer and Grosell, 2014; Cattano et al.,

2018) with possible combining effects with temperature (Domenici et al., 2014; Laubenstein et al., 2018).

Since the 2010's, studies have revealed mixed effects of ocean warming and acidification on biochemical, physiological and behavioural aspects of oviparous elasmobranchs, ranging from no response to lethal ones (Wheeler et al., 2021; Santos et al., 2021). These studies revealed intra- and interspecific variations on survival, development, metabolism (Rosa et al., 2014; Musa et al., 2020), physiology (Di Santo, 2015, 2016, 2019; Rummer et al., 2022), and behaviours (Green and Jutfelt, 2014; Pistevos et al., 2015, 2017). The variability in response could potentially stem from differences in exposure times, which need to be standardised according to the pressures experienced throughout the different life stages. For example, adults of the small-spotted catshark (Scyliorhinus canicula) increased their lateralization when exposed to acidified water for 4 weeks (Green and Jutfelt 2014), while neonates of the blacktip reef shark (Carcharhinus melanopterus) did not exhibit

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similar changes within seven days (Bouyoucos et al., 2020). Rearing large species under different climate change scenarios can also be particularly difficult (Smith et al., 2017), generally involving reduced numbers of experimental subjects and an increased overall variance caused by the predominance of inter-individual variations (Araujo and Frøyland, 2005). Rather than a limitation, this constraint calls for new experimental approaches that combine experimental groups and individuals responses to climate change. By considering the inter-individual variation in recruits, we can better assess the future ecological success of species in the face of climate change (Forsman and Wennersten, 2016).

In the Northeast Atlantic, elasmobranch studies carried out so far focused on the impacts of temperature on the small-eyed skate (Raja microocellata; Hume, 2019) and the small-spotted catshark embryos (Brüggemann, 2013; Ripley et al., 2021) either alone or combined with deoxygenation (Musa et al., 2020). In these studies, average annual temperatures ranging from 12.5  $^{\circ}\text{C}$  to 22  $^{\circ}\text{C}$  were used to assess temperature impacts on embryonic development from 4 to 7 months. However, to our knowledge, climate change studies on elasmobranchs have yet to consider the monthly temperature variations. These variations, marked by extremes, can significantly impact teleost fish embryos (e.g. reduction of body size, Spinks et al., 2019; developmental anomalies, Murray and Klinger, 2022). This is due to small differences between their maximum and minimum critical temperatures (i.e., a narrow temperature range) and because their optimal temperature for development differs from the average habitat temperature (Dahlke et al., 2020; Slein et al., 2023). Temporal variations of environmental parameters represent a particularly acute threat to embryos of oviparous elasmobranchs with anchored eggs, directly facing potential unfavourable environmental conditions. In particular, the impact of temperature variations on the developmental sequence is a critical factor requiring assessment. Furthermore, studies of the effects of acidification on oviparous elasmobranchs are scarce (but see Claiborne and Evans, 1992; Green and Jutfelt, 2014, Pegado et al., 2020a) although pH could explain a significant part of the variation in these species distribution (Coulon et al., 2024). The acidification has generally been studied independently of temperature rise, despite evidence demonstrating exacerbation of effects of elevated CO2 with warming on the embryos of the little skate, Leucoraja erinacea (Di Santo, 2015) and the brownbanded bamboo shark, Chiloscyllium punctatum (Rosa et al., 2014). Moreover, elevated temperature and acidification had detrimental effects on sharks, increasing their energetic demands while decreasing metabolic efficiency and their ability to locate food through olfaction (Pistevos et al., 2015).

The small-spotted catshark is an abundant, widespread oviparous elasmobranch across a wide range of latitudes (OBIS, 2021). In the Northeast Atlantic, it spawns from shallow waters to depths greater than 200 m (Ellis et al., 2004). It is also a species with relatively short generation time compared to other oviparous elasmobranchs of the Northeast Atlantic (Coulon et al., 2023). Furthermore, *S. canicula* females produce eggs year round with a peak in early summer around the British Isles (Ellis and Shackley, 1997), anchoring their eggs to macroalgae and other solid structures (Wheeler, 1978). Adults of these species also present limited dispersal abilities and high site fidelity (Rodríguez-Cabello et al., 2004; Kousteni et al., 2015). The inability of embryo to disperse once fixed to a substrate and adult limited dispersal abilities make this species a good model for studying the effects of summer temperatures on embryos.

We analysed the effects of ocean warming (+2 and +4 °C), within monthly temperature variations, combined with water acidification ( $\Delta pH = -0.2$  and -0.4) over the first ten months of early life (from July to April) of *S. canicula*. Temperatures and pH values used in this study followed three predicted climate change scenarios ranging from actual conditions to extreme ones. Using a broad-scale approach at the individual and group levels, we evaluated (i) embryo growth pattern, freezing behaviour and associated metabolism (ii) hatching success and

(iii) juvenile growth. One of the originalities of our study is to combine two recently developed frameworks, originally designed for ecological assessment and species extinction risk, to highlight inter-individual differences across developmental sequences according to the climate scenarios tested.

