The effects of elevated temperature on development and gas exchange in embryonic sharks

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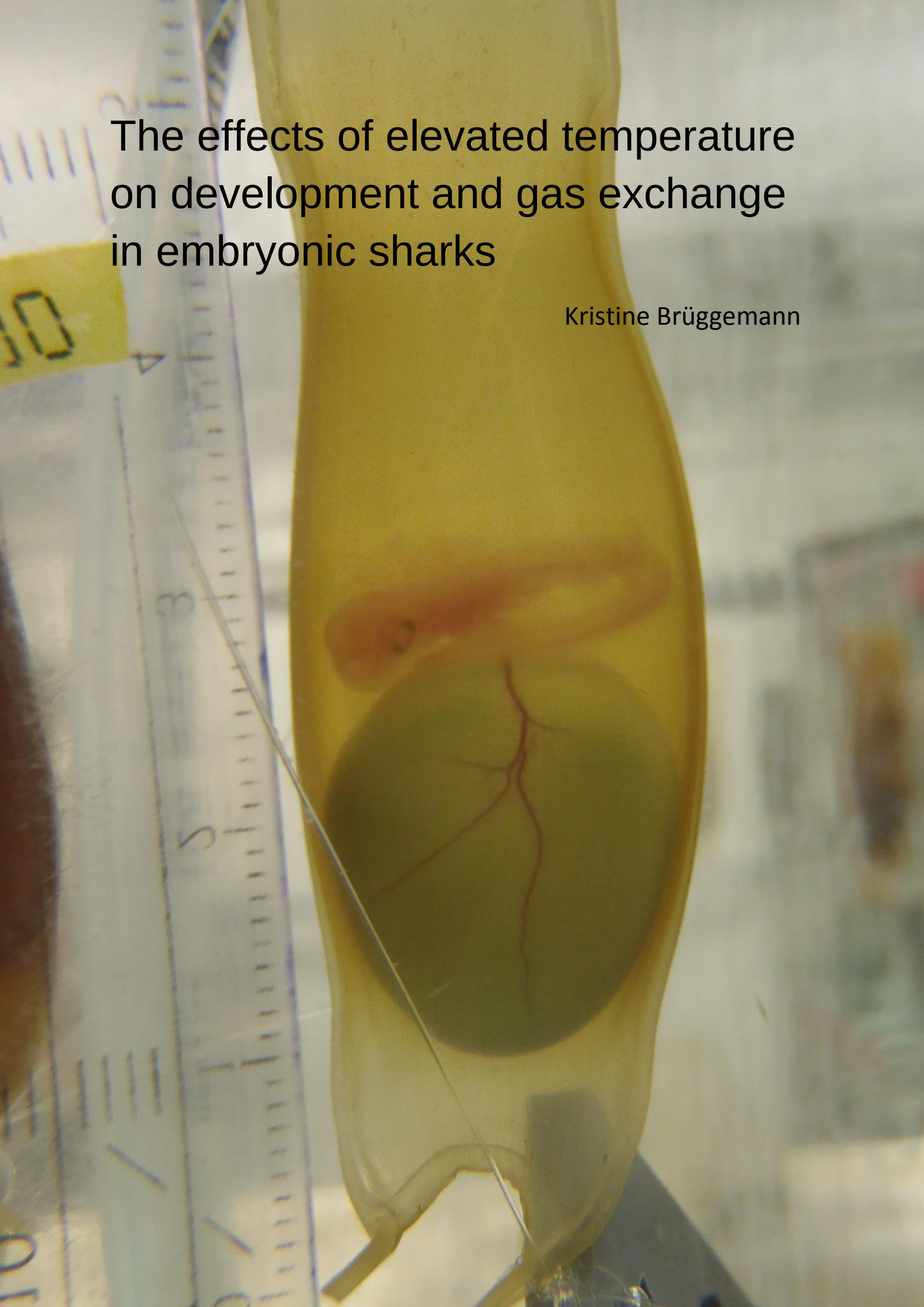

GEOMAR- HELMHOLTZ-ZENTRUM FÜR OZEANFORSCHUNG KIEL

The effects of elevated temperature on development and gas exchange in embryonic sharks

Master thesis in Biological Oceanography

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Picture 1. Juvenile *Scyliorhinus canicula*

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1. Summary

1.1 Abstract

Gas exchange, including measurements of both pO_2 and pCO_2, at the different developmental stages of *Scyliorhinus canicula* was observed at a control temperature of 15°C. Partial pressures of oxygen and carbon dioxide showed a strong inverse relationship. Oxygen partial pressure during the first developmental stages was high and stable (19±0.1 kPa) and decreased towards the opening of the slits in the egg case (6±1.5kPa), increasing later but staying very variable once the slits in the egg case have been opened (17±3.5 kPa) and increased even further, stabilizing during the last phase of development (21±1 kPa). Carbondioxide partial pressure was inversely related to pO_2, showing a strong hypercapnia during class 2A (Maximum value=0.34 kPa).

Three different temperature treatments, 15°C, 19°C and 22°C, were utilized. Embryos had significantly lower survival rate at higher temperatures (63% at 19°C and 56% at 22°C). Time in the egg was significantly shorter with increasing temperature. Embryos acclimated to 15°C and 19°C displayed a high pO_2 during the last development stage, embryos acclimated to 22°C were excluded from this analysis. Oxygen partial pressure did not vary significantly between embryos acclimated over a long time period at 19°C and those acutely warmed to 19°C after being acclimated at 15°C.

1.2. Introduction

Sharks are top predators in most marine ecosystems worldwide and have been the focus of the news lately in connection with the CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) meeting in March 2013, when several Chondrichthyan species were added to Appendix I and II, thus internationally increasing the level of control over their commercial trade. Chondrichthyans, the class containing Elasmobranchii and Holocephali, meaning sharks (including *S. canicula*), skates, rays and chimaeras, are typical k-strategists, being slow in development and late in sexual maturation. Slow growth and late maturity cause them to often be caught before maturity, decreasing the size of the gene pool and decreasing the number of reproducing adults. In addition any adaptation to climate change will take longer and take larger tolls on the number of individuals than in highly reproductive, fast growing r-strategists. This makes them vulnerable to extinction due to fishing pressures and climate change. In fact, most chondrichthyan species are listed on the IUCN red list; 25 species are listed as critically endangered, 41 as endangered, 116 as vulnerable and another 133 as near threatened (IUCN, 2013). The catalogue of life lists 1199 species of Chondrichthyes (Roskov,Y. et al.,2013), so compared to the number of those listed as threatened (critically endangered, endangered, vulnerable, near threatened and least concern) almost 15% of all

chondrichthyans are threatened. Several species of this group are thought of as “living fossils”, meaning their morphology has not changed over a long period of time. Examples of living fossils in sharks are Elephant Sharks, *Callorhinchus milii*, Frilled Sharks, *Chlamydoselachus sp*, and Goblin Sharks, *Mitsukurina owstoni* (www.sharkproject.org/haiothek accessed 25.03.2013).The oldest accepted shark teeth were found in Spain, originating from the early Devonian time period, about 411 million years ago, but fossils originating from earlier times are disputably also assigned to sharks. The earliest neoselachian, meaning modern shark, fossil is *Mcmurdodus whitei* from the early Devonian (Turner and Miller, 2005).

Modern sharks are present in many marine ecosystems, including coastal, demersal and pelagic (Ferretti et al., 2010) although they are mainly absent below 3000m (Priede et al., 2006). More than 90% of elasmobranch species are demersal (Ferretti et al., 2010). Despite the high economic and ecological value, especially of the fins and cartilage and as a connection between widely spread ecosystems (Ferretti et al., 2010), a lot remains unknown about these species.

The lesser spotted dogfish, *Scyliorhinus canicula* (Linnaeus, 1758), has been the focus of many studies as it is easy to obtain samples and uncomplicated to cultivate. Its native range spans from Norway, south to West Africa in the Atlantic ocean and into the North Sea and Mediterranean Sea (Compagno, 2001), where it is commonly found between 3-110m. Animals from the Mediterranean population are smaller in size at all important life history events (Mellinger et al., 1984). Mating occurs in late summer and eggs are deposited throughout the year. Different peaks in egg laying have been described, but most agree on a peak during winter and spring for the Irish Sea and English channel populations (Wearmouth et al., 2013). Females lay one egg per oviduct approximately every 11 days when fertilized, the species possesses two oviducts (Wearmouth et al., 2013). The eggs are deposited in shallow water, attached to macroalgae or sessile invertebrates via the posterior tendrils (Compagno, 2001). The female dogfish repetitively swim in circles around the base of the structure, tangling the tendrils around it and pulling the egg from the oviduct via the tendrils (Dodd, 1983). The deposition of eggs in pairs is beneficial for research as pairs can be separated into one egg per laying event to undergo treatment and keeping the other as a control (Diez and Davenport, 1987). The different stages of embryonic development have been described in detail by Ballard et al. (1993), including specific drawings. Fertilized eggs are retained in the uterus prior to deposition for up to 4 days, they are deposited at Ballard’s stage 4 to 5. The egg has four slits, one below each of the attachment sites of the anterior and posterior tendrils. These slits are covered during the first half of development by mucopolysaccharide plugs, keeping the embryo sheltered from bacteria and fungi in the surrounding sea water. This is why oxygen partial pressures in the perivitelline fluid are solely reliant on oxygen diffusion through the egg case during the first phase of development. Diffusion rate

is mathematically described by the Fick equation: $V_{O_2} = G_{O_2} \times (P_{O_{2out}} - P_{O_{2in}})$

