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Effect of ocean acidification on marine fish sperm (Baltic cod: *Gadus morhua*)

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Abstract

Ocean acidification, as a consequence of increasing marine pCO_2, may have severe effects on the physiology of marine organisms. However, experimental studies remain scarce, in particular concerning fish. While adults will most likely remain relatively unaffected by changes in seawater pH, early life-history stages are potentially more sensitive – particularly the critical stage of fertilization, in which sperm motility plays a central role. In this study, the effects of ocean acidification (decrease of pH to 7.55) on sperm motility of Baltic cod, *Gadus morhua*, were assessed. We found no significant effect of decreased pH on sperm speed, rate of change of direction or percent motility for the population of cod analyzed. We predict that future ocean acidification will probably not pose a problem for sperm behavior, and hence fertilization success, of Baltic cod.

1 Introduction

Atmospheric CO_2 levels are currently rising faster than at any time in the previous 21 million years, driven largely by anthropogenic activities such as burning of fossil fuels and changes in land-use (IPCC, 2007). As the oceans are in a slow but continuous equilibrium with the atmosphere, it is projected that corresponding increases in CO_2 absorption by the ocean from the atmosphere will lead to a drop in pH of $\leq$0.4 units by the year 2100 (Caldeira and Wickett, 2003; IPCC, 2007). This will in turn cause an under-saturation of calcium carbonate (Feely et al., 2004), which could have pervasive effects on calcifying marine organisms such as molluscs, cnidarians, and echinoderms (Fabry et al., 2008; Doney et al., 2009). In addition, elevated CO_2 concentrations can disturb the acid-base regulation, blood circulation, and respiration, as well as the nervous system of marine organisms, leading to long term effects such as reduced growth rates and reproduction (Pörtner et al., 2004).

BGD

7, 5859–5872, 2010

Adult fish are thought to be relatively insensitive to low ambient seawater pH because they have effective acid-base regulatory systems, whereas early life stages such as eggs and larvae have not yet fully developed these regulatory processes and are therefore likely to be affected (Morris, 1989; Sayer et al., 1993). The majority of fish are external fertilizers, and sperm are activated by seawater as they are expelled into the open ocean during a spawning event (Westin and Nissling, 1991). The effect of CO_2-induced ocean acidification on sperm motility and fertilization success in fish is not known, although this has been tested in several marine invertebrates with different results even between closely related species (Kurihara and Shirayama, 2004; Kurihara et al., 2007, 2009; Havenhand et al., 2008; Havenhand and Schlegel, 2009). Studies of the effects of acidification by mineral acids on sperm swimming in fish (e.g. rainbow trout; Baynes et al., 1981) have found motility to be inhibited below pH 7.8 (an effect that can be overcome by addition of Ca^{2+} and Mg^{2+} ions). Note that acidification by mineral acids has markedly different effects to CO_2-induced acidification, (Kurihara and Shirayama, 2004; Gattuso and Lavigne 2009). Billard and Cosson (1988) found the beat frequency (proportional to speed) of spermatozoa to be dependent on the pH of the swimming solution in trout, while for other species such as carp (Marian et al., 1997) sturgeon (Gallis, 1991; Linhart et al., 1995) and paddlefish (Cosson and Linhart, 1996) acidic conditions have been shown to reduce sperm motility. In contrast, Stoss (1983) found no effect of pH on motility but a reduction in short-term viability of salmonid sperm.

It seems likely therefore that reductions in seawater pH mediated by increasing levels of atmospheric CO_2 could have a substantial effect on the sperm motility – and hence fertilization success – of key marine fish species. Cod is one of the key fish species in the Baltic ecosystem. Stock recruitment strength of cod in the Baltic varies greatly from year to year as a result of variation in spawning efficiency and gamete and larval survival (Grauman, 1973; Houde, 2008). These early life-history stages are the most sensitive to abiotic conditions such as salinity and oxygen availability (Westin and Nissling, 1991). The extent to which ocean acidification might influence sperm

BGD
7, 5859–5872, 2010

swimming behavior of Baltic cod is examined in this study.

