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Research article

Impact of Ocean Acidification and Warming on the bioenergetics of developing eggs of Atlantic herring *Clupea harengus*

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Atlantic herring (*Clupea harengus*) is a benthic spawner, therefore its eggs are prone to encounter different water conditions during embryonic development, with bottom waters often depleted of oxygen and enriched in CO_2. Some Atlantic herring spawning grounds are predicted to be highly affected by ongoing Ocean Acidification and Warming with water temperature increasing by up to +3°C and CO_2 levels reaching ca. 1000 µatm (RCP 8.5). Although many studies investigated the effects of high levels of CO_2 on the embryonic development of Atlantic herring, little is known about the combination of temperature and ecologically relevant levels of CO_2. In this study, we investigated the effects of Ocean Acidification and Warming on embryonic metabolic and developmental performance such as mitochondrial function, respiration, hatching success (HS) and growth in Atlantic herring from the Oslo Fjord, one of the spawning grounds predicted to be greatly affected by climate change. Fertilized eggs were incubated under combinations of two PCO_2 conditions (400 µatm and 1100 µatm) and three temperatures (6, 10 and 14°C), which correspond to current and end-of-the-century conditions. We analysed HS, oxygen consumption (MO_2) and mitochondrial function of embryos as well as larval length at hatch. The capacity of the electron transport system (ETS) increased with temperature, reaching a plateau at 14°C, where the contribution of Complex I to the ETS declined in favour of Complex II. This relative shift was coupled with a dramatic increase in MO_2 at 14°C. HS was high under ambient spawning conditions (6–10°C), but decreased at 14°C and hatched larvae at this temperature were smaller. Elevated PCO_2 increased larval malformations, indicating sub-lethal effects. These results indicate that energetic limitations due to thermally affected mitochondria and higher energy demand for maintenance occur at the expense of embryonic development and growth.

Key words: Atlantic herring, embryonic development, mitochondrial capacity, Ocean Acidification, Ocean Warming, respiration

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Introduction

The atmospheric CO_2 concentration has increased dramatically since the preindustrial era, from ca. 280 µatm to ca. 410 µatm nowadays causing an increase in average ocean surface temperatures of about 0.83°C and a decrease in surface water pH of 0.1 units (Bopp *et al.*, 2013). If the rate of emissions does not change, the level of atmospheric CO_2 is expected to rise to ca. 851–1370 µatm by the year 2100 causing an average warming of 3.15°C and a decrease of 0.41 pH units in the ocean surface waters (Henson *et al.*, 2017).

While juvenile and adult fish appear to tolerate CO_2 levels far beyond average climate change predictions (>2000 µatm, Ishimatsu *et al.*, 2008), early life stages, such as developing embryos and lecithotrophic larvae, appear to be more vulnerable to both, Ocean Acidification and Warming (Baumann *et al.*, 2011; Chambers *et al.*, 2014; Pimentel *et al.*, 2014; Frommel *et al.*, 2016; Stiasny *et al.*, 2016; Dahlke *et al.*, 2017; Sswat *et al.*, 2018). This is probably due to simple modes of respiration (dermal vs. gills) during development and insufficient acid–base regulation before the formation of gills, a situation possibly exacerbated by the higher surface to volume ratio of the early stages compared to adults (Kikkawa *et al.*, 2003; Ishimatsu *et al.*, 2008). Moreover, developing embryos and lecithotrophic larvae are entirely dependent on parental provisioning of resources (yolk) and molecular defence mechanisms (Kamler, 2008) which may become limiting in a changing environment. Exposure to elevated PCO_2 (CO_2 partial pressure) has been found to adversely affect embryonic development (Tseng *et al.*, 2013; Dahlke *et al.*, 2017), larval growth and survival (Baumann *et al.*, 2011) and tissue/organ health (Frommel *et al.*, 2016) in some fish species. However, in other species, studies have not detected any effect on embryogenesis (Franke and Clemmesen, 2011; Maneja *et al.*, 2015), hatching (Frommel *et al.*, 2012) or growth and development (Munday *et al.*, 2011; Frommel *et al.*, 2012; Hurst *et al.*, 2012, 2013; Bignami *et al.*, 2014). Thus, it is important to understand which are the mechanisms underlying the sensitivity towards Ocean Acidification and Warming in the early life stages to assess which fish species will be affected more by the ongoing climatic changes.

Thermal acclimation and acid–base regulation may increase the metabolic costs of development, causing the reallocation of the yolk-limited resources from development and growth to maintenance (Rombough, 2011; Dahlke *et al.*, 2017). Studies of energy metabolism in developing fish eggs have been concerned mainly with measuring levels of potential energy reserves, metabolites and relevant metabolic enzyme systems (Tocher *et al.*, 1985; Finn *et al.*, 1996; Finn and Fyhn, 2010), and only recently, studies have begun to address how Ocean Acidification and Warming affect the metabolism of fish embryos (Flynn *et al.*, 2015; Pimentel *et al.*, 2016; Dahlke *et al.*, 2017).