In which V_{O_2} is the oxygen consumption of the embryo, G_{O_2}represents the conductance of the egg capsule, $P_{O_{2out}}$ is the oxygen partial pressure outside the egg and $P_{O_{2in}}$ is the oxygen partial pressure inside the egg (Cronin and Seymour, 2000). G_{O_2} is dependent on the effective surface area of the egg case (ESA), the thickness of the egg case (X) and Krogh's coefficient of diffusion (K_{O_2}):

$$G_{O_2} = K_{O_2} \times \frac{ESA}{X}$$

These equations show that diffusion is mainly limited by the ESA (effect surface area), the thickness of the egg case, the conductance of the egg case and the gradient between inside and outside pO_2. To increase the rate of oxygen diffusion, different strategies can be employed: the gradient between internal and external pO_2 can be increased by increasing internal pO_2, the effective surface area can be increased by changing the shape of the egg case or by decreasing the thickness of the egg case. ESA and reduced thickness of the egg case can be modulated by egg swelling, as observed in different molluscan species with elastic egg covers (Cronin and Seymour, 2000, Kress, 1972, Gutowska and Melzner, 2009). For example, in the cephalopod *Sepia apama*, ESA increases by 79%, while egg case thickness decreases by 72% (Cronin & Seymour 2000). Egg swelling is enabled by secretion of osmotically active substances into the PVF, which then draws water into the egg capsule and leads to swelling (Cronin and Seymour, 2000). Movements of the embryo within the perivitelline fluid produce convective currents in fish eggs (Rombough, 1988) and in molluscan eggs (Cronin and Seymour, 2000), thus aiding in maintaining high diffusion gradients between PVF and seawater. Despite these adaptations, pO_2 in many species still reaches very low values during development to ensure a strong gradient over the egg case wall to enhance diffusive flux. PVF pO_2 values in *Sepia officinalis* declined from above 12 kPa to below 5 kPa (Gutowska and Melzner, 2009). Due to embryonic respiration taking up O_2 and exhaling CO_2, PVF pCO_2 follows a contrary pattern to PVF pO_2, rising from 0.13 to 0.41 kPa during the development of *Sepia officinalis* (Gutowska and Melzner, 2009).

In *Scyliorhinus canicula,* no egg swelling occurs, as the egg capsule is rigid in comparison to that of cephalopod molluscs. However, ventilatory movements have been observed during all phases of development (Diez and Davenport, 1987). During the closed–egg phase, the embryo displays first trunk motility at stage 17 at an age of approximately 16 to 20 days (all observations at 16°C). By stage 27, the yolk sac is completely vascularized, additionally supporting oxygen uptake of the embryo. The external gills in pharyngeal clefts C2 to C5 may have blood flowing through them at this stage, also increasing the possible oxygen uptake rate of the embryo. The hatching gland becomes visible on the

protruding surface of the head. From this stage on the embryo has been cultured successfully outside the egg case. At stage 31, at an age of 60 to 80 days, pre-hatching occurs. This process includes the dissolution of the plugs, covering the four slits in the egg case, by an enzyme released from the hatching gland. This opens the egg case to circulating sea water, mediated by the movement of the embryo. This exposes the embryo to bacteria and fungi for the first time, requiring a functional immune system. It also removes one diffusion barrier for oxygen and carbon dioxide, enabling the embryo to maintain the same oxygen uptake ratio as before at higher blood oxygen partial pressure or alternatively to increase oxygen uptake ratio if blood oxygen partial pressure is kept at the same level. This is of great importance, as oxygen demand of the embryo increases exponentially over the course of development (Diez and Davenport, 1987). At this stage of development (60 to 80 days), the external gills have reached their maximal size. In the following stage 32, at a body length of approximately 65mm, pharyngeal respiration begins. The external yolk sac shrinks as it is relocated to an internal yolk sac during stage 33 (115-155days). At stage 34 (145-175 days) the embryo is ready for hatching, as all yolk has been internalized and the yolk sac and vitelline duct have degenerated. The shark can remain in the egg for further 8 to 18 days. All stages, stage descriptions and sizes for *S. canicula* in this paragraph were recorded by Ballard et al. (1993).

The deposition of eggs in shallow coastal areas exposes them to high diurnal and seasonal variation in ambient pO_2 and pCO_2, as well as in pH (Saderne et al., 2013). Coastal seas are typically not in equilibrium with the atmosphere with respect to pCO_2 and pO_2, as heterotrophic degradation of organic material is of high importance in these ecosystem (Melzner et al., 2013). *Scyliorhinus canicula* embryos must therefore be adapted to high variability of these factors, e.g. having a high tolerance to hypoxia (Diez and Davenport, 1987). In addition coastal seas will be strongly impacted by climate change (Melzner et al., 2013). It has been shown that temperature has increased by 1.13°C during the last 40 years in the North Sea (Wiltshire and Manly, 2004) and further increases by 0.5 to 1°C are expected in North Sea surface temperature by 2020 and up to 2.5°C by as soon as 2050 (Perry et al., 2005). This poses a strong challenge to any species living in these areas but especially to species with a relatively late maturity and high longevity such as *Scyliorhinus canicula* and to those species, using these areas as hatching grounds.

Thermal acclimatization is required when temperature changes in the habitat due to seasonal variance. Different changes to metabolic machinery, such as an adjustment of enzyme concentrations (Lucassen et al., 2006) and proliferation of mitochondria (Tyler and Sidell, 1984) , are made, depending on the direction of temperature change and on the duration of the temperature variation. Precht (1958) describes full and optimal acclimatization to a long term change in the ecosystem, type 2 adaptation, when Q_{10} values are close to 1, meaning the metabolic rate, does not

change with increasing temperatures. As a contrast, the author explained type 4 acclimation, where Q_{10} values range between 2 and 3, where changes in metabolic rate directly follow the temperature pattern of the ecosystem. As *S. canicula* is adapted to a variable environment naturally and long term temperature changes were used in this study, type 2 acclimatization was expected to occur.

The combination of these two factors, general adaptation to a variable environment and the prospect of encountering acute effects of climate change in the near future, makes *Scyliorhinus canicula* an interesting model organism to study the effects of climate change on a Chondrichthyan species.

This study focused on gas exchange in developing dogfish embryos and the effect of elevated temperatures on development by using three different experimental temperatures to which eggs were acclimated for several weeks. The perivitelline fluid (PVF) was analyzed for oxygen partial pressure, PVF pO_2, PVF pH, carbon dioxide partial pressure, PVF pCO_2, and the embryo's ventilation frequency, v_f, to test the following hypotheses:

1. **Perivitelline fluid (PVF) oxygen partial pressure (pO_2) will decrease during development.**
   During the development oxygen demand in the dogfish embryo increases with time (Diez and Davenport, 1987), this should lead to lower PVF pO_2 values in order to enhance diffusion of oxygen into the PVF.
2. **PVF pCO_2 and pO_2 are inversely related, embryos will experience severe hypercapnia during development.**
   PVF pCO_2, PVFpO_2, PVF pH and temperature are potentially important factors determining the development of all embryos within the egg case. The role of PVF pCO_2 and consequently pH has so far been neglected by most researchers but has been found to be of great importance during the development of other marine organisms, such as teleosts (Munday et al., 2012).
3. **Increased temperatures, as predicted to occur due to climate change, will lead to a decrease in PVF pO_2, an increase in ventilation effort and a shorter development time.**

   An increase in temperature leads to an increase in oxygen demand by the embryo, which might be counteracted by an increase in ventilation frequency, v_f. An increase in Q_{10} values for heart rate, cardiac output and respiratory frequency was observed at higher temperatures in embryonic *S. canicula* by Butler and Taylor (1975). Shorter development time has been observed at higher temperatures by Thomason et al. (1996a). Type 2 acclimatization is expected to occur due to the long acclimation period, which would lead to a similar v_f in those embryos, which have been acclimated to the higher temperature, and

those acclimated to control temperature of 15°C and a higher value for those acutely warmed.

2. Material and Methods

2.1. Egg collection and maintenance

Eggs were obtained from the SEALIFE aquarium in Berlin, Germany, where an adult population, originating from Weymouth, UK, has been kept since 2004. A known egg deposit site in the main dogfish tank at SEALIFE was cleared of eggs. Two weeks later all eggs from the site were collected for sampling thus providing a two weeks' time frame for the time point of egg deposition. The samples were then transported to Kiel, Germany, within three hours submerged in plastic bags of well aerated sea water in thermally insulated boxes. Subsequently eggs were placed randomly in the tanks.

2.2. Embryonic culturing systems

All groups were kept at 15°C, pH= 8.1 and in a salinity of 30-31 until the start of the experiment. Each temperature treatment was kept in a separate closed recirculating aquarium system equipped with a nitrification filter (Eheim, Deizisau, Germany) and a 15W UV sterilization unit (HW - Aquaristik, Germany). Temperature was controlled using a 250W heater (Eheim, Germany) and a steering unit (Biotherm 2000, Aquatico Ltd., Macroom Ireland) in a header tank. The systems were filled with filtered Baltic Sea water adjusted to the higher salinity using artificial sea salt (SeequaSal GmbH, Münster, Germany). Water was pumped from the filter sump (Figure 1, S, 250 liters), through the nitrification filter (F) and the UV sterilizer (UV) into the header tank (HT, 20 liter), from which the water passed via gravity feed to two animal tanks (AT1 and 2, 20 liters each). Water streamed back to the filter sump via overflows in the animal tanks. Aeration was provided in all tanks using diffusor stones (Dohse, Grafschaft-Gelsdorf, Germany). All eggs were suspended by the anterior tendrils and labeled using a small piece of PVC attached to the posterior tendrils. Tanks were checked daily for hatchlings and the approximate age at hatching was recorded. The three temperature levels used in the experiment were 15°C, 19°C and 22°C. The temperatures were raised 1°C every 2 days until the experimental temperature of 19°C and 22°C, respectively, were reached. Ammonia and nitrite concentrations in the recirculating unit were maintained at safe levels (<0.1 mgL^{-1}), pH, salinity and temperature were maintained within 0.05 (pH) and 0.1 (S, T) of target values.