2 Methods

Adult cod were caught from the RV Alkor in several hauls in the spawning grounds of the Bornholm Basin, Baltic Sea, during a cruise in August 2009. Ripe males were picked out immediately after catch and stripped just prior to analysis. Sperm were collected in dry glass vials and held on ice. Contamination of sperm with urine or feces was carefully avoided. The seawater used in experiments was filtered (0.1 µm), UV sterilized and had a salinity of 17.4. All water was pre-bubbled with the respective CO_2 partial pressure – control water with 380 ppm and acidified water with 1400 ppm – and kept in closed Nalgene© containers until use a week later (preliminary trials showed that this retained the water chemistry for at least one week). The pH of the water was measured directly before use with a pH-meter (WTW) calibrated with NBS buffers. The probe was later cross-checked with SWS pH reference materials (made by Vincent Sardene, IFM-GEOMAR) and the Dickson standard revealing an accuracy of 97%. A subsample of the water was measured for total alkalinity (A_T) and dissolved inorganic carbon (C_T) in order to calculate the carbonate system of the water using CO_2SYS (Lewis and Wallace, 1998). All experiments were conducted at a room temperature around 8 °C (with drift up to 9.5 °C), while the sperm was held on ice before use.

Sperm suspensions were created by activating 10 µl "dry" sperm from a single male in 4 ml of filtered seawater (control or acidified). One drop of sperm suspension was then placed onto a glass microscope slide inside an O-ring of 1 mm thickness and covered with a coverslip, to minimize wall-effects (Havenhand et al., 2008). To prevent sperm from adhering to the glass surfaces, slides and cover slips were pre-coated with bovine serum albumin (see Bolton and Havenhand, 1994). Sperm swimming behavior was recorded (within 10 s after activation) using a digital camera (Canon IXUS, 3.0 × digital zoom) mounted onto a microscope (Leitz Laborlux K, 10 × objective). For each fish, video was obtained from each of 5 replicate slides using control (pH_{NBS}=8.1)

BGD
7, 5859–5872, 2010

and acidified seawater (pH_{NBS}=7.5). Video clips (3 s duration) were analyzed using CellTrak1.3© (Motion Analysis Corporation, Santa Rosa, CA). The average swimming speed and the percentage of motile sperm direction was determined for each replicate of all 18 males. Sperm concentrations were calculated post-hoc from a subsample of sperm from each of the males using a hemocytometer.

2.1 Data analysis

Assumptions of normality and homogeneity of variance were checked using Kolmogorov-Smirnoff and Levene's test, and yielded no difference among variances. The effect of pH across different males was tested by two-factorial mixed-model ANOVA, using Statistica 6.1 (StatSoft, Inc.). The mean pH was calculated ± the standard deviation.

3 Results

Mean pH in the controls treatments was 8.056±0.065, and in acidified treatments was 7.554±0.004. Males ranged in size from 36–50 cm (356–991 g; Table 1). Average sperm swimming speeds and percent sperm motility differed greatly between the males, as did the difference between the treatment and controls (Table 1). Sperm swimming behavior in control treatments was marginally faster and of higher motility in controls than in treatment pH, however these differences were very small (Fig. 1). ANOVA showed that responses of sperm to reduced pH differed significantly between 20 males for all parameters measured, however there was no significant effect of pH on either speed or percent motility, and no significant interactions between male and treatment (Table 2). Therefore, we cannot reject the null hypothesis of CO_2 having no effect on the sperm in the parameters measured.

BGD
7, 5859–5872, 2010

4 Discussion

The results of ANOVA found no significant effect of CO_2-induced reductions in seawater pH on Baltic cod sperm behaviour (speed and percent motility; Table 2). Some authors have interpreted equivalent non-significant results in ocean acidification experiments as evidence for no effect of acidification (e.g. Byrne et al., 2009). This is inappropriate statistical practice as it is well established that non-significant results are simply inconclusive (Fisher, 1935; Nakagawa and Foster, 2004). Valuable insight on the likelihood that the null-hypothesis (of no effect) is correct can, however, be provided by confidence intervals around statistical effect sizes (Nakagawa and Foster, 2004). Using the methodology of Hedges and Olkin (1985) we calculated effect sizes and 95% confidence intervals around effect sizes for sperm speed and percent motility. These showed clear overlap with zero, (effect size (±95% CI) for sperm speed = −0.165±0.654, and for percent motility = −0.247±0.656). Consequently we conclude that sperm swimming behaviour (and therefore fertilization success) in Baltic cod is likely to be robust to near-future changes in ocean pH arising as a result of increasing atmospheric CO_2.