In an early study, Boulekbache (1981) described the energetic charge of ATP in the developing embryos of rainbow trout (*Oncorhynchus mykiss*). He found a decrease of ATP/ADP during cleavage (morula–blastula), then a slight increase during gastrulation followed by a plateau. This profile could represent a heavy utilization of pre-existing ATP during the early stage followed by a neosynthesis in parallel to increased embryo cell movement and diversification (Boulekbache, 1981; Tocher *et al.*, 1985). Since the ATP consumption profile seems to be correlated with specific moments of embryonic development and since at these stages ATP production may be limited by the endogenous resources (yolk), ATP production pathways, mitochondrial metabolism in particular, could play a key role in the embryonic sensitivity to ocean acidification and warming.

Atlantic herring (*Clupea harengus*) is a benthic spawner and schooling pelagic fish. It is widely distributed throughout the North Atlantic shelf regions from the East coast of North America to the West coast of Europe and the Baltic Sea Herring populations represent a major resource not only for other fish species, birds and whales (Lynam *et al.* 2015), but also for commercial fisheries, with annual catches of more than 2 million tones (FAO, 2018). Herring population dynamics are known to be sensitive to changes in water temperature: in the last centuries cool periods promoted the increase of herring biomass and the southward expansion of the distribution areas, while warming events exerted the opposite effect (Alheit and Hagen, 1997). Moreover, herring spawn over extended periods with a wide range of spawning locations specific to seasons and populations (Geffen, 2009). Therefore, herring populations spawning in areas predicted to be severely affected by climate changes may be more vulnerable to Ocean Acidification and Warming, especially those spawning during the summer/autumn season. For example, PCO_2 values above 4000 µatm could be reached in the future at important herring spawning grounds in the Baltic Sea such as the Kiel Fjord (Melzner *et al.*, 2013; Frommel *et al.*, 2014) and at higher latitudes including the Skagerrak and North Sea. PCO_2 is predicted to double with an increase in temperature of more than 3°C (Henson *et al.*, 2017; van Vuuren *et al.*, 2011: RPC 8.5). Studies of developing herring embryos from the Baltic Sea found no significant effect of high PCO_2 (4600 µatm) in hatch rate, development, or otolith size (Franke and Clemmesen, 2011). However, no studies have so far addressed the combined effects of increasing temperature and PCO_2 on the embryonic development of this fish.

It is thus important to understand the effects of ocean acidification and warming on the development of herring embryos under the conditions predicted to occur at the spawning grounds by the end of the century. To do so, we incubated developing embryos of Atlantic herring from the Scandinavian coast within a cross-factorial combination of three temperatures (6–10–14°C) and two PCO_2 levels

(400–1100 µatm), to mirror present conditions and the conditions projected for the end of the century (RCP. 8.5; van Vuuren *et al.*, 2011; Henson *et al.*, 2017) for the entire developmental period, from fertilization to hatch. We analysed mitochondrial respiration and the oxygen demand of late-stage embryos (50% eye pigmentation) to investigate mitochondrial function in relation to embryonic energy demand, respectively. Furthermore, we observed hatching success (HS), larval deformities and larval length at hatch to identify constraints on performance at the whole-organism level.

Methodology

This study was conducted at the Sven Lovén Centre for Marine Science, Kristineberg Biological Station (University of Gothenburg, Sweden) between April and May 2013 in accordance with the legislation of the Swedish Board of Agriculture (Permit: 332–2012).

Experimental animals

Ripe Atlantic herring, *C. harengus*, were caught with gill nets during the spawning season in April 2013 in the inner Oslo Fjord (Norway). Selected fish were caught and killed with a blow on the head and were stored on ice and transported to the Sven Lovén Centre. Gametes of males ($n = 3$) and females ($n = 3$) used for *in vitro* fertilizations were obtained by strip spawning approximately four hours after the fish had been caught.

Experimental design

A full-factorial design with three temperatures (6, 10 and 14°C) and two PCO_2 (400 µatm and 1100 µatm) was used for fertilization and incubation of herring eggs. Treatment conditions were selected to encompass ambient spawning temperatures (6–10°C and the PCO_2 recorded in the year 2013) as well as water warming and acidification projected for the end of this century according to IPCC's business-as-usual scenario (RCP 8.5, van Vuuren *et al.*, 2011). Eggs produced by different females were incubated separately and 'females' were treated as biological replicates ($n = 3$). Each female was represented by two incubators at each treatment combination (2 × 3 females × 6 treatments = 36 incubators in total). In order to avoid biased survival estimates, only one of both incubators was used to collect samples for measurements of oxygen consumption rates and mitochondrial capacities. The second incubator was used to evaluate HS and larval morphology at hatch. Incubator classification and arrangement within experimental units was done randomly.