#### 2. Materials and methods

#### 2.1. Experimental design

We chose the Shared Socioeconomic Pathways (SSP) scenarios 'SSP2: Middle of the road' where CO2 emissions stay around current levels until 2050, then falling but not reaching net-zero by 2100 (Fricko et al., 2017) and 'SSP5: Fossil-fueled Development (Taking the Highway)' where CO<sub>2</sub> emissions triple by 2075 (Kriegler et al., 2017). The two experimental treatments (SSP2-4.5 and SSP5-8.5) were compared to the control treatment corresponding to median water temperature and pH measured from 1995 to 2014 (AR6 1995-2014). The pH and temperature data were obtained from forecasts for Western and Central Europe and downloaded from http://interactive-atlas.ipcc.ch (Gutiérrez et al., 2021; Iturbide et al., 2021, Table 1). We subjected embryos to monthly median values for temperature, but we were unable to do so for monthly variation of pH, for which only annual averages were available (Gutiérrez et al., 2021; Iturbide et al., 2021, Table 1). Seawater tank supply was pumped from the Dinard coast (English Channel, France). Since the coastal context of the study implies more acidic water than the raw pH values predicted in the global ocean (Hönisch et al., 2012), we applied predicted annual pH differences between the scenarios and the control ( $\Delta$ pH <sub>AR6 1995-2014-SSP2-4.5</sub>: -0.2, and  $\Delta$ pH <sub>AR6 1995-2014-SSP5-8.5</sub>:

Eggs used in this study originated from 65 mated females of *S. canicula* fished in the Tregastel Bay and in the Morlaix Bay (35 km apart). Females can store sperm from different males for several months (up to 214 days for *S. canicula*, Ellis and Shackley, 1997), and no population structure was shown for this species in the English Channel (Manuzzi et al., 2019). After their capture, all females were reared at  $16\,^{\circ}$ C. Eggs were laid in captivity at the Aquarium Marin de Trégastel (n = 55) and the Station Biologique de Roscoff (n = 70) from June to July 2022 due to the biological constraints of the species ( $\sim$ 2 eggs laid per week per female). Dinard, the location of the experiments performed in this study, and the Tregastel Bay and Morlaix Bay are all located within a radius of less than 200 km on the English Channel coast (Fig. S1). We thus postulated that the water chemistry of these three sites are similar.

The 125 eggs were individually identified (laying date known to within a day) before being introduced four weeks after in the nine 112-L biosphere tanks (three replicates per treatment; Table S1). From the fourth week, the embryo has a long tail that can be observed noninvasively (stage 3; Musa et al. (2018)); making it easy to differentiate between alive from unfertilized and dead embryos. Tanks were fully aerated to create a normoxic (>95% air saturation) environment with independent cascade filtration systems (Eheim Pro4+ 250; 950 L h<sup>-1</sup>). Salinity (34 ppt), nitrite ( $<0.05 \text{ mg.L}^{-1}$ ) and nitrate ( $<40 \text{ mg.L}^{-1}$ ) were monitored weekly and 1/3 of the volume of tanks were renewed weekly to ensure good water quality. Additional water changes were also carried out in case of embryo death. Temperatures (Table 1) were individually maintained with a heater connected to a temperature control unit with a reliability of  $\pm$  0.2 °C (Greisinger GIR 300). For each tank, the first monthly temperature applied was always that of June, whatever the spawning month (between June and July). Temperatures were also checked independently when measuring salinity, with the device providing both information (Greisinger GLF 100). Monthly changes in temperature were achieved by modifying the desired threshold values and allowing the water to change gradually over the following 48 h. The pH (Table 1) was controlled with an aquarium computer (IKS Aquastar) which allows measuring the pH and adjusting it independently in each tank by bubbling  $CO_2$  with a reliability of  $\pm$  0.01. The natural

Table 1 Temperatures (  $\pm$  0.2 °C) and pH (day-night) applied on embryos and juveniles.

Scenario	Variable	$\sim$ embryos (n = 125)				$\sim$ juveniles (n = 63)					
		Jul.	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.
AR6 (1995–2014)	Temperature (°C) pH	18.4 7.90–7.80	19.4	18.4	16.3	14.0	12.1	10.8	10.0	9.9	10.7
SSP2-4,5 (CMIP6)	Temperature (°C) pH	20.3 7.90–7.80	21.4	20.4 7.70–7.60	18.2	15.6	13.5	12.1	11.3	11.2	12.1
SSP5-8,5 (CMIP6)	Temperature (°C) pH	21.9 7.90–7.80	23.1	22.1 7.50–7.40	19.6	16.9	14.8	13.3	12.5	12.3	13.2

oscillations in pH between day and night were allowed for by applying a variation of 0.1. The pH probes were recalibrated every month after temperature changes, and independent measurements were taken before and after recalibration with a pH-metre (Orion 3-Star). The pH variations, following the pH expected under the SPP2-4.5 and SSP5-8.5, was modified from September, when 90% of all the embryos were at stage 6 and had developed fully developed gills, to the end of the experiment (i.e., six months after hatching). The egg is pre-open from the 4th stage (Jeffree et al., 2007) but the 6th stage was chosen because of the presence of functional gills and active water pumping (Leonard et al., 1999), which indicates a significant exchange of water and ions between the embryo and its environment (i.e., the pH inside the egg should be the same as outside).

# 2.2. Experimental procedure and follow-up of embryos and juveniles

Embryos were recorded and photographed weekly until hatching (Canon PowerShot G12) by candling, a method that involves shining a light through the eggs to observe their internal development noninvasively. Developmental stage timings were quantified weekly following Musa et al. (2018) description. If the observed stage was more than one step ahead of the last observation, it was assumed that the intermediate step lasted for less than one week. Total length and yolk surface were measured twice by two people from the randomly sorted photographs using tpsDIG (Rohlf, 2015) to minimise observer bias and keep constant the number of points used to make the measurements. This ensures that each measurement was made with the same accuracy regardless of the embryo positioning (see Supplementary Appendix S2 for details). The growth rate (cm.week<sup>-1</sup>) of each individual was then calculated using a generalised linear model (GLM) using the 'glm' function from the 'stats' R package. As yolk sac consumption was negligible in the first weeks of survey, we identified the point at which it significantly began using the Pettitt test implemented in the 'trend' R package (Pettitt, 1979; Verstraeten et al., 2006) to calculate the yolk sac consumption rate (cm<sup>2</sup>.week<sup>-1</sup>; Wheeler et al., 2021) over the remaining portion of the curve using a GLM. Embryos were checked daily to accurately determine the timing of their death, if any.