Changes in ionic concentration and osmolality are known to trigger sperm motility in teleost fish sperm (Morisawa ans Suzuki, 1980). Once sperm are released into the environment, the external pH is of crucial importance as it influences intracellular proton concentration, depolarization of the cell membrane, and therefore sperm motility (Alavi and Cosson, 2005, 2006). The pH of cod seminal fluid lies between 7.9 to 8.4 (Suquet et al., 2005) and therefore, lowering the pH of the surrounding activating fluid to 7.55 should prevent the triggering of sperm motility (Cosson et al., 2008; although the review by Alavi and Cosson, 2005, states that the pH of the swimming medium has little influence on sperm motility).

The methods by which pH was manipulated in these studies can have substantial influence on the results: reducing pH by the addition of NH_4Cl had little effect on the activation of fish sperm (Alavi and Cosson, 2005) whereas CO_2 induced pH reductions

BGD
7, 5859–5872, 2010

in the seminal fluid of flat fish inhibit flagellar movement of the sperm (Billard et al., 1993; Dreano, 1995; Inaba et al., 2003). This latter effect is due to carbonic anhydrase (CA), which is involved in the conversion from CO_2 to HCO_3^- and acts as an inhibitor for fish sperm. While CA is present in abundance in flatfish, most other teleost species do not have major proteins in this their seminal fluid and therefore do not have this problem (Inaba et al., 2003).

The question why Baltic cod sperm are seemingly unaffected by acidified seawater is an intriguing one. High CO_2 (and correspondingly low pH) levels are common in their environment and it is therefore probable that they have adapted to these extreme conditions. The Baltic Sea is a unique system in that it is an enclosed low-salinity sea with little exchange of water with other oceans and relatively poor circulation. Further, it is highly eutrophic, such that the deep waters are often oxygen depleted (high CO_2), which dramatically decreases the pH of these layers (Kuss et al., 2006; Omstedt et al., 2009). In the Baltic Sea, cod spawn at a depth of around 65 m where their eggs are neutrally buoyant. At these depths newly spawned gametes and eggs may already be experiencing the acidification conditions predicted in future scenarios for the open ocean. The next step is to test whether North Sea cod, which spawn in well-oxygenated, low CO_2, surface waters are affected in their sperm motility by acidified seawater. The results will be central to determining the adaptive capacity of cod, and the likelihood that the crucial process of fertilization will be robust to future changes in ocean pH.

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BGD
7, 5859–5872, 2010

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BGD
7, 5859–5872, 2010

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BGD
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BGD
7, 5859–5872, 2010

Table 1. Summary of carbon system parameters: A_T = total alkalinity, C_T = total dissolved inorganic carbon (both measured), pCO_2 = partial pressure of CO_2 and pH (calculated with CO2SYS) at Temperature (T) and Salinity (S).

treatment	A_T (µmol kg^{-1} SW)	C_T (µmol kg^{-1} SW)	pCO_2 (µatm)	pH	T (°C)	S
acidified	2024.4	2055.2	1364.8	7.558	9.4	17.4
control	2068.8	1973.4	390.8	8.080	9.4	17.4

BGD
7, 5859–5872, 2010

Table 2. Sperm swimming behaviour in control (C) and acidified (A) conditions. Speed = mean sperm swimming speed ($\mu m\, s^{-1}$); % mot. = percent motile sperm.