Fertilization protocol

The eggs of each female were stripped onto 12 plates of Polyethylene mesh (500 µm mesh size, 10 cm diameter). To optimize fertilization success and oxygenation during development, care was taken to arrange the eggs in single layer. Two out of 12 egg-plates were fertilized at each of six different temperature × PCO_2 treatment combinations (Table 1) following a wet-fertilization protocol (Geffen, 1999). Individual egg-plates were placed in Petri dishes and incubated for 10 min with a milt-seawater dilution of 1:500 (10 ml, produced with milt aliquots from $n = 3$ males). After being carefully rinsed, the egg-plates were transferred into hatching jars filled with 1 l of filtered (0.2 µm) and UV sterilized seawater (adjusted to the respective treatment combination). The percentage of fertilized eggs on each plate (i.e. fertilization success) was determined by visual inspection under a stereomicroscope after 12 h of incubation. Mean values are shown in Table 1.

Incubation

The incubation set-up is shown in Supplementary Figure S1. Herring eggs adhered to mesh-plates were incubated within transparent, bottom tapered hatching jars (Imhoff sedimentation cones, 1000 ml volume, Supplementary Fig. S1A), which were submerged into 400 l seawater baths thermostatted to different temperatures (6, 10 and 14°C, Supplementary Fig. S1B). Each incubator was sealed with a Styrofoam lid to prevent outgassing of CO_2. Eggs received dim light with a daily rhythm of 12 h/12 h light/darkness. Every 24 h, 80% of the water volume of each incubator was replaced by filtered (0.2 µm) and UV-sterilized seawater (33 PSU) to avoid oxygen depletion and bacterial or fungal infestation. Herring eggs were not exposed to air during water exchange. Each water bath contained two 60-l reservoir tanks, which were used to pre-adjust exchange-seawater to the corresponding temperature and PCO_2. Water temperatures of the different water baths were recorded automatically every 15 min (±0.1°C, Table 2) by a multi-channel aquarium computer (IKS-Aquastar, IKS Systems, Germany).

Elevated PCO_2 conditions were administered by injection of pure CO_2 gas into the submerged 60 l reservoir tanks by bubbling through large aeration stones (20 cm length). A multi-channel feedback system (IKS-Aquastar), connected to individual pH-probes (IKS-Aquastar) and solenoid valves were used to adjust PCO_2 values. Pure CO_2 was infused via perforated silicone tubes until the desired pH/PCO_2 was reached. The PCO_2 of the reservoir tanks was measured *in situ* prior to every second water exchange with an infrared PCO_2 probe (Vaisala GM70, manual temperature compensation, ±10 µatm accuracy; Vaisala, Finland). The probe was equipped with an aspiration pump and sealed with a gas-permeable membrane to measure PCO_2 in air equilibrated with dissolved CO_2 in the water, as described by Munday *et al.* (2013) and Jutfelt and Hedgarde (2013). Factory calibration was confirmed by measurements of seawater previously bubbled with a technical gas mixture (1010 µatm CO_2 in air; AGA Sweden). Prior to the daily water exchange, pH-values of the reservoir tanks were measured with a lab-grade pH-electrode to three decimal places (Mettler Toledo InLab Routine Pt 1000 with temperature compensation, Mettler Toledo, Switzerland), which was connected to a WTW 3310

pH-meter. A two-point calibration with NBS-buffers was performed on a daily basis. To convert NBS to the free proton concentration scale for seawater pH (Waters and Millero, 2013), the electrode was recalibrated with Tris-HCl seawater buffers (Dickson *et al.*, 2007), which were acclimated to the corresponding incubation temperature prior to each measurement. Seawater pH-values refer to the free proton concentration scale throughout this manuscript (for summary see Table 2), Individual values for each measured parameter are available in the Open Access library PANGAEA (see 'Data availability' section).