After hatching, total incubation time was quantified (  $\pm$  1 day), and each individual was weighed and measured (see Supplementary Appendix S2 for details). Individuals were fed ad libitum with thawed shrimp and squid every day until they were four months old, then every two days. Substrate, enrichment, and hiding places were offered. Juveniles (n = 63) were sexed at the age of 1 month (Table S2), weighed and measured monthly (see Supplementary Appendix S2 for details). Over the juvenile follow up, external stress was kept at minimum, so that juveniles were not individually-identified, avoiding tagging or repeated photographic identification.

In this study, we individually followed the development of embryos until their hatching as juveniles, as inter-individual variation in development is expected to be important at this stage (e.g., developmental failure due to chromosomal defects, gene interactions; VanRaden and Miller, 2006). At the juvenile stage, we carried out replicate-scale tracking (i.e., juvenile data is pooled from each treatment), as we hypothesised that inter-individual variation in growth would be smaller

between juveniles of the same tank than between tanks.

# 2.3. Freezing capacity and embryo recovering

Embryos are subjected to intense predation pressure (Powter and Gladstone, 2008) to which they respond by stopping ventilatory behaviour and ceasing to move, a so-called freezing response (Kempster et al., 2013). When the embryo holds its breath, it accumulates a 'metabolic debt' with respect to aerobic metabolism which it will have to recover (Leonard et al., 1999; Kempster et al., 2013). The rate of oxygen consumption measured after the expression of the freezing behaviour is a proxy of the cost involved. We assessed embryos freezing capacity and recovering throughout oxygen uptake measures with a static respirometry method. We focused on the pre-hatching stage (~October). Each egg was handled underwater to mimic the physical disturbances caused by a potential predator investigating the egg (Ripley et al., 2021) and placed in a 120 mL glass respirometry chamber directly capped into experimental tank (n = 55). We quantified the duration of the freezing response from handling in the tank to resumption of buccal pumping or uncoiling of the tail using a chronometer (Ripley et al., 2021) (n = 42). The oxygen saturation was measured using a mini oxygen sensor with a dip probe connected to a Witrox 4 oxygen metre (LoligoSystems) coupled with a temperature probe to automatically adjust oxygen saturation values to water temperature. Embryos were left in the respirometry chamber until we could calculate a rate of oxygen consumption (~15-20 min) taking care to have an oxygen saturation always above 80% in the chamber (Syendsen et al., 2016). The rate of oxygen consumption  $(mg.O_2.embryo^{-1}.h^{-1})$  was calculated by multiplying the rate of decrease in oxygen saturation by the volume of the tank (Wheeler et al., 2021). We validated the measurements by following the recommendations of Chabot et al. (2021), with a R<sup>2</sup> threshold of 0.85. Respirometry measurements with a fresh empty egg, from an embryo not involved in the experiment, were carried out before and after the respirometry measurements on the embryos and did not reveal any significant microbial respiration rates (<1%).

# 2.4. Embryos and juveniles growth patterns

We anticipated a higher mortality under the two scenarios. Hence, our objective was to delve into the causes of embryo death, particularly emphasizing individual growth divergences over time. Embryos growth was analysed individually in two ways: (i) each week, we quantified the difference between the growth state of an embryo from an experimental treatment and each of the control embryos, (ii) at the end of embryonic development, we compared the whole growth trajectory of an embryo from an experimental treatment with the set of growth trajectories of the control embryos.

We used the ecological quality assessment (EQA) framework developed by Sturbois et al. (2023), to summarise the inter-individual differences in each growth state belonging to individuals trajectory between embryos under control condition compared to the two treatments. EQA is a statistical method based on a dissimilarity matrix projected on a multivariate space, and measures deviations from a set of reference conditions contained in a state-based or trajectory-based

reference envelope. EQA was originally used for assessing and reporting the quality of ecosystems, but it can be applied to any traits based matrix. In our experiment, the space was defined with a distance matrix calculated from embryos length, yolk surface and developmental stage using the R package 'gawdis' (de Bello et al., 2021) and was used to assess the quality of embryos growth trajectories. A state-based EQA was first performed to quantify the impact of the two treatments at each growth state (i.e. weeks) compared to each growth state belonging to the control conditions (squared distance). It was followed by a trajectory-based EQA to compare the whole growth trajectories of individuals which have experienced the two treatments with the control trajectory reference envelope ( $Q \ge 0.5$ : inside the trajectory reference convex hull; Q < 0.5: outside the trajectory reference convex hull). EQA was performed using the 'ecotraj' R package (De Cáceres et al., 2019; Sturbois et al., 2023).

For juveniles, for which we did not have individual trajectories we calculated average growth rates for all the juveniles in a tank and differences in growth rates (length and weight) between experimental treatments were computed using Dunn's tests.