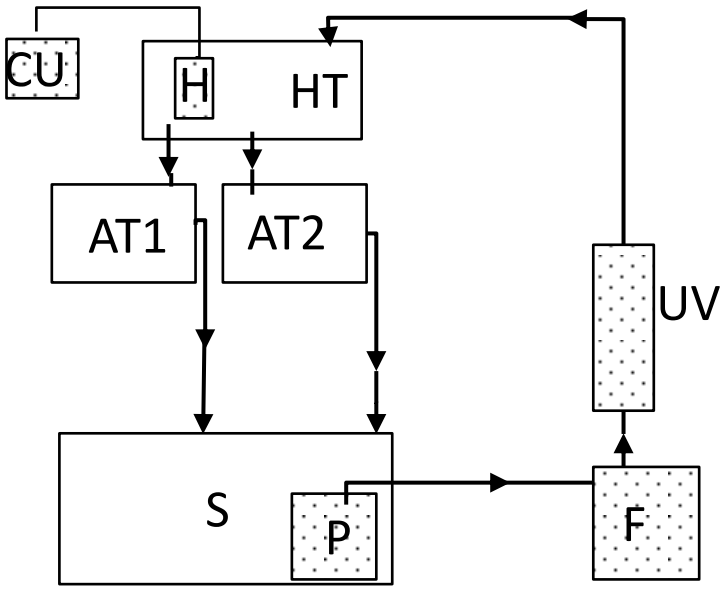


Figure 1. Exemplary set-up of one of the recirculating aquarium systems used to hold the eggs

2.3. Experimental setup and measurements

Ventilation frequency (v_f), PVF pO_2, PVF pH (NBS scale) and PVF dissolved inorganic carbon (C_T) were measured in a separate experimental system, consisting of a nitrification filter (Eheim, Deizisau, Germany), a 15W UV sterilization unit (HW - Aquaristik, Krefeld, Germany) attached to a filter sump which supplied a 20 liter experimental tank. Temperature was controlled via a cooler (Aqua Medic, Bissendorf, Germany) as the room temperature ranged around 22°C at all times. The light in the room was dimmed and limited to a single 60W light bulb facing the wall to avoid disturbance of the embryos. All embryos were measured at their acclimatized temperature first to avoid additional stress. 2 eggs were measured simultaneously. Eggs were gently transferred from the embryonic culturing system to the experimental system within a small plastic container, always submerged in water to avoid air exposure and trapping of air bubbles. When air bubbles were trapped, they were gently removed via a small syringe inserted through the open slits in the egg case. Eggs were fixed in position in the experimental tank by inserting the needle-type oxygen optode through the central anterior part of the egg casing wall. Due to the elasticity of the egg casing, a tight seal was formed around the syringe that was secure enough to suspend the entire egg casing vertically in the tank using the oxygen optode holder (Picture 2). The oxygen optode tip (diameter 140 µm) was inserted ca. 1 cm into the egg casing, as close as possible to the shark's ventilatory apparatus. Embryos were allowed to acclimatize for at least an hour before measurements were taken.

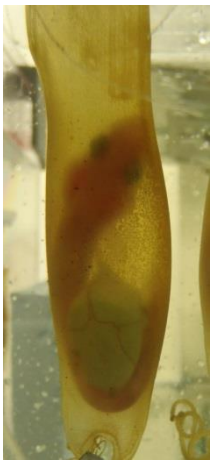
Picture 2. Class 3 embryo in the experimental system during PVF pO_2 measurement. The embryo can be seen in the transparent egg case, suspended from the needle, inserted approximately 1 cm through the top of the egg case without touching the embryo. From this needle the oxygen opthode is ejected. On the posterior tendrils a plastic label for egg identification is visible.

2.3.1. Ventilation frequency and PVF pO_2

Prior to any measurements the embryo was classified using an extended version of the three age classes as described in Thomason et al. (1996b). A fourth class was added to provide a higher resolution of the difference before and after the dissolution of the polysaccharide plugs.

Class 1: Egg case is still closed and the embryo is less than 1cm in length. The fluid inside the egg case is circulated only by the body movement of the embryo. This corresponds to stage 4 through to stage 24 by Ballard et al. (1993).

Class 2A (added): The embryo is less than 4 cm small and does not show typical regular ventilation movement patterns such as a continuous tail beat yet. Movements appear irregularly, varying from a complete curling of the whole body to short periods of fast tail beats and complete stillness. This class contains stages 25 to 30 (Ballard et al., 1993).



Class 2B: The egg case has been opened. The medium sized embryo (approximately less than 6.5 cm) is mobile in the egg case and has developed external gill filaments. Motility is used to enhance the water flow through the egg case. Typically, regular ventilatory movements are alternating between regular tail beats, whole body movements along the longitudinal axis and times of stillness. This class contains embryos assigned to stage 31 and 32 (Ballard et al., 1993).

Class 3: The embryo's body length exceeds the size of the egg case and its motility is strongly restricted. The gills have been fully developed and have been internalized. The embryo relies on buccal/ pharyngeal pumps to move the sea water through the egg case. The external yolk sac is visibly shrinking and disappears during the progress of this class. This class refers to embryos in stage 33 and 34 (Ballard et al., 1993).

All eggs were sampled at their acclimatized temperature first and then acutely warmed to 19°C (15°C acclimated eggs). No measurements were conducted using the 22°C experimental group. Experiments followed the scheme in Figure 2.

Ventilation frequency (v_f) was assessed visually three times in a row every hour between 10am and 5pm for 30 seconds. Posterior body movements per minute for class 2A and B embryos or number of ventilation cycles for class 3 embryos were counted. Preliminary tests showed that the presence of the observer did not influence ventilation rate. Care was taken to avoid unnecessary movements of the observer during measurements.

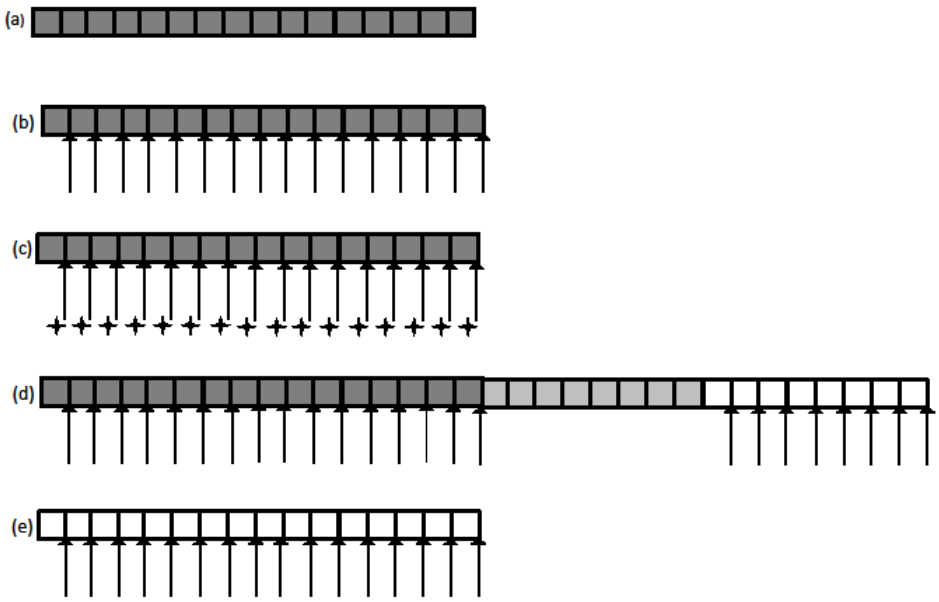


Figure 2. Timeline displaying the order of experimental procedures during continuous PVF pO2 measurements, each square representing one hour, (■)meaning one hour at 15°C, (■) meaning one hour of warming and (□) meaning one hour at 19°C. Arrows (↑)represent three measurements of v_f. Stars (✦) represent one measurement of pH and C_T. (a) displays embryos of class 1, (b) shows class 2A, (c) displays embryos of class 2B raised at 15°C, (d) shows class 3 embryos acclimated to 15°C and (e) displays class 3 embryos acclimated at 19°C

Oxygen partial pressure in the perivitelline fluid (PVF pO_2) was measured for 16 continuous hours using a needle-type oxygen optode (Presens, Precision Sensing GmbH, Germany) attached to a 4-channel microsensor oxygen meter (OXY-4 micro PreSens, Precision Sensing GmbH, Germany), which was inserted through the top of the egg case without touching the developing embryo (Picture 2).

This method does not consume oxygen as it is based on the dynamic luminescence quenching by molecular oxygen (Gatti et al., 2002). The oxygen sensor consists of a glass fibre cable inserted into a syringe, which extended from the tip after insertion into the egg case. The tip of the fiber is coated in an excitable layer of dye working as a sensor spot. At each measurement the sensor spot is excited by a blue LED and emits fluorescence. If the sensor encounters an oxygen molecule, a non-radiative energy transfer occurs and the resolving decrease of luminescence is measured. This decrease is called quenching. The mathematical relation between the intensity of fluorescence without a quencher I_f^0 , the fluorescence in the presence of a quencher I_f, the quenching rate coefficient k_q, the lifetime of the emissive excited state of the sensor dye without a quencher τ_0 and the concentration of the quencher [Q] is given in the Stern-Volmer equation:

$$\frac{I_f^0}{I_f} = 1 + k_q\,\tau_0 \times [Q]$$

The software automatically uses the implemented Stern-Volmer equation to calculate the output value oxygen saturation in percent.