Data collection

Whole-embryo oxygen consumption

Oxygen consumption rates (MO_2) of late-stage embryos (at 50% eye pigmentation) were measured in closed, temperature-controlled respiration chambers (OXY0 41 A, Collotec Meßtechnik GmbH, Germany, Supplementary Fig. S2) following methodologies described by Schiffer *et al.* (2014). All measurements were performed in duplicates (with two respiration chambers) at the same developmental stage (~50% eye pigmentation) and treatment as during incubation. Staging was done by visual inspection during the daily water exchange. Development times until 50% eye pigmentation (and hatching) did not differ between PCO_2 treatments (Supplementary Fig. S3 and Table S1), as was demonstrated for Baltic herring under more extreme PCO_2 conditions (4 600 µatm, Franke and Clemmesen, 2011). The stage at 50% eye pigmentation was selected because it represents a clearly discernible developmental landmark (Hill and Johnston, 1997) at which the embryonic cardiocirculatory system, and thus metabolic capacity, is already well-developed (Hill and Johnston, 1997). For each run, ~20 (±3) eggs were loaded into each of the two respiration chambers. The chambers were previously filled with a volume of ~2 ml sterilized seawater, whereby each chamber was alternately used for different PCO_2 treatments. The eggs were placed on a polyethylene mesh (500 µm mesh size) with a magnetic micro-stirrer (3 mm) underneath to avoid oxygen stratification within the respiration chamber (see Supplementary Fig. S2). The change in oxygen saturation was detected by micro-optodes (fiber-optic microsensor, flat broken tip, diameter: 140 µm, PreSens GmbH, Germany) connected to a Microx TX3 (PreSens GmbH, Germany). Recordings were stopped after 60 min (at 6°C) or as soon as the oxygen saturation declined below 80% air saturation (20–40 min at 10 and 14°C). After each run, the wet mass per egg and the exact water volume of the respiration chamber was determined by weighing on a precision balance (±0.01 mg). Bacterial oxygen consumption (always below 5%) and optode drift (always below 1%) was determined by blank measurements before and after three successive runs with eggs. Given that egg masses did not differ between temperature and PCO_2 treatments (Supplementary Fig. S3), MO_2 was expressed as (nmol O_2 egg^{-1} h^{-1}) according to the following formula: $MO_2 = DO_2 * Vol/N_{Eggs}$, where DO_2 is the decline in oxygen saturation (nmol l^{-1} h^{-1}), Vol is the water volume of the respiration (ml) chamber and N_{Eggs} is the number of eggs.

Table 1: Mean ± SEM fertilization success of Atlantic herring (*Clupea harengus*) eggs fertilized at different levels of temperature and PCO_2. Differences between temperature and PCO_2 treatments were statistically not significant ($F = 1.9$, $P = 0.192$ and $F = 0.97$, $P = 0.344$, respectively)

Temperature (°C)	Fertilization success (%)	
	Control PCO_2	High PCO_2
6	88.8 ± 2.8	79.4 ± 4.3
10	76.0 ± 9.0	72.2 ± 8.6
14	88.9 ± 8.5	85.3 ± 8.0

Mitochondrial function

Mitochondrial function was measured in a cellular suspension of late-stage eggs (at 50% eye pigmentation) as described in Dahlke *et al.* (2017). Briefly, one hundred eggs from $n = 3$ females were gently ground on ice in a glass potter filled with 2-ml ice-cold modified mitochondrial respiration medium MiR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 160 mM sucrose, 1 g l^{-1} bovine serum albumin, pH 7.4, 380 mOsmol l^{-1}) (Iftikar and Hickey, 2013; Gnaiger *et al.*, 2015). The resulting suspension was collected avoiding the collection of the eggshells and mitochondrial respiration was analysed using Oroboros Oxygraph-2k™ respirometers (Oroboros Instruments, Innsbruck, Austria). The

Table 2: Summary table of the water parameters measured during the incubation of Atlantic herring (*Clupea harengus*) eggs until hatch. Data are presented as mean ± SD

Duration (days)	Nominal T (°C)	Measured T (°C)	Oxygen (%)		PCO_2 (µatm)		pH_F	
			Control PCO_2	High PCO_2	Control PCO_2	High PCO_2	Control PCO_2	High PCO_2
27	6	6.15 ± 0.06	94.40 ± 0.71	94.40 ± 0.61	415 ± 10	1101 ± 47	8.15 ± 0.02	7.77 ± 0.02
16	10	10.04 ± 0.06	94.40 ± 0.63	94.40 ± 0.49	408 ± 10	1050 ± 46	8.17 ± 0.02	7.79 ± 0.03
11	14	14.07 ± 0.20	95.00 ± 0.00	95.00 ± 0.00	403 ± 12	1050 ± 29	8.18 ± 0.02	7.78 ± 0.02

oxygen flux (nmol O_2 (egg* h)$^{-1}$) was recorded and calculated in real-time using Oroboros DatLab 5.2.1.51 (Oroboros Instruments, Innsbruck, Austria). Measurements were conducted in MiR05 buffer equilibrated to atmospheric PCO_2 and acclimation temperature of the eggs. The cO_2 ranged from atmospheric saturation (ca. 370 nmol ml^{-1}) to 150 nmol ml^{-1}. A substrate–uncoupler–inhibitor titration (SUIT) protocol was used to investigate the capacities of the single components of the electron transport system (ETS) measured as oxygen consumption attributable to each component (nmol O_2 (egg* h)$^{-1}$). In detail: ETS capacity was measured by step-wise (1 µM each) titration of carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone (FCCP) in the presence of Complex I (CI) and Complex II (CII) substrates (10 mM glutamate, 2 mM malate, 10 mM pyruvate and 10 mM succinate). CI, CII and Complex III (CIII) were inhibited by the addition of 0.5 µM rotenone, 5 mM malonate and 2.5 µM antimycin a, respectively. All chemicals were obtained from Sigma-Aldrich (Germany).