#### 2.5. Hatching success and developmental traits

The impact of treatments on the hatching success was analysed with a GLM with a binomial distribution and a logit link function, with the hatching success (0: no hatching; 1: hatching) as a response and experimental treatments as predictors. Conversely, the treatment impacts on mortality were assessed through weekly survival outcomes, implemented in a Cox's proportional hazards model, with tanks as a random effect, using the 'survival' R package (Therneau and Grambsch, 2000). The analysis included a total of 1183 observations, with 49 events (i.e., occurrences of mortality) observed (see Supplementary Appendix S2 for details). A Wald Test was used to assess whether treatments contributed significantly to the model, and a Score (Logrank) Test was used to assess global differences between treatments (Agresti, 2007). We also performed a Pearson's Chi-squared test for count data to test whether embryo mortality is evenly distributed between the different embryonic stages.

Then, we aimed to identify the key characteristics contributing to the success of the embryonic development. We studied the embryos' developmental trait combinations involved in the different probability of hatching. Adapting the method used in Carmona et al. (2021) and Coulon et al. (2023) that links the extinction risk of species to their traits, we chose nine developmental traits: growth rate, yolk consumption start, yolk consumption rate, stages duration (from 4 to 7), freezing, and O<sub>2</sub> consumption rate (Table S3; see Supplementary Appendix S2 for details) for studying the probability of embryo hatching. A distance matrix was calculated from these developmental traits using the 'gawdis' R package (de Bello et al., 2021). Then, a principal coordinate analysis (PCoA) was performed on the dissimilarity matrix to summarise the inter-individual dissimilarities in a biplot called 'development space'. In parallel, differences of each developmental trait between experimental treatments were computed using Dunn's tests.

We calculated the functional dissimilarity between individuals in a treatment (e.g. control) and all other individuals (for this example: SSP2-4.5 and SSP5-8.5) to test the difference between experimental treatments (based on developmental traits dissimilarities), using the 'dissim' R function of the 'TPD' R package (Carmona et al., 2019). It reflects the degree of functional dissimilarity between the probabilistic distributions of individuals in the development space between the two treatments, and ranges from 0 (complete overlap) to 1 (no overlap). We then tested whether this functional dissimilarity was lower or higher than expected by chance given the number of individuals in each treatment.

We estimated the occurrence probability of developmental trait combinations within the developmental space using kernel density estimation with unconstrained bandwidth using the 'funspace' R

package (Carmona et al., 2024; Duong, 2007). We then compared the observed functional dissimilarity to a null model where the experimental treatments were randomly assigned to individuals, keeping the number of individuals in each treatment constant using the 'TPD' R package. We drew 999 simulated assemblages and compared simulated and observed functional dissimilarities. Standardised effect sizes (SES) were calculated as the difference between the observed values and the mean of the simulated values after standardisation by their standard deviations: P-values higher (lower) than 0.975 (0.025) indicate that the observed functional dissimilarity is significantly lower (higher) than expected by chance (using a 5% threshold).

Hatching probabilities were then mapped in the developmental space, and tested with a generalised additive model (GAM), using the individual hatching success (0: no hatching; 1: hatching) as a response, and the dimensional position in the developmental space (i.e. PCoA axes) as predictors using the 'funspace' R package (Carmona et al., 2019; Duong, 2007).

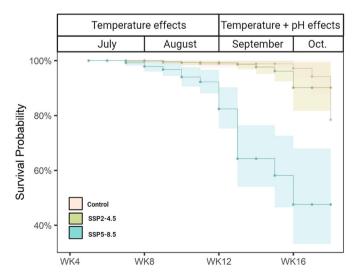
The flowchart of the methodology developed for our analyses is presented in Fig. S2.

#### 3. Results

# 3.1. Embryo hatching success

The GLM and Cox's proportional hazards models showed that the hatching success and, conversely, risk of mortality (Table S4) were highly dependent on the experimental treatments (Wald test:  $\chi^2=66.1$ , df  $=2,\,p<0.001$ ; Score (logrank) test:  $\chi^2=111.2$ , df  $=2,\,p<0.001$ ). Values ranged from 81% (26 out of 32) and 83% (34 out of 41) of hatching for the control and SSP2-4.5 treatments respectively (GLM, p=0.85), to 11 % for the SSP5-8.5 treatment (5 out of 45; GLM, p<0.001) (Fig. 1; Fig. S3a).

The survival probability dropped in the SSP5-8.5 treatment between the last week of August and the first week of September, at the end of the developmental stage 5 (Fig. 1; Fig. S4). This mortality was preceded by embryos departing growth trajectories highlighted by increasing squared distance from the state reference growth envelope (i.e., from control individuals; Fig. 2). SSP2-4.5 embryos also deviated from the



**Fig. 1. Embryos survival analysis.** Kaplan-Meier survival plot illustrating the survival probabilities of control (beige; n=35), SSP2-4.5 (green; n=44) and SSP5-8.5 (blue; n=45) embryos based on a Cox proportional hazards regression analysis. The x-axis and y-axis represent the time in weeks and the estimated survival probability, respectively. The shaded regions indicate the 95% confidence intervals. The log-rank test yielded a significant difference in survival between the groups (p < 0.001). Censored observations are denoted by tick marks on the survival curves. Created with BioRender.com.