2.3.2. Perivitelline fluid pCO_2 determination

In order to calculate carbon dioxide partial pressure (pCO_2) in the perivitelline fluid (PVF), dissolved inorganic carbon (C_T) and pH (NBS scale) was measured in discrete water samples taken from class 2 A and B embryos. The pH was measured first in the surrounding sea water near the inflow of the egg using a pH probe (WTW, Weilheim, Germany). A gas tight syringe (Hamilton Bonaduz AG, US) was used to extract a sample of 200 µl of water through one of the upper slits in the egg case. The sample was carefully transferred into an Eppendorf tube (0.5ml) in a temperature controlled water bath adjusted to 15°C. The pH of the sample was measured using the same pH electrode which was used to sample the surrounding water. The value was accepted when pH was stable for five continuous seconds. The sample (10 µl) was then transferred into a Corning 965 Carbon Dioxide Analyzer (Olympic Analytical, Malvern, UK). This instrument uses a lactate solution to liberate C_T in a sealed reaction chamber. Subsequently, C_T can be measured as CO_2 in the gas phase using a thermal conductivity sensor. The analyzer was calibrated using a dilution series of sodium-bicarbonate standard (Sigma-Aldrich, Hamburg, Germany).

2.3.3. Water movement through the egg case

A class 2B egg was placed in a shallow dish submerged in seawater taken from the same culturing system as the egg. When the water had calmed, a concentrated calcein solution was injected into the water column adjacent to one of the four openings in the egg case. The spreading of the calcein stain was observed visually. This was repeated for each of the four openings and three different embryos.

2.4. Data processing and statistical treatment

The collected data was formatted using Microsoft Office- Excel 2010 and loaded into "R" statistical computing software (Version: R 2.15.2, Copyright: 2012 The R Foundation for Statistical Computing) for statistical treatment. Packages used were "pgirmess," "car," "pastecs" and "ggplot2," all were downloaded using the Cran mirror "Germany (Falkenstein)."

2.4.1. Age at hatching

Data was entered into R and checked for normality using a Shapiro-Wilk test, histograms and a qqplot. A Bartlett-test was applied to test for homogeneity of variances. An analysis of variances and post-hoc testing was used.

2.4.2. PVF pO_2

Mean PVF pO_2 and standard deviation over 16 hours was calculated for each egg using MS Excel. This data was analyzed by group for normality using Shapiro-Wilk's test and tested for homogeneity of variances using Levene's test. A Kruskal-Wallis test was used to analyze differences between groups.

2.4.3. Ventilation rates in relation to PVF pO_2

Ventilation rate (v_f) was entered into MS Excel, grouped according to development stage and temperature, and entered into R with the corresponding PVF pO_2 values. Due to the non-normal distribution of the pO_2 and v_f data sets, a nonparametric test for Spearman's rank correlation coefficient for ventilation frequency to PVF pO_2 was used. The influence of temperature was assessed using a Kruskal-Wallis test on the data for class 3 embryos.

2.4.4. PVF pCO_2

The C_T in µmol*kg^{-1} seawater was calculated from the corresponding calibration curve, which was measured daily. Partial CO_2 pressure was derived from the C_T and corresponding pH (NBS) value using CO2Sys (Lewis and Wallace, 1998). Spearman's rank correlation coefficient was calculated for pH to pO_2 and pH to pCO_2.

3. Results

3.1. Developmental changes at 15°C

3.1.1. PVF pO_2

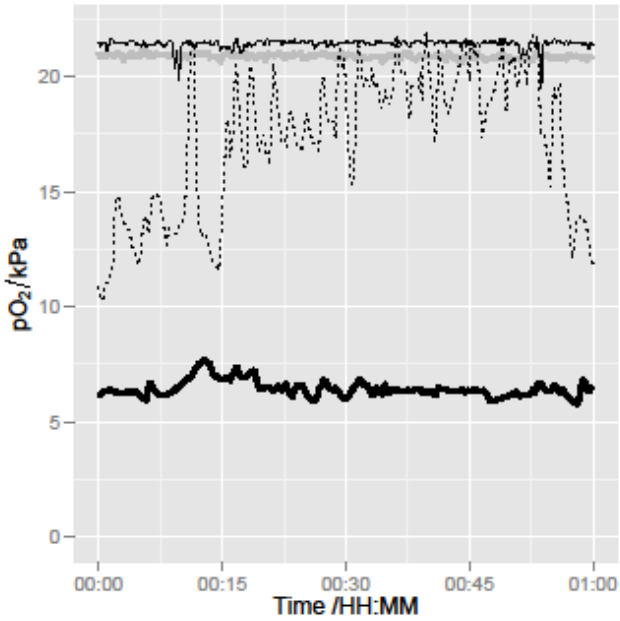


Figure 3. Exemplary PVF pO_2 over the course of one hour in the different age classe, class 1 (▬), class 2A (▬), class 2B (⋯⋯) and class 3 (▬).

Mean PVF pO_2 was compared between the different age classes of embryos raised at 15°C. Mean pO_2 was 19.3 kPa for class 1, 6.1 kPa for class 2A, 16.8 kPa for class 2b and 21.1 kPa for class 3, respectively. PVF pO_2 was normally distributed for class 2A, class 2B and class 3 embryos at 15°C. Oxygen partial pressure of class 1 was not normally distributed. Variances were not homogeneous as shown by Levene's test, $F(3.24)= 3.6981$, p=0.026. A Kruskal-Wallis test demonstrated a significant difference in the mean PVF pO_2 of different developmental stages, $H(3)= 21.19$, $p=9.63*10^{-5}$. Focused comparisons of the mean ranks between development stages showed that mean PVF pO_2 of class 2A embryos differed significantly from class 3 embryos (*obs. difference*=18.89, *critical difference*=10.94). The difference between class 2A and class 1 was close to the critical difference (*obs. difference*=12.50, *critical difference*=13.60) and when the number of tests was reduced by comparing the open egg stages only to the class 1 embryos, it was found to be a significant difference (*obs. difference*=12.5, *critical difference*=10.97).

Table 1. Mean PVF pO_2, standard deviations and coeffcients of variation in the different age classes

Age class	Mean PVF pO_2 / kPa	Mean standard deviation	Mean coefficient of variation/ %	N	Temperature
Class 1	19.3	0.1	0.8	4	15°C
Class 2A	6.1	1.5	24.4	7	15°C
Class 2B	16.8	3.5	22.1	8	15°C
Class 3	21.1	1.0	4.5	9	15°C

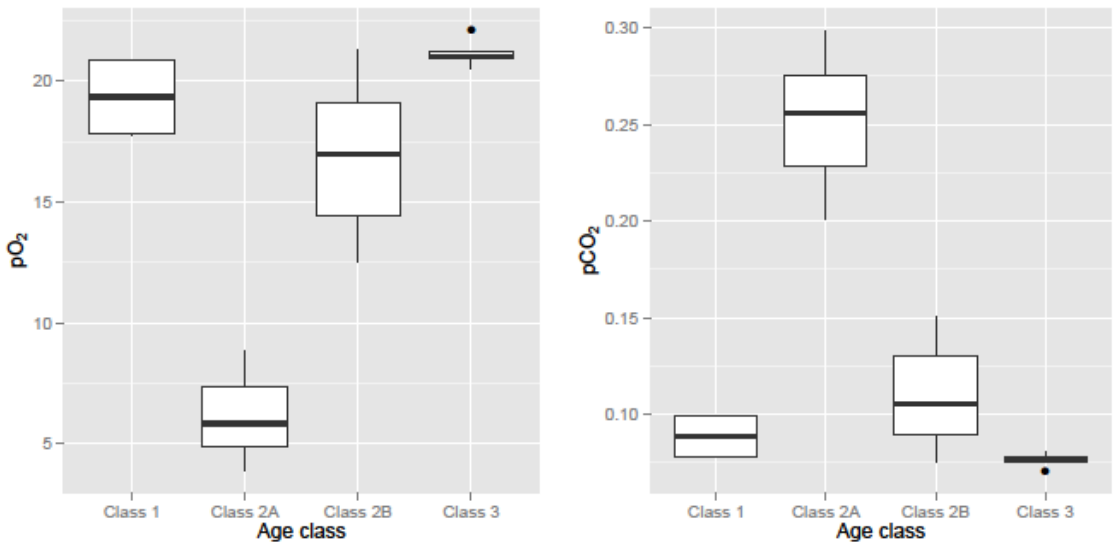


Figure 4. (a) Changes in PVF pO_2 in kPa in relation to the different age classes. (b) Changes in PVF pCO_2 in kPa in the different age classes estimated based on the trendline equation of PVF pO_2 to PVF pCO_2 in class 2B embryos.

3.1.2. PVF pCO_2 and pH

PVF pCO_2 was inversely correlated with PVF pO_2 and reached up to 0.34 kPa at the minimum PVF pO_2 of 2.3 kPa observed in class 2A egg cases (Figure 5). Simultaneously, pH decreased from values >8.0 to values as low as 7.3. There was no significant change in PVF C_T with decreasing PVF pO_2 (Figure 5).

Spearman's rank correlation coefficient was $1.301*e^{-9}$ for pH to pO_2 and $2.738*e^{-11}$ for PVF pO_2 to PVF pCO_2.