Fish number	Length (cm)	Weight (g)	pH C	pH A	Speed C	Speed A	Δ speed	% mot. C	% mot. A	Δ % mot.
1	36	356	7.858	7.508	66.81	58.68	8.12	89.14	84.35	4.79
2	37	382	7.858	7.508	67.54	68.46	−0.93	85.88	81.63	4.25
3	47	779	7.858	7.508	37.49	34.57	2.92	71.07	70.96	0.11
4	46	782	7.858	7.508	49.71	51.91	−2.19	74.43	75.29	−0.85
5	39	429	7.858	7.508	52.00	40.48	11.52	80.44	76.85	3.59
6	38	445	7.858	7.508	42.92	45.89	−2.97	79.54	81.64	−2.10
7	40	541	7.858	7.508	55.59	62.28	−6.69	78.85	82.99	−4.14
7	40	533	7.858	7.508	63.04	64.97	−1.93	73.97	75.10	−1.12
9	46	887	7.985	7.501	73.25	70.19	3.06	78.14	80.11	−1.97
10	41	511	7.985	7.501	44.87	39.97	4.90	76.81	69.02	7.78
11	41	636	7.985	7.501	60.90	54.91	5.99	85.19	82.60	2.59
12	43	571	7.985	7.501	68.72	61.76	6.97	83.59	76.75	6.84
13	40	550	7.985	7.501	63.88	62.01	1.87	85.61	89.83	−4.22
14	48	759	7.985	7.501	56.55	62.30	−5.75	81.27	81.82	−0.56
15	48	809	7.985	7.501	56.10	45.43	10.68	87.49	83.97	3.52
16	40	462	7.985	7.501	88.71	85.75	2.96	92.07	88.76	3.31
17	50	991	7.985	7.501	51.59	49.70	1.89	83.97	79.94	4.03
18	41	537	7.985	7.501	55.23	59.51	−4.27	85.72	56.48	−0.76
Mean	42	609	7.929	7.504	58.61	56.60	2.01	81.84	80.45	1.39
S.D.	4	183	0.065	0.004	12.14	12.63	5.40	5.65	5.63	3.59

BGD
7, 5859–5872, 2010

Table 3. ANOVA results of effect of CO_2 "treatment" (fixed) and male (random) on sperm swimming speed and % motility along with the interaction of treatment × male.

average speed	MS Effect	df Error	MS Error	*F*	*p*	*r*
treatment	140.79	18.62	73.41	2.02	0.18	0.32
male	1437.29	16.72	69.48	20.69	0.00	0.73
treatment × male	69.64	139.00	94.97	0.73	0.76	0.20
% motility						
treatment	93.29	17.57	31.88	2.99	0.10	0.38
male	281.94	16.77	31.16	9.05	0.00	0.58
treatment × male	31.18	139.00	34.63	0.90	0.57	0.22

BGD
7, 5859–5872, 2010

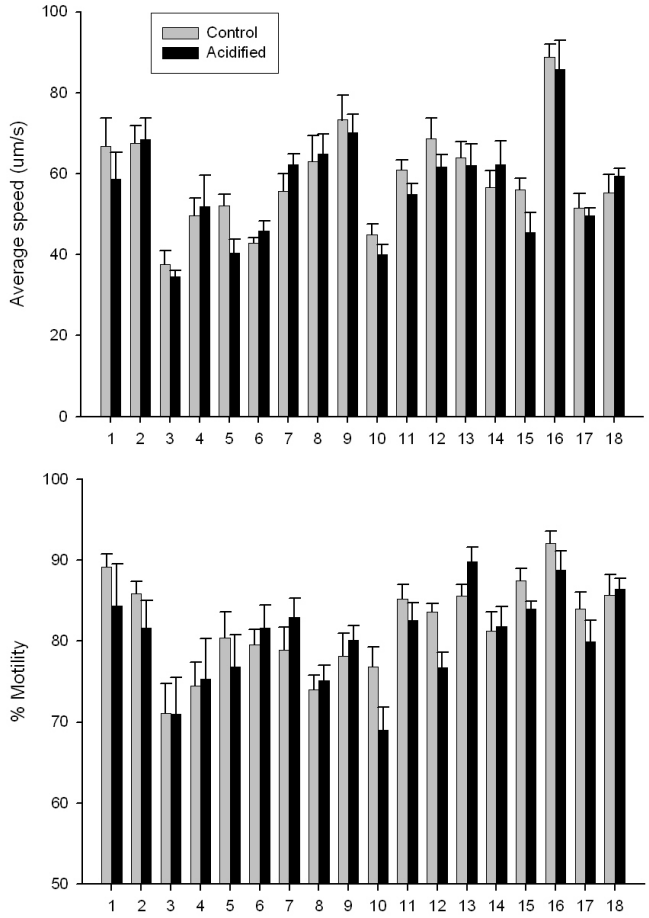

Fig. 1. Individual variability between males (1-18) in control (grey) and acidified (black) treatments for sperm swimming speed and percent motile sperm. Error bars denote the standard deviation from the mean between the five replicates.

BGD
7, 5859–5872, 2010