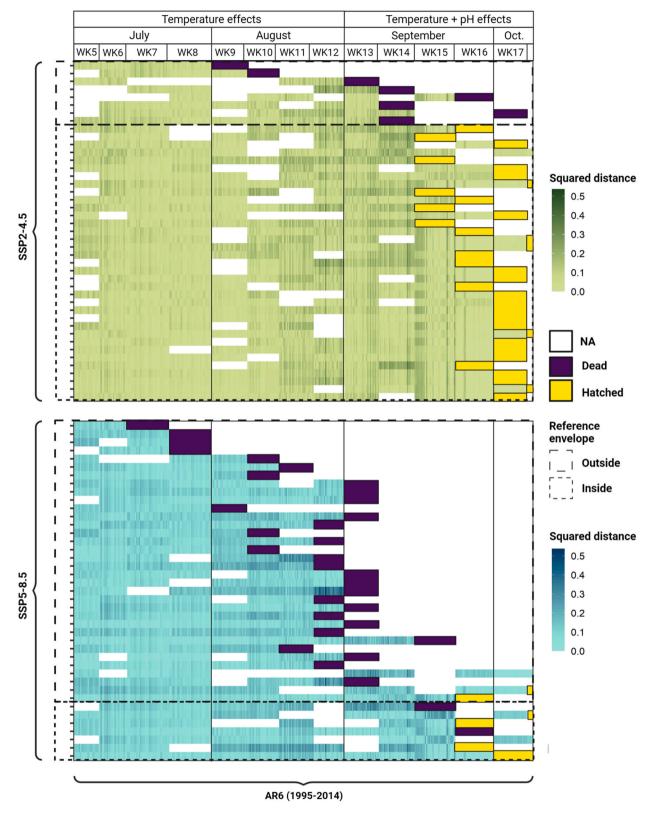


Fig. 2. Embryo growth pattern. Heatmaps showing inter-individual differences at each state (i.e., weeks) belonging to the growth trajectory of SSP2-4.5 (n = 41) and SSP5-8.5 embryos (n = 39) (y-axis; one raw for each embryo) when compared to AR6 (1995–2014) control embryos (x-axis; on column for each embryo for each week) (i.e. the state reference of the growth envelope). Light colours indicate a low distance to the state reference of the growth envelope, while dark colours indicate a high distance. Embryos inside or outside the trajectory reference growth envelope are framed by dotted boxes. Photos did not always make it possible to determine the embryo total length, yolk area and developmental stage, and certain inter-individual differences at each stage (i.e., weeks) could not be assessed (i.e. NA values). Embryo death or hatching are represented by dark purple and yellow boxes, respectively, but are not involved in the calculations. Created with BioRender.com.

state reference growth envelope in August and were able to recover towards the state reference growth envelope in September (stage 6; Fig. S4; Fig. 2). Trajectory based-EQA performed at the scale of the whole individual trajectories pointed out that 81 % (35 out of 43) of SSP2-4.5 embryos trajectories were included in the trajectory reference envelope, against only 17 % (7 out of 41) for SSP5-8.5 embryos (Fig. S5). This analysis also highlights inter-individual differences in growth trajectories within the same treatment. In the SSP5-8.5 treatment, two individuals were outside the trajectory reference envelope but still managed to hatch and, conversely, although inside the trajectory reference envelope, two embryos did not hatch and died at the 7th stage of development (Fig. 2). They weighed 0.8 g and 0.9 g and measured 6.9 cm and 5.9 cm respectively.

# 3.2. Developmental traits and probability of hatching

The first two axes of the PCoA (80% of total variance; Fig. 4A) showed that embryos with the lowest probability of hatching (Fig. 4B) were also the most dissimilar ones in the developmental space (SES = 0.48), namely SSP5-8.5 embryos, a dissimilarity greater than random expectation (SES = 5.62, p=1) (Fig. 4B; Table S5). This means that SSP5-8.5 embryos had the lowest probability of hatching and the most different combination of developmental traits.

Axes summarising the inter-individual differences between embryos (i.e., PCoA axes) and testing for the relationship between each developmental trait and the treatments (Fig. 5), show that embryos with the lowest probability of hatching (i.e., SSP5-8.5 embryos; Fig. 3) had a lower yolk consumption rate, with a lower growth rate than SSP2-4.5 and control individuals, and yolk consumption started earlier than control individuals. Stage 4 was shorter for SSP5-8.5 embryos than for control and SSP2-4.5 embryos (Fig. 4). The stage 5 was particularly critical for SSP5-8.5 embryos, with 52% mortality (17 out of 33, Fig. S3b; Pearson's Chi-squared test for Count Data, p < 0.01) coinciding with the mortality peak observed in August and the first week of September (Fig. 1). Stage 7 was halved for SSP2-4.5 and SSP5-8.5 embryos compared with control embryos (Fig. 4). The duration of the freezing behaviour of SSP5-8.5 embryos in pre-hatching stage (stage 7) was reduced by more than half compared with that of control and SSP2-4.5 embryos (Fig. 4). The oxygen consumption rates after freezing behaviour was 1.5 times higher in SSP5-8.5 embryos (Fig. 4).

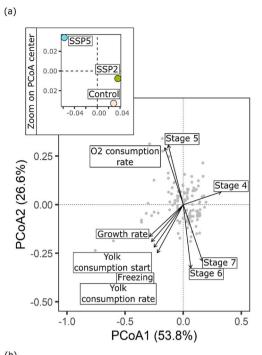
# 3.3. Neonates condition and juveniles growth

Incubation time was about 10 days shorter for SSP2-4.5 (n=36) than for control individuals (n=30). Moreover, inter-individual variability in incubation time was significantly higher in SSP5-8.5 treatment so that individuals had an intermediate incubation time (n=5; Fig. 5).