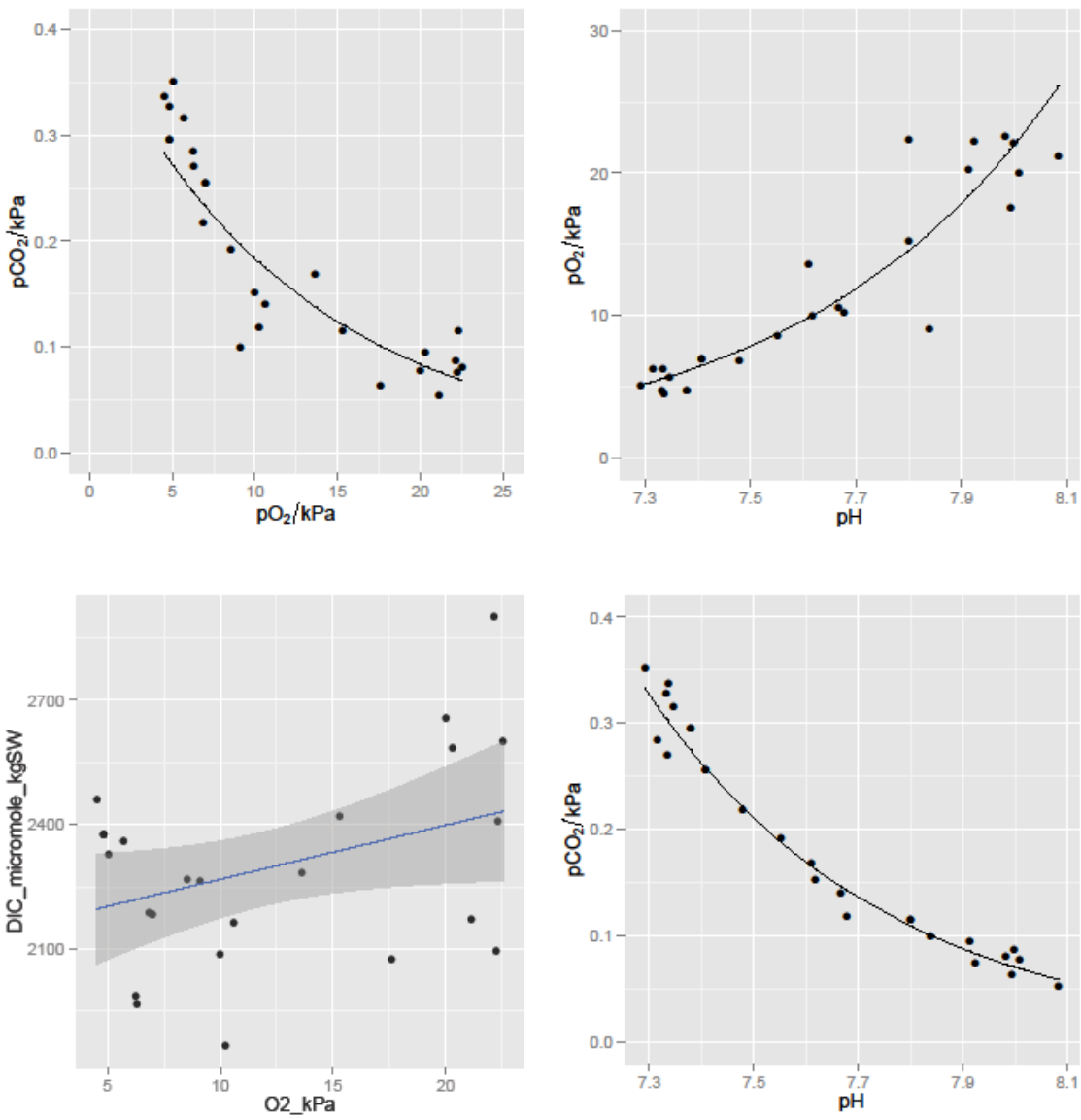


Figure 5. All figures are comprised of data over all age classes acclimated at 15°C. (a) The inverse correlation of PVF pCO_2 and PVF pO_2. (b) The correlation between PVF pO_2 and PVF pH (NBS scale). (c) C_T plotted against PVF pO_2, showing no clear trend. (d) The inverse correlation between PVF pCO_2 and PVF pH.

3.1.3. Ventilation

Ventilation rates (v_f) were recorded at discrete intervals every hour along with continuous PVF pO_2 measurements. Measurements were tested for correlation of v_f and PVF pO_2 in subsets according to age class. Spearman's rank correlation coefficient was not significant for any age class but the oldest. For class 2A the test calculated a p-value of 0.3062, for class 2B the p-value was 0.1611. The correlation coefficient was positive and significant for class 3 embryos at 15°C with a p-value of 0.01677.

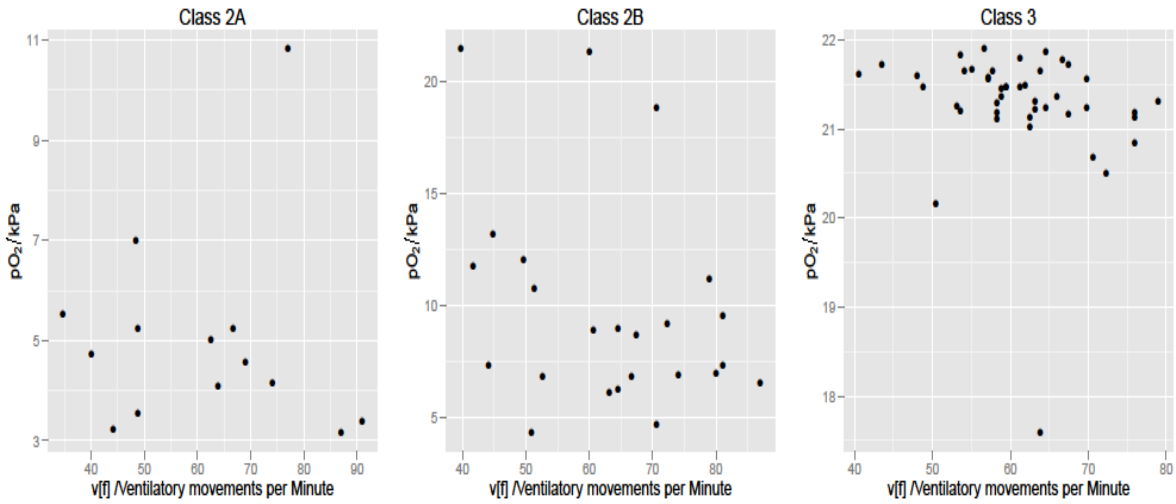
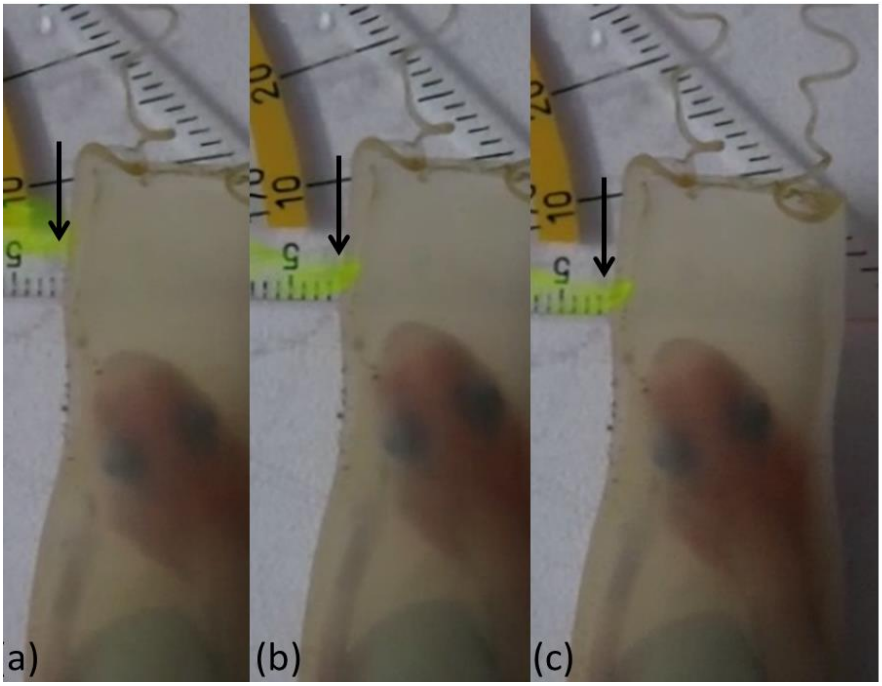


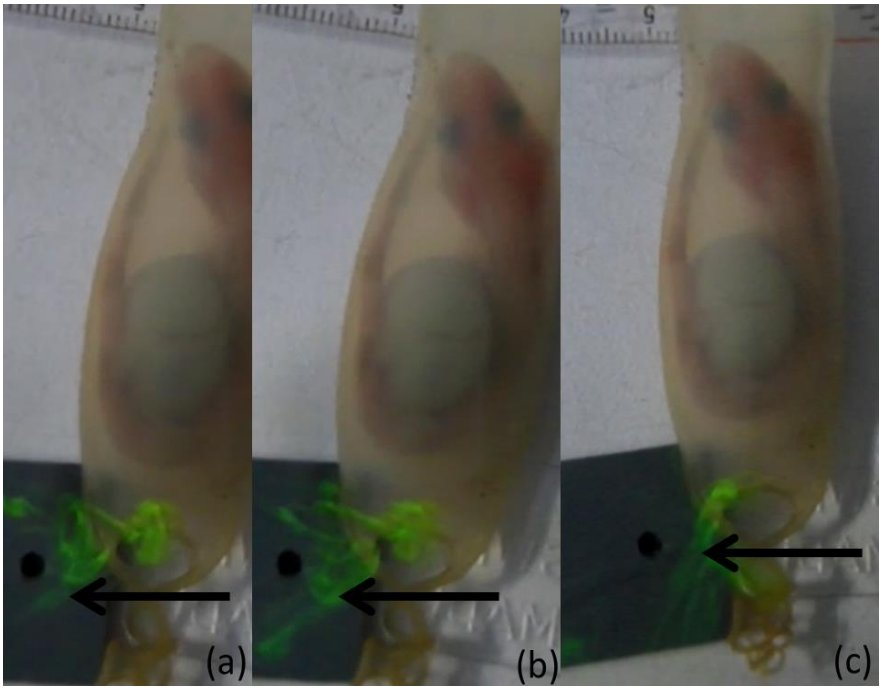
Figure 6. Ventilation frequency of the different age classes and its effect on PVF pO_2, showing no significant correlation in age class 2A and 2B.