Hatching success

Once hatching started, free-swimming larvae were collected in the morning, euthanized with an overdose of tricaine methane sulphonate (MS-222) and counted after visual examination for morphological deformities under a stereomicroscope. The incidence of larval deformities was quantified as the percentage of hatchlings that exhibited severe deformations of the yolk sac, cranium or vertebral column. HS, defined as the percentage of non-malformed larvae that hatched from fertilized eggs, was calculated as:

$$HS = (L_i - L_d)/E_f * 100$$

where L_i represents the number of hatched larvae, L_d is the number of deformed larvae and E_f is the number of fertilized eggs (shown in Table 1).

Larval size at hatch

Subsamples of 10–30 non-malformed larvae of three females at each treatment combination were photographed for subsequent measurements of larval standard length (SL) using Olympus image analysis software (Stream Essentials©, ± 1 µm). Only samples obtained from the same daily cohort (during peak-hatch at each temperature treatment) were used for statistical comparison between PCO_2 treatments.

Data analysis

All data are presented as mean ± SEM.

Statistical analyses were conducted using R 3.2.0 (R Core Team, 2015) and the level of statistical significance was set at $P < 0.05$ for all the statistical tests.

Normal distribution and homoscedasticy of the data were assessed by Shapiro–Wilk and Bartlett's tests, respectively.

Multi factorial analyses of variance (two-way ANOVA) including the female parent ID as covariance were used to evaluate whether temperature and PCO_2 and the combination of both factors had an effect on the parameters object of this study. The two-way ANOVA was followed by Tukey's HSD test for temperature and Student's *t*-test for CO_2.

In addition, MO_2 data were analyzed with the female-specific egg mass included in the model as covariate.

The temperature coefficient Q_{10} was calculated according to the equation:

$$Q_{10} = (R_1/R_2)^{10/(T2-T1)}$$

R: respiration rate
T: temperature at which the respiration rate was measured.

Results

Mitochondrial function and whole-embryo respiration

The *in vivo* oxygen consumption rates (MO_2) of Atlantic herring embryos were affected only by temperature ($F = 173.87$, $P < 0.001$, Fig. 1b). In general, MO_2 increased with temperature in a non-linear fashion: expressed as Q_{10}, the increase in MO_2 between 6 and 10°C (Control PCO_2: 2.30 ± 0.23; High PCO_2: 2.00 ± 0.20) was lower than between 10 and 14°C (Control PCO_2: 2.27 ± 0.14; High PCO_2: 3.17 ± 0.27).

In vitro, the mitochondrial oxygen flux corresponding to the maximum capacity of the ETS was affected only by temperature ($F = 5.61$, $P = 0.019$, Fig. 1a) and increased between 6 and 10°C ($P = 0.04$), but, unlike whole-embryo MO_2 (Fig. 1), reached a plateau between 10 and 14°C ($P > 0.05$). CI and CII contributed differently to the ETS according to temperature ($F = 17.28$, $P < 0.001$, Fig. 2). CI contribution declined with increasing temperature while CII contribution increased (Fig. 2). At 14°C, only 37% of the ETS capacity was contributed from CI, compared with 62% at 6°C (Fig. 2).

Viable hatch and length at hatch

HS (Fig. 3) was significantly affected by temperature ($F = 14.07$, $P = 0.001$) with a reduction of hatched larvae at 14°C compared with the other acclimation groups (6–14°C: $P = 0.005$; 10–14°C: $P = 0.001$; Fig. 3a). Elevated PCO_2 had no significant effects on HS but caused a significant reduction ($P = 0.02681$) of the HS in the group incubated at 6°C (64.26 ± 1.72%) compared to the group incubated at the same temperature but under control PCO_2 (75.43 ± 2.85%).

The proportion of larvae hatching with severe morphological malformations was higher in the groups incubated under high PCO_2 ($F = 13.03$, $P = 0.004$, Fig. 3b) with percentages almost doubled compared with the groups incubated under control PCO_2 (tab. 1). Larval malformations

