Supplementary Information to:

Elevated carbon dioxide affects the plasma composition and behaviour of a shark

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Supplementary Materials and Methods

Ethics statement
Permits for handling, rearing and experimental procedures were obtained from the ethical committee on animal experiments of Gothenburg, Sweden (ethical permit: Jutfelt 100-2010 and 151-2011).

Experimental animals
The study was conducted at the Sven Lovén Centre for Marine Sciences, Kristineberg, University of Gothenburg, Fiskebäckskil, Sweden, from August to November of 2012. *S. canicula* is a common local species of nocturnal benthic shark that feeds on invertebrates and mainly inhabits depths of 10-100 m [1]. The animals used in this experiment were bred and reared in a flow-through system with water pumped from 30 m depth in the Gullmarsfjord (58.283°N, 11.433°E) at the public aquarium *Havets Hus*, Lysekil, Sweden. The study was replicated over time instead of with multiple tanks, and the position of the treatment was varied between the experiments. A total of 20 animals (ages 4-24 months) were obtained for the experiment and conditioned during two separate occasions (August to September and September to November). At the start of each treatment period, the animals were sexed and weighed, and their lengths were measured. The small juveniles (young of the year) weighed 15.7g ($\pm$ 5.1) and their length was 177 mm ($\pm$ 14); the larger juveniles (> one year) weighed 366.2g ($\pm$ 61) and their length was 485 mm ($\pm$ 28). The males and females were randomly distributed into two different 1125 L fiberglass tanks. These tanks were fitted with natural shell gravel substrate and flow-through seawater pumped from 30-meters depth. The flow was 9 L min^{-1} through each of two aerated 200 L header tanks. Unsexed small juveniles (n = 5) were distributed into two separate plastic 30 L bins suspended inside the fiberglass tank (2 to 3 sharks in each; only on the second experiment). The bins were fitted with shell gravel substrate; a 4-watt aquarium pump provided circulating water taken from the main fiberglass tank, and the bins had holes for overflow. The animals were maintained at a 12 hour:12 hour light:dark cycle and fed *ad lib* every other day (juveniles every day) with peeled, previously frozen shrimp (*Pandalus borealis*).

Experimental treatments

The pCO_2 of the CO_2 treatment tank was maintained at a target value of 990 µatm by dosing 100% CO_2 gas (AGA, Gothenburg, Sweden) into the aerated 200 L header tank through a solenoid valve (M-ventil standard, Aqua Medic, Bissendorf, Germany) regulated by a pH stat computer (Aqua Medic, Bissendorf, Germany). The pCO_2 levels of both header tank systems were measured daily using an infrared CO_2 probe (GMT 222, Vaisala, Helsinki, Finland) connected to a submerged closed-loop, gas-permeable silicone membrane equilibrated with dissolved pCO_2 in the respective tanks [2-6]. Probe accuracy was confirmed on several occasions during the experiment by measuring thoroughly bubbled water with a controlled gas mixture of 1010 ± 10 ppm pCO_2 in air (AGA, Gothenburg, Sweden), as well as factory calibration (detecting 0.7% drift) before and after the experiments. Water salinity, oxygen saturation, temperature and pCO_2 were measured daily, and alkalinity was measured weekly. Oxygen saturation remained above 90% in all measurements. The water temperature was 12.7 ºC ± 1.45 (SD), and the average salinity was 31.6 PSU ± 0.5 (SD). The pCO_2 of the control system was maintained at 401.3 µatm $\pm$ 13.0 (SD), and the pCO_2 of the treatment system was maintained at 993 µatm ± 66.4 (SD). Carbonate system speciation was calculated using CO2calc (Hansen, USGS, Reston, Virginia, USA) with pCO_2, alkalinity, temperature and salinity as factors and using the constants from Roy et al. 1993 [7] and Dickson 1990 [8]. The relevant water chemistry is summarised in table S1.

The animals were maintained in treatment for a minimum of four weeks prior to the experiments to provide adequate time for acclimation to the water carbonate chemistry. The two testing periods lasted three weeks each, and the animals were returned to the public aquarium in Lysekil after testing. Any animal showing signs of emaciation or sickness (n $CO_2 = 2$, n control $= 3$) was removed for medical treatment and excluded from the experiment.