3.1.4. Water movement through the egg case

The calcein stain showed that the water was pulled at both anterior slits but more strongly at the one closer to the mouth (Picture 3). It was ejected at the posterior slits (Picture 4). The stain could not be traced in one continuous line as it was diluted too strongly when it passed the gills. However the pull at the anterior slits and push at the posterior slits was observed clearly, when calcein was injected close to the respective slit. Two videos taken during the experiment are included on a CD-Rom at the back of this thesis, showing the inhalation of water at the anterior slits (Video 1) and expulsion of water at the posterior side (Video 2).



Picture 3. Screenshots taken from a video, starting shortly after injection of a calcein stain close to one of the anterior slits in the egg case (a),20 seconds later (b) and 40 seconds after the first (c). The progress of the stain is indicated by the arrow, showing that the dye is pulled into the egg through the anterior slits.



Picture 4. Screenshots from a video taken during a calcein experiment, showing class 2B embryo in the egg case and the movement of the stain away from the posterior slits in the egg case. At (a) directly after injection of the dye, (b) approximately 5 seconds after injection and (c) circa 12seconds after injection. The arrow is used to track the movement of the dye. In video 2 (see attached CD-Rom) the movement of the embryo is clearly visible and causing expulsion of water from the posterior slits.

3.2. Temperature effects

3.2.1. Age at hatching and survival

Age at hatching was distributed normally for all three temperature levels as indicated by a non-significant result in a Shapiro-Wilk test, histograms and qq-plot (Figure 7).

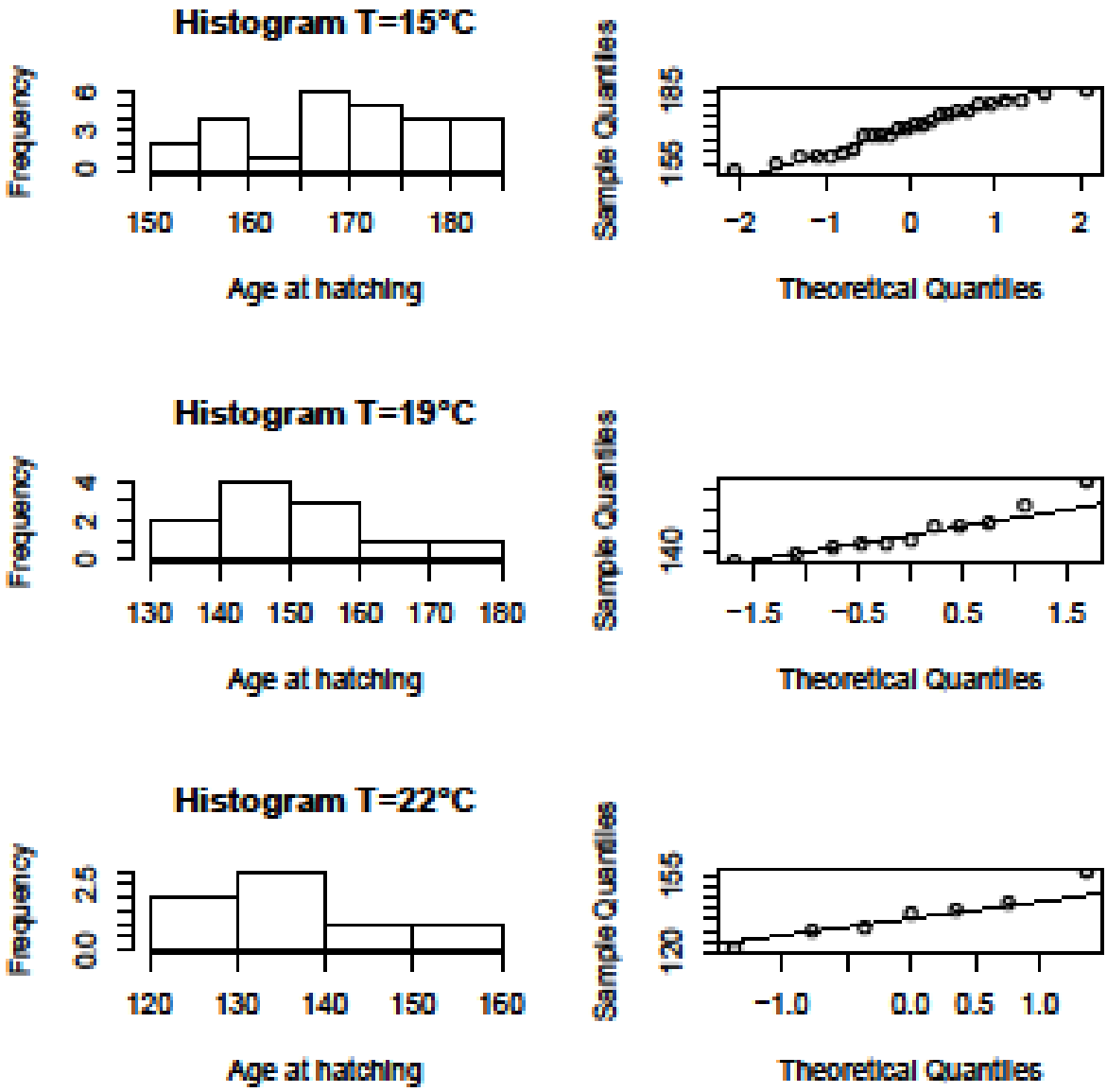


Figure 7. Distribution and sample quartiles of days in the egg until hatching in response to temperature**.** N(15°C)= 26, N(19°C)= 11 and N(22°C)= 7.

Variances were homogeneous and an ANOVA showed a significant difference in age at hatching for the different temperature levels (Figure 8). Mean time until hatching was 169.58 ±9.49 days at 15°C, 149.45±11.33 days at 19°C and 136.57±11.43 days at 22°C.

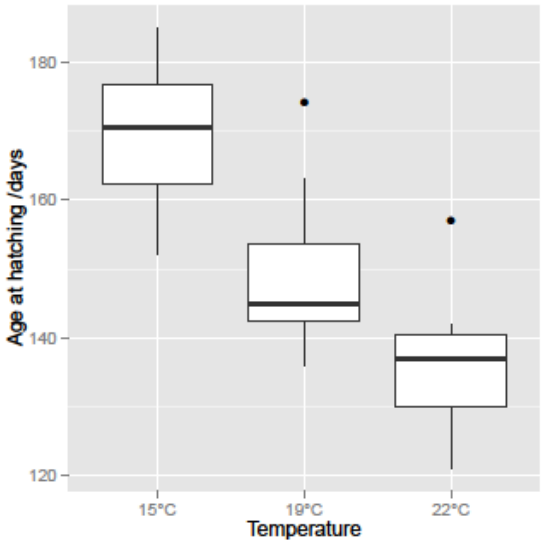


Figure 8. Relationship between temperature (°C) and time spent in the egg case after deposition.

Table 2. Survival rate of embryos acclimated to different temperatures.

Temperature (°C)	15	19	22
Survival (%)	97.0	63.2	56.3
Sample size (n)	33	19	16

A Pearson's Chi-squared test of independence was performed to examine the relation between temperature and survival. The result showed a significant relation, $c^2(df=2,\ N = 68) = 13.7436$, p=0.001037. Survival was significantly lower at higher temperatures (Table 2).

3.2.2. PVF pO_2 for class 3 embryos

Due to high losses at 22°C and the fast development time this temperature level had to be excluded from the analysis as sample size was too small. Data were distributed normally for embryos raised at 15°C but not for those at 19°C as shown by the result of a Shapiro-Wilk test with W=0.88 and p=0.16 at T=15°C and W=0.82 and p=0.04 at T=19°C. Embryos raised at 15°C were subjected to a temperature increase to 19°C over the course of approximately 8 hours and measured again at this temperature. Data from these measurements were distributed normally with W=0.91 and p=0.41. Levene's tests result was non-significant, variances were homogenous between the groups,

F(2.21)=2.5096, p=0.11. Kruskal-Wallis testing indicated a significant difference between the three groups of measurements, *H*(2)=8.85 and p=0.01195. The mean oxygen content of the eggs kept at 15°C differed significantly from the oxygen content in the eggs kept at 19°C, *obs. difference*=9.67 and *critical difference*=7.98. The comparison between the eggs kept at T=15°C and those kept at T=15°C but measured at 19°C (*obs. difference*=7.06, *critical difference*=8.92) and the comparison between those kept at T=19°C and those kept at T=15°C but measured at 19°C (*obs. difference*=2.61, *critical difference*=8.92) were both non-significant.

Table 3. Effect of acclimatization temperature on mean PVF pO_2 and the respective standard deviations and coefficients of variation.

