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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_T 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).

Adult fish are thought to be relatively insensitive to low 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 could therefore 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). While there is not much knowledge on the effects of ocean acidification on fish sperm, some experiments have been performed on invertebrate sperm, with differing results even between closely related species (Kurihara and Shirayama, 2004; Kurihara et al., 2007, 2009; Havenhand et al., 2008; Havenhand and Schlegel, 2009; Kurihara et al., 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). 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 the short-term viability of salmonid sperm.

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It therefore seems likely 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. Its recruitment strength in the Baltic varies greatly from year to year as a result of variation in spawning efficiency as well as 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 swimming behavior of Baltic cod is examined in this study.

2 Methods

Adult cod were caught from the R/V *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. Seawater was pre-bubbled in order to reach a CO_2 partial pressure (pCO_2) of 380 and 1400 µatm and kept in closed Nalgene© containers (preliminary trials showed that the carbonate chemistry was unaltered for at least a week). The pH of the water was monitored throughout each trial with a pH-meter (WTW) calibrated with National Bureau of Standards (N.B.S.) buffers. Total alkalinity (A_T) and dissolved inorganic carbon (C_T) were measured on subsamples and the carbonate system was calculated from C_T and A_T 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 (without previous contact to any water) from a single male in 4 ml of filtered seawater (control or elevated pCO_2). 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 x digital zoom) mounted onto a microscope (Leitz Laborlux K, 10 x objective). For each fish, video was obtained from each of 5 replicate slides using control (pH_T = 8.08) and acidified seawater (pH_T = 7.55). Video clips (3 s duration) were analyzed using CellTrak1.3©(Motion Analysis Corporation, Santa Rosa, CA). Looking at an average of 200 tracks per slide, the average swimming speed and the percentage of motile sperm was determined for each replicate for all 18 males.

3 Data analysis

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

4 Results

Mean pH (calculated with CO_2SYS) in the control and elevated pCO_2 conditions was 8.080 and 7.558, respectively (Table 1). Males ranged in total length from 36 to 50 cm (356 to 991 g; Table 2). The average sperm swimming speeds and percent sperm motility differed greatly between the males, as did the difference between the treatment and controls (Table 2), however these differences were size independent. Sperm swimming behavior was marginally faster and of higher motility in control treatment than at low pH, but these differences were very small (Fig. 1). ANOVA showed that responses of sperm to reduced pH differed significantly between 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 3). Therefore, we cannot reject the null hypothesis of CO_2 having no effect on the sperm in the parameters measured.

5 Discussion

No effect of CO_2-induced ocean acidification was found on the sperm behaviour of Baltic Sea cod (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 [$\pm$ 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

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, calculated pH_T (total scale), T, Temperature and S, Salinity.

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

Table 2. Sperm swimming behavior in control (C) and acidified (A) conditions. Speed = mean sperm swimming speed (µm s^{-1}); % mot. = percent motile sperm.

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

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

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 and 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; Alavi and Cosson, 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 could inhibit the triggering of sperm motility, as the optimum pH of the activation medium has been found to be about one unit higher than the pH of the seminal fluid in other fish species (Alavi and Cosson, 2005).

The methods by which pH was manipulated in these studies can have substantial influence on the results: reducing

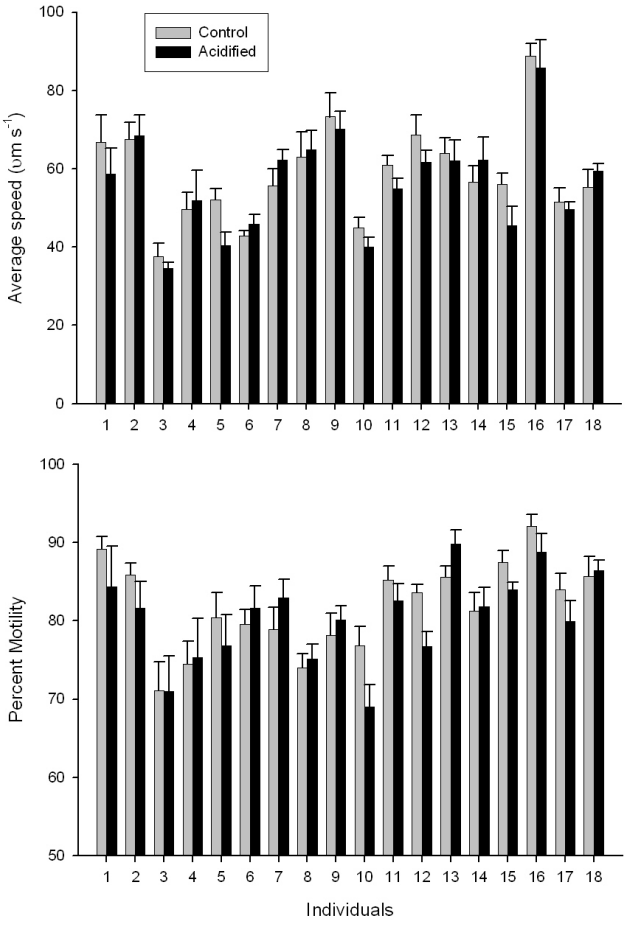

Fig. 1. Individual variability between males (1–18) in control (grey) and acidified (black) treatments for sperm swimming speed and percent motile sperm. Error bars denote the standard deviation from the mean between the five replicates.

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 in the seminal fluid of flat fish inhibit the flagellar movement of the sperm (Billard et al., 1993; Dreano et al., 1995; Inaba et al., 2003). This latter effect is due to carbonic anhydrase (CA), a strong pH regulator, which catalyzes the conversion of CO_2 to HCO_3^-, which in turn acts as an inhibitor for fish sperm. The CO_2/HCO_3^- mechanism possibly evolved in flat fish to prevent spontaneous activation of sperm in the seminal fluid (Inaba et al., 2003). While CA is present in abundance in flatfish, most other teleost species do not have major proteins in their seminal fluid and therefore do not have this problem of concentrating motility inhibiting ions (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: it is enclosed with relatively poor circulation, has a low salinity and does not exchange much with the North Sea. Further, it is highly eutrophic, such that the deep waters are often depleted in oxygen and exhibit high pCO_2 and hence low pH (Kuss et al., 2006; Omstedt et al., 2009). During the spawning season, Baltic cod spend most of their time in waters with a salinity of at least 12 and oxygen levels above $4\,ml\,l^{-1}$ (Schaber et al., 2009), limits that also determine egg survival. In August 2009, these conditions corresponded to depths between 50 and 64 m where we measured pH_{NBS} values between 7.4 and 7.9. Therefore, 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 pCO_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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