Hatchlings had similar total length whatever the treatment, but SSP5-8.5 and SSP2-4.5 individuals had a lower weight than control ones (Fig. 5). The SSP5-8.5 juveniles (n=5) were characterised by a higher growth rate than those from the control treatment (n=26), however the high variability in their weights did not translate in a significant increased weight gain (Fig. 5).

#### 4. Discussion

The purpose of this study was to investigate, at the individual and group levels, responses of early life stages of a temperate oviparous elasmobranch model species (*S. canicula*) to two conditions of ocean warming and acidification expected for the late 21st century. We showed that effects of increased temperature combined with decreased pH are highly dependent on the climatic scenario applied. The use of the EQA and TPDs frameworks made it possible to determine, from the comparison of individual development trajectories, which embryos did not hatch, and at what time they diverged from the others. Through our weekly follow-up and integrative study, we can also infer hypotheses on



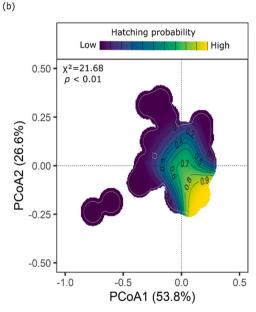


Fig. 3. Hatching success and developmental traits. (a) Projection of embryo developmental traits on the two principal axes of the principal coordinate analysis (PCoA). Annotations in the zoom on PCoA centre refer to the centroid of each experimental treatment, SSP2 stands for SSP2-4.5 and SSP5 for SSP5-8.5. (b) Probability of embryos hatching according to the generalised additive model (GAM) with binomial distribution, using the position of embryos in the developmental space as predictors. Yellow to purple gradient tones indicate a high to low probability of hatching, respectively. The grey line indicates the 0.99 quantile of the spectra of each group.

the causes of mortality. While embryos of the 'SSP2: Middle of the road' scenario were weakly impacted in terms of hatching success and growth, almost all embryos (i.e. 89%) exposed to the 'SSP5: Fossil-fueled Development (Taking the Highway)' scenario never hatched. Mortality peak was recorded in August, when the 5th developmental stage of embryos (Musa et al., 2018) experienced the highest temperatures. At hatching, SPP5-8.5 and SPP2-4.5 neonates were slimmer than control ones, but SPP5-8.5 juveniles subsequently had a higher growth rate than

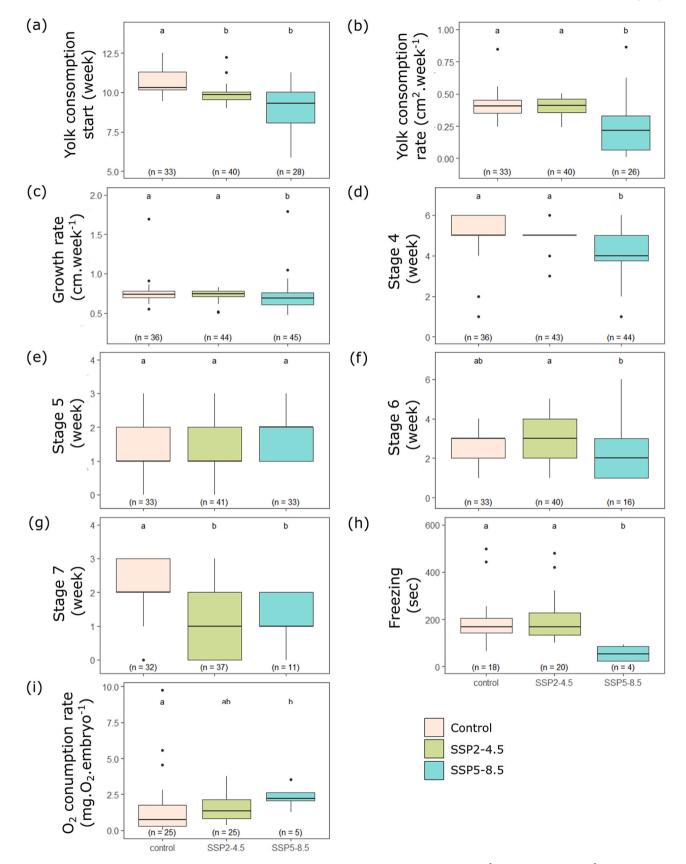
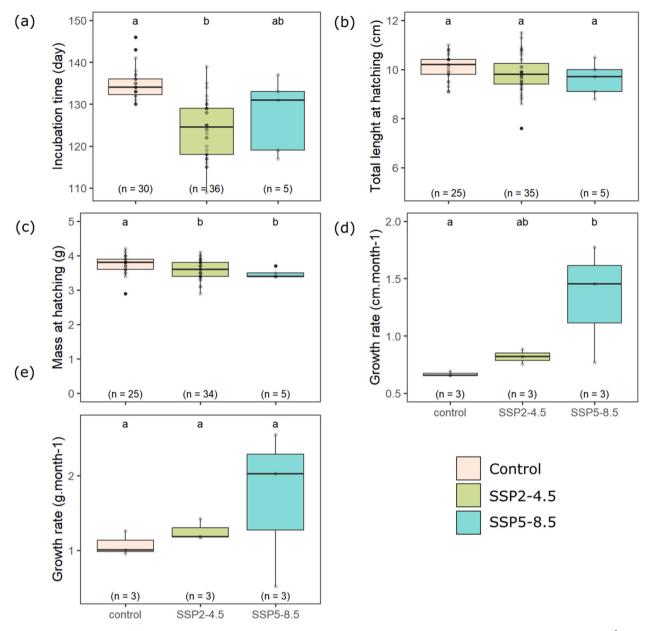


Fig. 4. Embryos developmental traits. (a) Yolk consumption start (week); (b) Yolk consumption rate (cm.week $^{-1}$ ); (c) Growth rate (cm $^{-1}$ ); (d) Stage 4 duration (week); (e) Stage 5 duration (week); (f) Stage 6 duration (week); (g) Stage 7 duration (week); (h) Freezing duration (sec); (i) O2 consumption rate (mg.O<sub>2</sub>.embryo $^{-1}$ . h $^{-1}$ ). Boxplots are filled according to treatments (beige = Control; green = SSP2-4.5; blue = SSP5-8.5). Letters above boxplots denote significant differences between developmental traits attributes (Dunn's test).