Age class	Mean PVF pO2 / kPa	Mean standard deviation	Coefficient of variation (%)	Number of eggs	Temperature (°C)
Class 3	21.1	1.0	4.5	9	15
Class 3	20.4	1.3	6.7	6	15-19
Class 3	19.3	2.0	11.2	9	19

3.2.3. Ventilation in class 3 embryos

Spearman's rank correlation coefficient was positive and significant for ventilation frequency to pO_2 in class 3 specimens kept at 15°C, 19°C and those transferred from 15°C to 19°C with corresponding p-values of 0.01677, 0.001261 and 0.0306 respectively. Kruskal-Wallis testing showed a significant difference between ventilation frequency at 15°C to both 19°C and the embryos raised to 19°C (Table 4 and Figure 9). Ventilation frequency between the embryos kept at 15°C and measured at 19°C and those kept at 19°C did not differ significantly.

Table 4. Kruskal-Wallis test results for the significance of differences in v_f at different temperatures and different acclimatization temperatures.

Comparisons	Observed difference	Critical difference	Difference
19°C to 15°C	36.1	16.2	TRUE
19°C to 15-19°C	2.0	20.7	FALSE
15°C to 15-19°C	34.0	21.0	TRUE

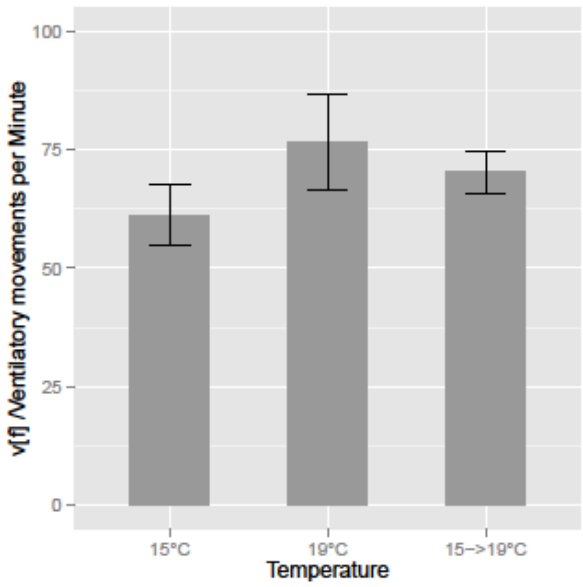


Figure 9. Relation between median v_f and acclimatization temperature, difference is significant for all, except between 19°C and the v_f of the embryos acclimated at 15°C and acutely warmed to 19°C.

4. Discussion

4.1. Does PVF pO_2 decline over development time?

The results of my experiment showed a clear difference in PVF pO_2 and ventilation frequency between the age classes. In early stage eggs, class 1, a high PVF pO_2 close to air saturation was measured (Figure 4), indicating that diffusion is sufficient to sustain the oxygen uptake of the embryo. This is in accordance with the results of Diez and Davenport (1987). When the oxygen need of the embryo increases exponentially with development (Diez and Davenport, 1987), a stage is reached when diffusion needs to be enhanced by strongly increasing the diffusion gradient between seawater and PVF, leading to very low PVF pO_2 of 2.3 kPa. The lowest PVF pO_2 found in this study occurred in class 2A eggs, prior to the opening of the slits and the change to class 2B. This indicates a possible causal connection between PVF pO_2 and the timing of the removal of the plugs covering the egg case slits. PVF pO_2 and ventilation frequency do not correlate significantly at this development stage, again corresponding with descriptions of Diez and Davenport (1987), indicating that movement in class 2A does not give an immediate response in PVF pO_2, thus hiding the influence of movement on PVF pO_2.

After opening of the egg slits at the step from class 2A to class 2B PVF pO_2 increases slightly but is still very variable and significantly below seawater pO_2. The PVF pO_2 of eggs, containing class 2B embryos, does not correlate significantly with embryonic v_f. This is most likely due to the strong PVF pO_2 and

ventilation frequency variability at this stage, as indicated by a high mean coefficient of variation of 22.1%, which is 5 times higher than in class 3 eggs.

When the embryos have developed to class 3, larger than the length of the egg case and strongly restricted in their mobility, PVF pO_2 stabilizes at 21.1 ± 1 kPa and there is a significant correlation between PVF pO_2 and ventilation frequency. These high values indicate that the buccal pumps and rhythmic movement of the tail are sufficient to provide a strong and directed sea water current through the egg case to satisfy the high oxygen demand of the embryo. The results of this study for this egg class are in disagreement with those of Diez and Davenport (1987), who found that PVF pO_2 decreased in older embryos (ca. 200 days at 11 - 15°C, see below). The authors reasoned that this was due to the strong restriction of movement of the embryo by the egg case. My measurements showed that there is no decrease in PVF pO_2 until hatching. The movement of the body is indeed restricted to slight movements of the tail fin and the buccal pumps, but the water volume surrounding the animal in the egg case is minimal and the distance of the mouth to the anterior slits, where water is inhaled, is very small (< 1 cm). Diez and Davenport (1987) did not report the exact developmental stage of the embryo they studied. However they note that they studied a well-developed embryo of ca. 200 days of age which could encompass all embryos in class 2B and above. Embryos in Diez and Davenport's experiment were acclimated at 11°C and transferred to 15°C approximately 2 weeks prior to the measurements. This means that due to the different duration of development at the different temperatures (refer to section 4.3), the given age of 200 days cannot be compared to the ages of animals in this experiment. However, it can be assumed that the embryos, observed by Diez & Davenport, can be roughly placed in class 2B or class 3. Based on the data presented in their paper (Figure 4), PVF pO_2 varied between 10 and 15 kPa , which is consistent with the wide variations of class 2A and B in this study(Diez and Davenport, 1987). An additional difference between our studies could be the positioning of the oxygen electrode within the egg case, which was not clearly described in the paper by Diez and Davenport (1987). Any site posterior of the mouth and gill area would most likely yield considerably lower PVF pO_2 values than the ones measured in this study. However, it seems that the electrode was placed anterior of the mouth opening (personal communication, Prof. Davenport, University of Cork).

It has been suggested that in several marine organisms, PVF pO_2 triggers hatching (de Wachter et al., 1988, Cronin and Seymour, 2000), indicating that PVF pO_2 has a strong impact on developmental timing. The main factors influencing the development of marine animals during the egg phase are temperature, oxygen and egg size (Blaxter, 1969, Kamler, 1992). My results show that hatching in lesser-spotted dogfish is not due to oxygen limitation as PVF pO_2 remain at similar levels as the pO_2 of surrounding water until hatching. Nevertheless the hatching gland of *S. canicula*, which enables

hatching in other marine organism, is active at a different development stage than in most teleost fish. While it releases an enzyme that dissolves the inner layer of the egg case in e.g. the teleost *Clupea pallasi* and leads to hatching of a free swimming larva (Alderdice et al., 1979), the shark hatching gland releases an enzyme at stage 31, which dissolves the mucopolysaccharide plugs which cover the anterior and posterior slits of the egg case (Ballard et al., 1993). In addition it prepares the egg case for hatching by opening the connection between the two layers of the egg case at the anterior, square edge, where the embryo will eventually hatch (Ballard et al., 1993). After this process, it has fulfilled its function and degenerates. The embryo is prepared to hatch at this stage and can in fact be cultivated outside the egg case, when it is carefully removed (Ballard et al., 1993). It uses the protracted pre-hatching period to grow further in the sheltered environment of the egg case. Larval Atlantic salmons (*Salmo salar*) use a similar strategy, they hatch at a similar developmental stage while still carrying an extensive external yolk sac and retreat into a sheltered environment between gravel on the river bed to grow further. Here, the benefit of premature hatching is the enhanced gas exchange efficiency (Wells and Pinder, 1996). Hatching in another salmonid species, the rainbow trout *Oncorhynchus mykiss,* is stimulated by low environmental oxygen (Latham and Just, 1989). Comparing the step from class 2A to 2B to the hatching of salmonids, it is a likely assumption that the opening of the slits, as the equivalent to hatching in salmonids, might also be causally related to a low PVF pO_2. This is supported by this data with the lowest PVF pO_2 values during class 2A. While hatching is apparently not caused by low PVF pO_2 in *S. canicula*, pre-hatching (Ballard et al., 1993) might be hypoxia induced. Retardation of opening of the shark egg case to a maximum degree is probably beneficial, as it delays contact with microbes and metazoan parasites, all of which would challenge the immune system of the embryonic shark (Ballard et al., 1993). It is likely that trade - offs between hypoxia costs and immune costs determine the opening process of the egg case.

Considering that *S. canicula* eggs are deposited in shallow water at the base of macroalgae (Compagno, 2001), where temperature, light as well as pO_2 and pCO_2 vary diurnally and seasonally (Saderne et al., 2013), hatching could be triggered by hypoxic hypercapnia, potentially in combination with thermal stress. Environmental pO_2 in coastal areas has been shown to vary seasonally between 0 and 400 µM from high summer to winter, respectively (Melzner et al., 2013). Adapted to this environment, *S. canicula* embryos have shown a high tolerance to hypoxia, which appears to be lost during development (Diez and Davenport, 1987). Another possible factor triggering hatching is space. During the last egg phase, the embryo is longer than the egg case and lies folded with strongly restricted mobility. As the last of the yolk is transferred from an external yolk sac to an internal one and will sustain the embryo for about ten days after hatching (Ballard et al.,

1993), hatching triggered by starvation or lack of proteins is also not likely. Further research is needed to clarify the trigger for hatching in this species.