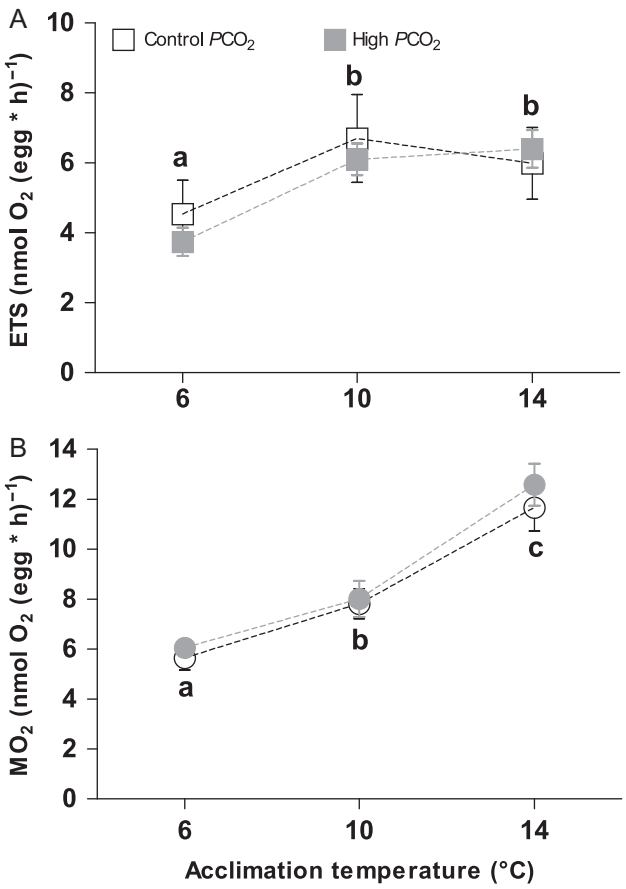

Figure 1: Respiration performance and mitochondrial capacity of Atlantic herring (*Clupea harengus*) embryos at 50% eye pigmentation stage. Values are reported as mean ± SEM. Panel **A**: Electron Transport System (ETS) capacity. Open squares: control PCO_2 (400 µatm), solid squares: high PCO_2 (1100 µatm). Panel **B**: Whole-embryo respiration. Open circles: control PCO_2 (400 µatm), solid circles: high PCO_2 (1100 µatm). Different letters within panels indicate significant differences ($P < 0.05$) between temperature treatments independent of the CO_2 treatment.

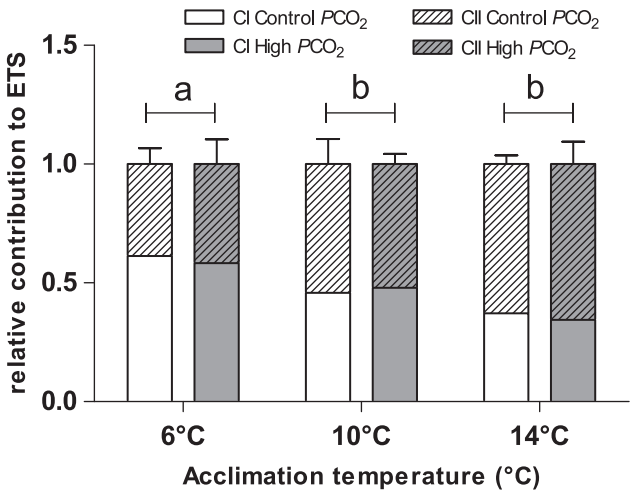

Figure 2: Contribution (%) of Complex I and Complex II to the electron transport system (ETS) in the embryos of Atlantic herring (*Clupea harengus*). Embryonic stage: 50% eye pigmentation. Values are reported as mean ± SEM. Open bars: Complex I, dashed bars: Complex II. Open bars: control PCO_2 (400 µatm), solid bars: high PCO_2 (1100 µatm). Different letters indicate statistical differences ($P < 0.05$) between temperature treatments independent of the CO_2 treatment.

were not significantly correlated with increasing temperatures ($F = 1.67$, $P > 0.05$) and there was no interactive effect between temperature and CO_2.

SL at hatch (Fig. 4) was significantly affected by temperature ($F = 43.12$, $P < 0.001$) with a trend toward reduction with warming. SL was not affected by elevated PCO_2 ($F = 9.11$, $P > 0.05$).

Discussion

In this study, we analysed the development and mitochondrial function of Atlantic herring (*C. harengus*) embryos that were incubated to either current water conditions, or to conditions projected for the end of this century in waters surrounding the Scandinavian coast; one of the main spawning grounds of this species in the North Atlantic.

In general, we found that elevated temperature reduced HS and high PCO_2 caused larval malformation. Mitochondrial function was not affected by elevated PCO_2; however, temperature played a major role in shaping mitochondrial respiration, with subsequent effects on embryonic respiration and body length at hatching; which is in line with other studies on Atlantic herring (Geffen, 2002; Peck *et al.*, 2012).