Swimming activity measurements

Activity measurements were collected by video recordings of individual animals as they moved freely in a 1500 mm by 500 mm tank, with a water depth of 500 mm. The tank used a flow-through of 1.5 L per minute of water from the system in which the tested animal had been acclimated. The sharks were fasted for 24 hours prior to filming. They were filmed for 16 hours (2 hours of light; 12 hours of dark; 2 hours of light) using a computer-controlled IR camera (DCS 932-L, D-Link, Taipei, Taiwan) and ambient IR light. Measurements were taken by playback and visual observation. The duration of time spent at the bottom and at the surface was recorded for each swimming bout. From these data, the following parameters were calculated and statistically analysed: average and maximum swimming time (per hour, in seconds, minutes and percentage; at the bottom, surface and total); average and maximum time per swimming bout (in seconds, minutes and percentage); number of swimming bouts per hour; and number of swimming bouts reaching surface. A frequency plot (figure S1) was calculated by dividing the number of swimming bouts per hour by the number of animals per treatment.

Lateralization

Lateralization, the degree of behavioural asymmetry, was measured in two different sizes

of square-shaped double t-mazes (large maze: 1000 mm length with 300-mm-wide channels; small maze: 500 mm length with 90-mm-wide channels) made from fiberglass and Plexiglas (PlastKapTek, Mölndal, Sweden) according to [9]. Each maze was evenly illuminated. Animals smaller than 200 mm in length were tested in the smaller maze, and animals larger than 200 mm in length were tested in the larger maze. Each animal was gently encouraged by hand to swim 10 times towards each T-intersection during two separate days for a total number of 40 turning decisions per shark. Each turn was recorded, and the relative and absolute laterazisation indexes were calculated according to [10].

Blood chemistry

Blood was sampled from each animal measuring over 250 mm in length, using a syringe with a needle (20 G $^1/_2$ 0.9 x 40 mm Microlance, BD, Franklin Lakes, New Jersey, USA) inserted at a 45-degree angle at the ventral midline into the caudal vessels (vein or possibly a mix of venous and arterial vessels) ventral to the spine. A total of 2 mL of blood was drawn, and the chemical properties were immediately measured. pH was measured within 30 seconds using a freshly calibrated pH meter fitted with a thermometer and small-volume pH electrode (CyberScan PC650, Eutech Instruments, Singapore). Plasma was extracted by centrifugation (7000 rcf) for one minute, and the dissolved inorganic carbon (DIC) of the plasma was measured using a carbon dioxide analyser (Corning 965, CIBA, Tewksbury, Massachusetts, USA), fitted to a standard curve, and verified using precision CO_2 standard vials with 2 g L^{-1} CO_2 (Raegecon Analytical, Shannon, Ireland). Blood plasma was stored frozen at -80 °C until the levels of K^+, Na^+, Ca^{2+} and Cl^- were measured using an ion radiometer biosensor system (EML 105, Radiometer Medical A/S, Copenhagen, Denmark). Haematocrit was measured using micro haematocrit glass tubes (Brand, Copenhagen, Denmark). Haemoglobin was determined using a haemoglobin analyser (HaemoCue Hb 201$^+$, Haemocue AB, Ängelholm, Sweden) and corrected according to [11]. pCO_2 isopleths and HCO_3^- scale for the Davenport diagram were constructed using the following modified Henderson-Hasselbalch equation: $HCO_3^- =10^{(pH-pK'+log(\alpha*pCO_2))}$. pK' and α constants for *S. canicula* were obtained from Albers and Pleschka (1967)[12] and Pleschka and Wittenbrock (1971)[13], respectively.