4.2. Are PVF pCO2 and pO2 inversely related and do embryos experience severe hypercapnia during development?

The results of this study show a strong inverse relation between PVF pO_2 and PVF pCO_2 in class 2B embryos. A strong inverse relation between pH and PVF pCO_2 has only been shown in one other marine species, the cephalopod mollusk *Sepia officinalis* (Gutowska and Melzner, 2009).The estimated PVF pCO_2 for other egg classes in this study indicated high PVF pCO_2 values in class 2A, thus indicating severe hypercapnia during development. To maintain a sufficient CO_2 excretion rate across the gill epithelia, blood pCO_2 must be significantly higher than the PVF pCO_2. The effect of higher atmospheric pCO_2, as predicted for the future due to anthropogenic CO_2 emissions, will cause an additive effect, increasing the pCO_2 in the ocean by equilibration, causing PVF pCO_2 of eggs deposited in shallow coastal waters to increase to ensure sufficient pCO_2 excretion rates, which in turn causes an increase in the blood pCO_2 of the developing embryo to ensure CO_2 excretion over the gills. Such an additive effect was demonstrated for cephalopod eggs (Hu et al., 2011). Holeton and Heisler (1983) reported that the blood pCO_2 in *Scyliorhynus stellaris* is approximately 0.18 kPa higher than that of the ambient seawater. If the ratio is similar in *S. canicula,* during development they might face values of up to 0.53 kPa in their blood, based on the maximum PVF pCO_2 values estimated in this study. This leads to the need of an extensive acid-base regulatory effort to stabilize blood pH. When fish are exposed to a high pCO_2, blood pH is buffered by an accumulation of HCO_3^- in the blood and an export of Cl^- from the blood (Ishimatsu et al., 2008). Nilsson et al. (2012) described that the strong effects of elevated blood pCO_2 during fish larval development are due to a disturbance of neurotransmitter function, caused by the alterations in plasma Cl^- and HCO_3^- concentrations. These probably cause GABA-A receptors (which are chloride and bicarbonate channels), the main inhibitory neurotransmitters in the vertebrate brain (Bormann et al., 1987), to become excitatory instead of inhibitory. This phenomenon could explain the drastic behavioral changes observed during exposure to elevated sea water pCO_2, such as a reversal of the avoidance reaction to an olfactory predator cue (Ferrari et al., 2011a). Other impacts that might also be related to extracellular acid-base disturbances are impairments in homing ability (Devine et al., 2012) and prey perception (Ferrari et al., 2011b). Jutfelt et al. (2013) demonstrated that strong reactions to increased seawater CO_2 partial pressure are not limited to tropical species but are also present in temperate, shallow water species with a broad temperature and salinity tolerance range such as three-spined stickleback (*Gasterosteus aculeatus*).

Extremely high natural variation in sea water pH have been measured at different coastal and near shore ecosystems, including kelp forests and coral reefs of the US coast (Hofmann et al., 2011). The macrophyte meadows in the Baltic sea, similar to those in the North sea where *Scyliorhinus canicula* eggs are deposited, show an extremely high natural variability of pCO_2 and pH and a strong daily oscillation due to the photosynthethic activity of the macroalgae during the day and respiration during the night (Saderne et al., 2013). *S. canicula* embryos have adapted to this by exhibiting a high hypoxia tolerance and, based on the results of this study, hypercapnia tolerance (Diez and Davenport, 1987).

Scyliorhinus canicula is already well adapted to coping with high pH and pCO_2 values in ambient seawater during the strong oscillations present in their natural habitat. However, while these oscillations are strong, they are short in duration time, so the effect of a long - term increase of pCO_2 in addition to the natural variation and the corresponding decrease of pH in ambient seawater has yet to be studied.

4.3. Does an increase in temperature lead to a decrease in PVF pO_2, increase in ventilation effort and a shorter development time?

Based on this study, an increase in temperature does not cause a significantly lower PVF pO_2 in class 3 eggs. Ventilation effort increased with temperature in large embryos from 61 to 70 and 76 ventilatory movements per minute, after acute and long term warming to 19°C, respectively. This apparently balanced the effect of decreased physical solubility of oxygen in seawater, as well as the increased oxygen demand of the embryo (Butler and Taylor, 1975). The results of this study demonstrated that *S. canicula* embryos hatch approximately 20 days earlier at 19°C than at 15°C. However all embryos hatched up to 130 days earlier (at approximately 170 days after egg deposition at 15°C) than those observed by some other authors at 11°C (Diez and Davenport, 1987) and at 16°C (Thomason et al., 1996a). Nevertheless, the hatching times are consistent with others, such as the 5 to 11 months, i.e. roughly 150 to 330 days described by Compagno (2001) in the wild and with the range of 145 to 179 days described by Ballard et al. (1993) at a temperature of 16°C.

An increase in temperature has been described to cause an increase of the critical oxygen tension in teleosts (Spitzer et al., 1969) and also in adult *Scyliorhinus canicula* (Butler and Taylor, 1975). Butler and Taylor (1975) demonstrated that temperature has a high impact on the reaction to hypoxia in adult dogfish. In their study *S. canicula* behaved as an oxygen regulator at 7°C but showed characteristics of an oxygen conformer at 17°C. If the situation in embryonic dogfish is similar, this could be a strong challenge for embryos during class 2A , when PVF pO_2 is very variable and low even at 15°C. Embryos at this stage are apparently close to the limit of meeting their oxygen demands by means of diffusion at 15°C, so climate change with increased temperatures rising up to 19°C and

above might have lethal effects. This is supported by the observation that 93% of all deaths ocurred prior to reaching class 3.

Contrary to expectations, this study found that ventilation in acutely warmed embryos at class 3 did not differ significantly to those acclimated to the higher temperature over a longer time (compare Figure 7). One would expect that v_f is lowest at control temperature of 15°C, intermediate at 19°C after acclimation and highest at 19°C after an acute warming, following the two types of acclimatization first described by Precht (1958). However, the results of this study indicate that full type two acclimation does not occur at 19°C in *S. canicula* embryos and that this temperature is, in fact, in the suboptimal range for this species. This is supported by the decrease in embryonic survival rates in earlier age classes at 19°C (compare Table 2).Despite this, ventilatory frequency is still sufficient to provide a convective current through the egg case to keep PVF pO_2 almost at the same value as pO_2 of ambient sea water. A long term increase in oceanic temperature, as predicted to occur due to climate change (IPCC 2007), will cause a challenge for the embryonic stages of *Scyliorhinus canicula*, as temperatures might easily surpass the value of 19°C and climb to 22°C and above in late summer. A long term data series from Weymouth, the origin of the studied *S. canicula* population before captivity, shows monthly mean temperatures surpassing 19°C as early as 2003 (Joyce, 2004). The English coast has warmed by a mean 0.3°C per decade over the last century but in detail showing a warming trend of 0.5 to 0.75°C between 1984 and 2004 (Joyce, 2004). This could lead to an avoidance reaction, such as distribution shift north to colder waters or into deeper waters as has been described for different teleost and chondrichthyan species native to the North Sea , e.g. Atlantic cod, *Gadus morhua*, and cuckoo ray, *Leucoraja naevus* (Perry et al., 2005) . Another option is a change in depth of egg laying sites. Egg deposition at deeper sites would lead to colder temperatures but also in some coastal areas to an increased risk of hypoxia as well as a potential lack of suitable macroalgae.

More research is needed to better establish the response in embryonic *S. canicula* to a combined increase in temperature and pCO_2. So far, to my knowledge, no data is available on the role of pCO_2 during development or the effects of changes in pCO_2 during development of *S. canicula*. A similar experiment to Saderne et al. (2013) could provide a suitable range of ambient pCO_2 to conduct an experiment using different pCO_2 treatments. For a first experiment, only pCO_2 should be varied, using a standard temperature such as 15°C to provide a basic understanding of the response. Afterwards a combined temperature and pCO_2 experiment could be designed, based on the information gathered. Temperatures used should be in a similar range to those used in this experiment as they represent both a current mean temperature and a temperature already encountered by *S. canicula* in the wild but, as described in this study, at the edge of its optimum range. Due to the high variation in age at

hatching and size at different life stages between populations, it is important that both these experiments are done on the same population of *Scyliorhinus canicula*.

No predictions can be made to the effects of ocean acidification and a rising seawater pCO_2 on *S. canicula* yet. However, the species will be challenged by rising temperatures due to climate change, despite being very tolerant as adults to different forms of adverse conditions, such as hypoxia, air exposure and even trawling (Butler and Taylor, 1975, Revill et al., 2005). The eggs are sensitive to temperatures of 19°C and above, which are predicted to occur regularly due to climate change within the next decade and already occur in summer (Wiltshire and Manly, 2004). While *S. canicula* has a relatively high age at maturity in comparison to most teleosts, it matures relatively young, when compared to other elasmobranchs, such as spiny dogfish, *Squalus acanthias* (Compagno, 2001). This means, *S. canicula* has a higher chance to adapt to the rising temperatures and higher pCO_2, despite the risk of a decrease in recruitment. Donelson et al. (2012) described a rapid trans-generational acclimation to higher temperatures in teleosts, outlining that one generation can be enough to support drastic changes in temperature tolerance.

These findings show clearly that there are many aspects of climate change and adaptation not understood yet and that predictions on how well a species will cope with rising temperatures cannot be made upon one generation alone. Further insight could be gained from the progress of embryos raised at higher temperatures in contrast to embryos raised at control temperatures and potentially a trans-generational study. This would be work- and time-intensive but it would provide valuable information as *S. canicula* is probably the only shark species, which is easy to obtain in the numbers and ages needed, easy to keep and breed in captivity and simple to rear under controlled conditions. Experiments like this could provide important knowledge about sharks, their biology and how they might cope with the increasing temperatures, predicted for the near-future.

5. Acknowledgements

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7. Selbsterklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die eingereichte schriftliche Fassung der Arbeit entspricht der auf dem elektronischen Speichermedium.

Weiterhin versichere ich, dass diese Arbeit noch nicht als Abschlussarbeit an anderer Stelle vorgelegen hat.