**Fig. 5.** Neonates condition and growth. (a) Incubation time (day); (b) Size at hatching (cm); (c) Mass at hatching (g); (d) Growth rate (cm.month<sup>-1</sup>); (e) Growth rate (g.month<sup>-1</sup>). Boxplots are filled according to treatments (beige = Control; green = SSP2-4.5; blue = SSP5-8.5). Letters above boxplots denote significant differences between attributes (Dunn's test).

#### control and SSP2-4.5 juveniles.

In this study, we demonstrated that monthly temperature variations can have significant effects on early life stages of the small-spotted catshark. Between July and September (stage 4-6), embryos from treatments SSP2-4.5 and SSP5-8.5 were incubated at an average temperature of 20.0 °C and 21.6 °C respectively (Table 1), but with gradual increase or decrease of 1–3  $^{\circ}\text{C}$  depending on months. This temperature regime led to the survival of 83% (34 out of 41) and 11% (5 out of 45) of embryos for SSP2-4.5 and SSP5-8.5 respectively. In a similar study, but with constant temperature regimes of 19 °C and 22 °C, 63% (12 out of 19) and 56% (9 out of 16) of embryos survived respectively (Brüggemann, 2013). In our study, we obtained a higher survival rate of embryos exposed to a moderate increase of temperature. Therefore, we can hypothesize that gradual increase and small variations around the mean temperature of 20.0 °C could be less detrimental to the survival of embryos of the small spotted catshark than a long exposure to constantly high temperature. However, this does not hold for a higher increase of temperature. This can be explained by the temperature of 23.1 °C experienced by SSP5-8.5 embryos in August (Table 1) that appears to be critical for the embryonic development of *S. canicula*. The temperature causing embryo death is consistent with threshold temperatures for embryonic development ranging from 3  $^{\circ}\text{C}$  to 5  $^{\circ}\text{C}$  above the current temperature documented in C. punctatum (Rosa et al., 2014), the epaulette shark (Hemiscyllium ocellatum; Gervais et al., 2018), L. erinacea (Di Santo, 2015) and the Port Jackson shark (Heterodontus portusiacksoni; Vila Pouca et al., 2019). Our study highlights the importance of monthly temperature variations for the development of the small-spotted catshark embryos as compared to constant temperatures (Brüggemann, 2013), which comforts that these variations should be taken into account in experiments assessing the effects of global warming (Slein et al., 2023). Additionally, our study points to the risks of additional or synergetic effects of marine heat waves on the survival of early life stages in the Northeast Atlantic (Pegado et al., 2020b; Simon et al., 2023), related to a poor embryo tolerance to extreme high temperature. This

emphasises the need for new experimental approaches closely aligned with the environmental pressures experienced by marine organisms, particularly in coastal nurseries.

In parallel, we showed an increased risk of mortality at the 5th embryonic stage of the small-spotted catshark under the SSP5-8.5 scenario when they experienced high temperature in summer. This developmental stage is characterised by the shrinking of gill filaments (Musa et al., 2018) and the transition to internal gills (Pelster and Bemis, 1992). In our experimental set-up, we ensured a stable oxygen saturation, whatever the temperatures, to avoid a significant increase in embryonic mortality (Musa et al., 2020). Hence, the observed increased mortality could be due to the inability of embryos to consume enough oxygen while their gills are modifying. We also showed a lower but earlier yolk consumption rate, associated with a reduced growth rate. This may indicate that embryos exposed to the SSP5-8.5 scenario had earlier energy demands associated with a poor yolk to body conversion, resulting in starvation. Therefore, lipid metabolic pathways (Wen et al., 2013; Bernal et al., 2020), as well as gill development (Takata et al., 2018), particularly involved at the 5th embryonic stage (e.g., membrane biogenesis), would require further study. Weeks during which the growth trajectories of SSP5-8.5 embryos deviated from the state reference growth envelope should be particularly targeted. In addition, the individual approach showed that some embryos whose growth trajectories deviate from the state reference growth could still hatch. The origin of such phenotypic change is unknown, but it could be genetically based or the result of phenotypic plasticity (Crozier and Hutchings, 2014; Merilä and Hendry, 2014). Conversely, embryos with growth trajectories similar to those of control embryos do not necessarily hatch. This could be explained by the proportionality between size and weight, which remains similar to that of hatched embryos, but whose very low values do not allow the embryos to free themselves from their egg.