The capacity of the ETS increased with temperature between 6°C and 10°C without a further increase at 14°C; however, the relative contribution of CI and CII to the ETS changed with temperature (for the entire range 6–14°C), with the contribution of CI being negatively correlated to temperature. In developing teleost fish, embryos mainly rely on carbohydrates during the initial phase of development, until blastula (Kamler, 2008), then catabolize amino acids from protein (benthophils) or free amino acids (FAA, pelagophils), together with lipids (Finn and Fyhn, 2010). In a study on Atlantic cod (*Gadus morhua*) eggs, Fyhn and Serigstad (1987) showed that the FAA content of the yolk was depleted by ~90% during spawning to hatching, but without a corresponding increase in the protein content of the developing embryo. Moreover, they found that alanine, serine, leucine, isoleucine, lysine, and valine (in that order) quantitatively dominated the amino acids pool, and accounted for ~75% of the decrease. Alanine, serine, leucine, isoleucine and lysine enter the TCA cycle at the citrate synthase step, via pyruvate (alanine and serine) or via acetyl-coA and acetoacetyl-coA (leucine, isoleucine and lysine); both of which are fed into CI and CII. Only valine and isoleucine enter the cycle via succinyl-coA and feed directly into CII. Taking this into account, the reduction of CI contribution to

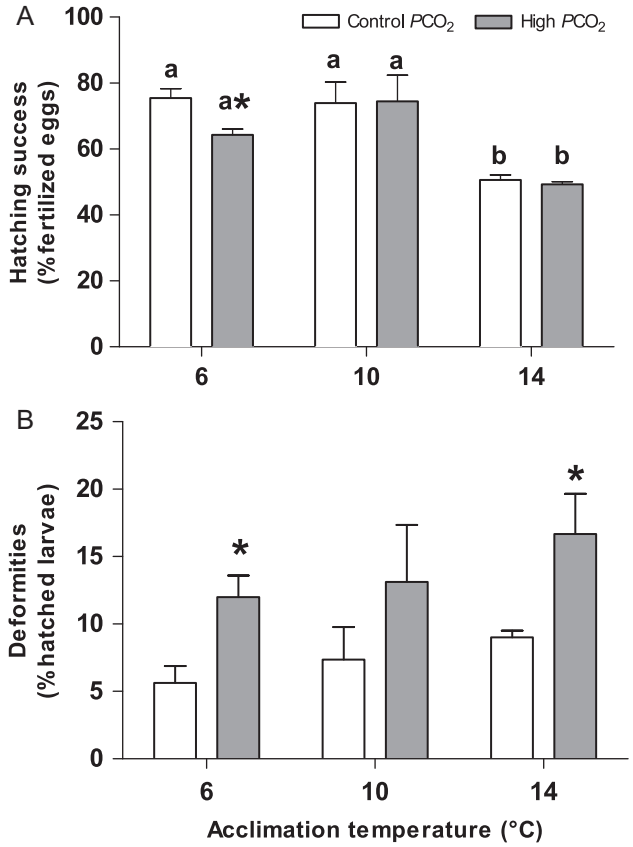

Figure 3: Viable hatch of Atlantic herring (*Clupea harengus*). Values are reported as mean ± SEM. Panel **A**: hatching success as percentage of fertilized eggs that hatch. Open bars: control PCO_2 (400 µatm), solid bars: high PCO_2 (1100 µatm). Panel **B**: Larval malformations as percentage of hatched larvae. Open bars: control PCO_2 (400 µatm), solid bars: high PCO_2 (1100 µatm). Different letters indicate statistical differences ($P < 0.05$) between temperature treatments, * indicates significant differences ($P < 0.05$) between CO_2 groups at the same temperature.

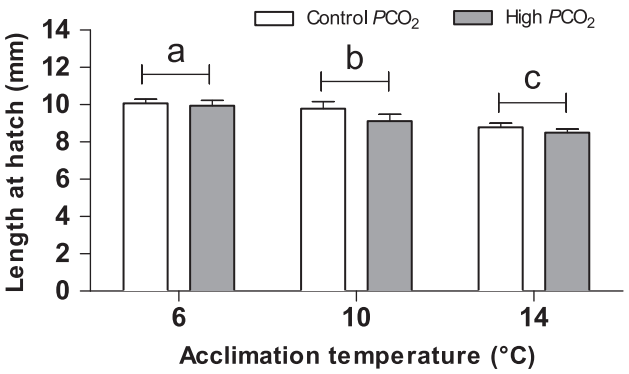

Figure 4: Length at hatch (mm) of Atlantic herring larvae (*Clupea harengus*). Values are expressed as mean ± SEM. Open bars: control PCO_2 (400 µatm), solid bars: high PCO_2 (1100 µatm). Different letters indicate significant differences ($P < 0.05$) between temperature treatments independent of the CO_2 treatment.

the ETS, in favour of CII, could indicate a shift in metabolic pathways from the preferred CI feeding amino acids (alanine, serine, leucine, isoleucine and lysine) to CII feeding amino acids (valine and isoleucine), as a result of increasing temperature. However, several studies have reported a reduced contribution of CI to the ETS with decreasing temperature in adult fish and embryos, with suggested causes being either a lack of substrates or a change in membrane fluidity (Hilton *et al.*, 2010; Iftikar *et al.*, 2015; Dahlke *et al.*, 2017). These two hypotheses are not contradictory, but complement (and even cause) each other.