Oxygen consumption

Intermittent flow respirometry was performed on all animals according to methods described by Clark et al. (2013)[14]. Animals were not fed for 48 hours (24 hours for small juveniles) prior to the experiment to eliminate residual specific dynamic action. Each experiment started with a chase exhaustion protocol where individual sharks were chased by hand in circular tanks for ten minutes or until the sharks were unable to lift their bodies when lifted into the air and held by their peduncle. After exercise, the animals were quickly placed in sealed recirculating Perspex respirometry chambers of suitable sizes. The chambers were fitted with a robust 3 mm oxygen optode (Firesting, Pyroscience, Aachen, Germany) and circulated in a closed loop. The chambers were kept at ambient temperature and pCO_2 exposure by suspending the chambers inside the respective treatment and control tanks. Measurements were started within one minute of closing the chambers. We continuously collected 5-minute MO_2 measurements with 5-minute flushing in between measurements for the first three hours to obtain the maximum oxygen consumption ($MO_{2\ max}$). The single highest 3-minute MO_2 time period for each shark was used as the $MO_{2\ max}$.

After the initial $MO_{2\,max}$ period, the animals were kept undisturbed and unfed in the respirometers for another 48 hours with continuous 10-minute measurements for animals measuring more than 200 mm and with 50-minute measurements for smaller animals. This process was consequently interrupted with 10 minutes of flushing. The mean of the lowest 10% of recorded MO_2 values is presented as the $MO_{2\,min}$. $MO_{2\,max}$ and $MO_{2\,min}$ were calculated using the standard formula for intermittent-flow respirometers [14]:

$$MO_2 = ((V_r - V_f) \cdot \Delta C_w O_2) / (\Delta t \cdot M_f)$$

where V_r is the volume of the respirometry chamber, V_f is the volume of the animal, $\Delta C_w O_2$ is the change in oxygen concentration in the chamber and Δt is the duration over which the O_2 concentration was measured. The absolute aerobic scope was calculated as $MO_{2\,max} - MO_{2\,min}$, factorial aerobic scope as $MO_{2\,max} / MO_{2\,min}$ (figure S2).

Growth measurements

Length and weight were measured at the beginning of each exposure period and after 30 days of exposure. Length was measured as total length (TL) in mm from the nose tip to the tip of the tail. The specific growth rate (SGR) was calculated based on the weight and length using the following formula:

$$SGR = (\ln (W_f) - \ln (W_i)) \cdot 100 / t$$

where W_f is the final weight in grams, W_i is the initial weight in grams and t is the time (in days).

Denticle measurements

Tissue samples were collected from the pectoral fins using sterilised scissors (n CO_2 = 3, n control = 3). Denticle micrographs were obtained using a scanning electron microscope (JSM T220A, JEOL, Tokyo, Japan), and the maximum width and length were measured using the ruler function in Google Sketchup (Trimble Navigation, Sunnyvale, California, USA). Measurements were obtained for 50 randomly selected denticles per sampled individual. The ultrastructure of the denticle surface was inspected for imperfections at high magnification (5000 x).

Statistics

Differences in the blood chemistry, growth, respirometry and lateralization results were analysed for statistical significance using Student's t-test, and mixed model analysis (IBM SPSS Statistics, IBM, Armonk, New York, United States) was used to analyse the swimming activity measurements and denticle morphology data. All values are shown as the mean ± SEM unless otherwise stated.

Dryad raw data

http://dx.doi.org/10.5061/dryad.6s713

Supplementary Results

Table S1. Water chemistry of the control and elevated CO_2 treatments. Temperature, salinity, pCO_2, and alkalinity (A_T) are measured data; pH_{tot}, $\Omega_{aragonite}$ and $\Omega_{calcite}$ were calculated using CO2calc (USGS, Reston, Virginia, USA). Data are presented as the mean ± SD.