Surviving embryos also suffered from limited ability to express freezing behaviour in the pre-hatching stage occurring in October (respectively at 16.3 °C; 18.2 °C; 19.6 °C). The duration of freezing behaviour was reduced by more than half for SSP5-8.5 embryos (n = 4) compared to that of control (n = 18) and SSP2-4.5 embryos (n = 20), which may translate in reducing their potential ability to hide from predators (Kempster et al., 2013). Additionally, their oxygen consumption rates after freezing behaviour were 1.5 times higher (n = 5) compared to the control. This result may imply that freezing was limited by a higher oxygen need to fulfil enhanced metabolic rate in these embryos (Leonard et al., 1999; Kempster et al., 2013). In a similar study conducted on embryos at stage 6 (with the vitellus not fully consumed), the freezing reaction duration was 7 times shorter for embryos reared and tested at 20 °C compared to 15 °C (Ripley et al., 2021). These differences may indicate a threshold effect at 20 °C or a greater effect of temperature on freezing behaviour at the sixth stage of development compared with the pre-hatching stage.

In our study, we deliberately confounded warming and acidification from the 6th embryonic stage of development of *S. canicula* (September), when a significant exchange of water and ions took place between the embryo within its pre-opened egg and the external environment due to embryo active water pumping. However, earlier in their development, in August when the tanks were not yet acidified, the growth trajectory of SSP5-8.5 embryos deviated from that of control ones. Consequently, the deaths observed in August were solely due to the warming. By contrast, mortality observed in September may have been triggered by acidification (Santos et al., 2021) on top of warming temperatures experienced in August; the August surviving embryos may have indeed reached their physiological thermal limits. This is consistent with previous analyses showing that the combined effects of acidification and warming are similar to those observed with warming alone (Santos et al., 2021), with acidification exacerbating global warming impacts, as described for L. erinacea embryos (Di Santo, 2015). It is worth adding that earlier exposure to acidification could have triggered these effects earlier in the development of S. canicula embryos.

At hatching, we showed as expected (Di Santo, 2015; Hume, 2019; Rosa et al., 2014; Wheeler et al., 2021) that SSP5-8.5 and SSP2-4.5 neonates had lower body weights than control ones. Additionally, all SSP5-8.5 juveniles (n = 5) survived over the six-month period after hatching, contrasting with previous studies where mortality was observed within 30 days after hatching (Rosa et al., 2014; Di Santo, 2015; Gervais et al., 2018). We also detected a lower but positive growth rate gradient as a function of increased temperatures and acidification, while Gervais et al. (2018) found the opposite. These contrasting results can be explained by the monthly temperatures applied during the juvenile stage, which were lower than the annual average temperatures chosen in these related studies. In addition, in our experimental set-up, juveniles were fed *ad libitum* to fulfil any potential increase in energy demand and prevent growth restriction (Cominassi et al., 2020).

Females of the small-spotted catshark produce and anchor eggs to a substrate (Wheeler, 1978) from shallow waters to depths greater than 200 m (Ellis and Shackley, 1997), rendering their embryos unable to move or escape the laying site. The loss of coastal habitats suitable for spawning due to global change is expected to slow down population turnover and may be linked to population size reduction (Lyon et al., 2011; Levy et al., 2015), if females cannot shift towards more suitable laying sites (Crear et al., 2020). Therefore, identifying and subsequently protecting deep spawning sites (e.g., from trawling) become critical (Kinney and Simpfendorfer, 2009; Sguotti et al., 2016; Wheeler et al., 2020). It should also be noted that we focused on the effects of climate change on summer spawning, as it is the period of the year the most intensely impacted in terms of temperature rise (Ellis and Shackley, 1997; ICES, 2022), but winter or spring spawnings could be positively impacted by a moderate increase in temperature (Salinas-de-León et al., 2018; Wheeler et al., 2020). In the SSP2-4.5 scenario, we observed a reduction of around 10 days in incubation time, demonstrating that the low temperature increase did not lead to significant effects, which might support this assumption. Time shift in spawning periods could also be possible, particularly through the selection of early-spawning individuals (McQueen and Marshall, 2017; Olmos et al., 2023). Northeast Atlantic populations suffering from increased temperature and acidification are expected to migrate towards higher latitudes and deeper depths in search of more suitable habitat, as observed recently for the small-spotted catshark (Coulon et al., 2024), and be replaced in their unsuitable habitat by more tolerant ones (i.e., from warmer latitudes) (Di Santo, 2016; Gervais et al., 2021). These hypotheses should be explored through spatial models of population dynamics incorporating data on species thermal tolerance (Neer et al., 2007; Levy et al., 2015).

Finally, a 'middle-of-the-road' scenario for the 21st century will not entail a decrease of embryo survival of the small-spotted catshark, but a 'fossil-fueled development' scenario could lead to population dynamic imbalances through very high embryo mortality. Our study attests the importance of analysing both individual and group responses in assessing the vulnerability of elasmobranch critical life stages to temporal variations in temperature. This new insight will provide responses closer to what species experienced *in situ*. This study highlights the detrimental effects of climate change on the fitness of embryos of a widely distributed and non-threatened species of oviparous elasmobranch. These results raise concerns about the future of species with higher distribution constraints and/or endangered ones.

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# **Ethics**

The experiments complied with the ARRIVE guidelines and have

been carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

# CRediT authorship contribution statement

Noémie Coulon: Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. Stanislas Pilet: Software, Investigation, Formal analysis. Anne Lizé: Writing – review & editing, Writing – original draft, Validation, Methodology, Conceptualization. Thomas Lacoue-Labarthe: Writing – review & editing, Validation, Resources, Methodology. Anthony Sturbois: Writing – review & editing, Software, Methodology. Aurèle Toussaint: Writing – review & editing, Software, Methodology. Eric Feunteun: Writing – review & editing, Supervision, Project administration, Funding acquisition. Alexandre Carpentier: Writing – review & editing, Validation, Project administration, Methodology, Funding acquisition, 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.

# Data availability

Data are shared in Appendices

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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.2024.106531.

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