A shift in ETS contribution from CI to CII results in a less efficient ATP production pathway, since each cycle of the TCA cycle theoretically produces ~7.5 ATP from CI, but only ~1.5 ATP from CII. The decreased ATP provision at higher temperatures would need compensation by an increase in embryonic respiration (MO_2) as seen at 14°C in this study. In addition to the shift in the contribution of the individual complexes to ETS, increasing temperature may also cause a rise in mitochondrial uncoupling, increasing oxygen demand to compensate for the increased proton leak (Weinstein and Somero, 1998; Hardewig *et al.*, 1999). Therefore, an animal's respiratory rate (MO_2) may increase in order to partially compensate for these constraints. However, a limit may be reached where the animal is no longer able to provide oxygen to mitochondria or aerobically produce enough ATP; which may lead to constraints on performance, the onset of anaerobic metabolism and eventually death (Hardewig *et al.*, 1999; Pörtner, 2002).

In this study, we identified several negative effects of decreased ATP production efficiency at a higher incubation temperature (14°C). There was a decreased HS at this elevated temperature and the larvae that hatched at 14°C were smaller than the larvae from other incubation temperatures, indicating that less energy was available for development. Therefore, high temperature (14°) may have limited mitochondrial function, which is mirrored at the whole-organism level, by the decreased length and HS. This provides a link between thermal sensitivity of energy metabolism and the effects of warming at the whole-organism level.

Elevated PCO_2 caused a significant increase in larval deformities. This is similar to the findings of Frommel *et al.* (2014), which showed that elevated PCO_2 caused significant organ damage and reduced growth in the larvae of Atlantic herring. However, in another study on Atlantic herring embryos, (Franke and Clemmesen, 2011) found no significant effect of elevated PCO_2 (levels up to 4635 µatm) on egg mortality or the occurrence of embryonic malformations. These contrasting findings could be partially explained by the different origins of the herring populations. The herring used in this study and the study by Frommel *et al.* (2014) came from the Scandinavian coast, while the herring used in the study by Franke and Clemmesen came from the Kiel Fjord in the Baltic Sea, where PCO_2 levels are above

2300 µatm due to upwelling events (Thomsen *et al.*, 2010). Atlantic herring display high plasticity in physiological tolerance (Geffen, 2009; Peck *et al.*, 2012), allowing different populations to spawn in different seasons and live in a broad range of temperatures and salinities (Geffen, 2009). Herring lay adhesive benthic eggs (Nash *et al.*, 2009; Schmidt *et al.*, 2009) and therefore encounter potentially challenging hydrographic conditions during egg development, since bottom waters are often depleted of oxygen and enriched in CO_2, relative to surface waters. These results contribute to the growing evidence of differences in the sensitivity towards Ocean Acidification and Warming between herring populations (Franke and Clemmesen, 2011; Sswat *et al.*, 2018) and compared to pelagic spawners such as Atlantic cod, flounder and many tropical reef species (Chambers *et al.*, 2014; Munday *et al.*, 2016; Dahlke *et al.*, 2017).

Conclusions

Our study assessed the effects of combined Ocean Acidification and Warming on developing eggs of Atlantic herring. By studying such effects at both the cellular level (e.g. mitochondrial functioning) and the organism level (e.g. body size at hatching), this study provides a link between the thermal sensitivity of an individual's energetic metabolism with the fitness of the individual as a whole.

Elevated temperature significantly affected mitochondrial function by shifting the relative ETS contribution from CI to CII. This may decrease ATP production, which could lead to a mismatch between the energy produced by the mitochondria and the energy requested by the organism for maintaining metabolism; which in turn could reduce the energy allocation to development indicated by reduced length at hatch.

Elevated PCO_2 did not affect HS; however, it did increase the occurrence of malformed larvae. This suggests that exposure to near future acidification levels may cause sub-lethal cellular damage that may not be reflected in vitality and survival rates. These sub-lethal effects of ocean acidification may present the largest risk to individuals and populations (Briffa *et al.*, 2012). For example, smaller larval size at hatch may increase the risk of predation and reduce foraging ability (Miller *et al.*, 1988).

Furthermore, herring populations experience high fishing mortality in addition to other environmental stressors such as pollution and hypoxia. Therefore, potential effects of ocean acidification and warming must be added to the list of anthropogenic perturbations leading to increased mortality in fish early life stages.

Data availability

The datasets containing the physiological and morphological parameters measured in this study and the data regarding the incubation physico-chemical parameters are available from the Open Access library PANGAEA (www.pangaea.de; https://doi.pangaea.de/10.1594/PANGAEA.884123 and https://doi.pangaea.de/10.1594/PANGAEA.884124).

Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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Author contributions

E.L., F.T.D., F.C.M., D.S. and H.-O.P. designed the experiment; E.L., F.T.D. and F.C.M. collected, analysed and interpreted the data; E.L., F.T.D., F.C.M., D.S. and H.-O.P wrote the article.

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