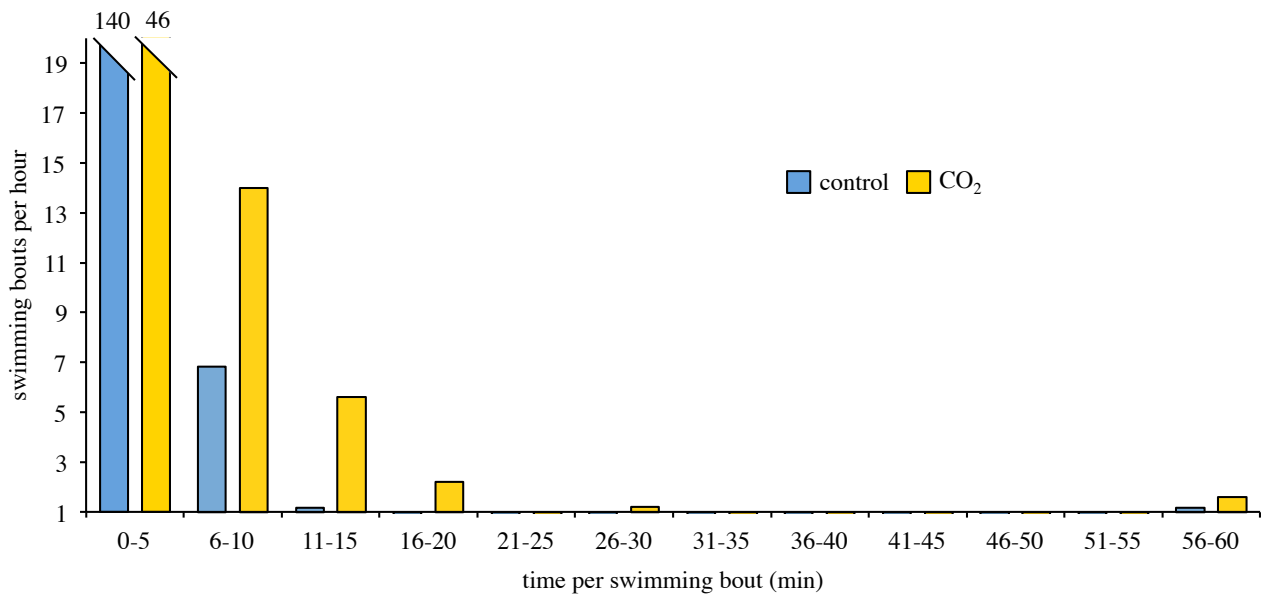| Parameter	Control	CO_2
pCO_2 (µatm)	401.3 ± 13.0	993.5 ± 66.4
Alkalinity (TA)	2350 ± 37.1	2363 ± 53.7
Salinity (PSU)	31.6 ± 0.5	31.6 ± 0.5
Temp (°C)	12.7 ± 1.45	12.7 ± 1.45
pH_{tot}	8.1 ± 0.02	7.72 ± 0.05
Ωaragonite (calc.)	2.41 ± 0.17	1.16 ± 0.12
Ωcalcite (calc.)	3.79 ± 0.26	1.83 ± 0.18

Figure S1
Effects of pCO_2 on swimming bout duration in *S. canicula*. Frequency plot of average swimming bout durations for sharks exposed to control water (blue bars) or high CO_2 water (yellow bars) for four weeks. The y-axis is broken for clarity, and the bar numbers for the 0-5 minute time period is written above. Control n = 6, CO_2 n = 5.
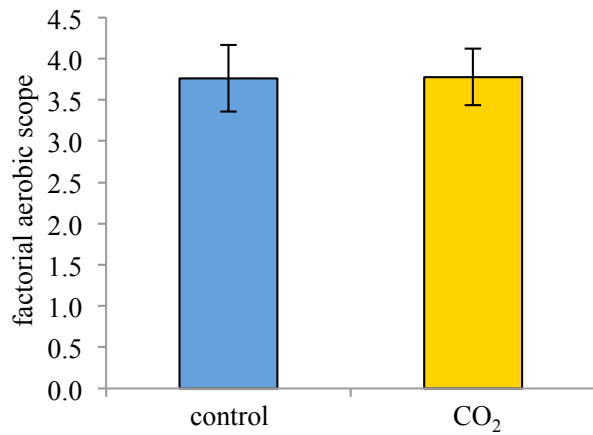
Figure S2

Effects of pCO$_2$ on factorial aerobic scope in *S. canicula*. Oxygen consumption rates presented as factorial aerobic scope of sharks exposed to control conditions (blue bars) and high CO$_2$ conditions (yellow bars) for four weeks. Control n = 7, CO$_2$ n = 9.

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