**Electronic supplementary material - 2**

**Robust quantification of fish early life CO2 sensitivities via serial experimentation**

Hannes Baumann, Emma L. Cross, and Christopher S. Murray

**Methods**

***Spawner collection and spawning methods***: All experiments used offspring derived from wild mature Atlantic silversides (*Menidia menidia*) that were repeatedly collected during the spawning season (May-July) from local sites with a 30 × 2 m beach seine (3 mm mesh). In 2012-2014, spawners for experiments 1-12 [[1-3](#_ENREF_1)] were collected from Poquot Cove on the central north shore of Long Island (New York, 40.95°N, 73.10°W). In 2016-2017, spawners for experiments 13-20 [[2](#_ENREF_2), [4](#_ENREF_4)] were collected from Mumford Cove (eastern Connecticut; 41.32°N, 72.02°W). The two sites are located on opposite shores of Long Island Sound approximately 100 km apart. Adults were transported to the laboratory (2012-2014: Flax Pond Laboratory, Stony Brook University; 2016-2017: Rankin Laboratory, University of Connecticut Avery Point) and held overnight in aerated tanks (60L) and at 20°C to promote egg hydration.

Strip-spawning occurred the next day and followed time-tried methods of experimental research on silverside species [[e.g., 1-12](#_ENREF_12)]. To ensure sufficient genetic diversity representative of wild populations, eggs were stripped from a minimum of 20 females (one experiment used only 12). Stripped eggs were gently mixed into shallow plastic dishes lined with 1-mm plastic window screening covered in seawater. Subsequently, milt from each of 20+ males was collected and pooled into 500-ml glass beakers, mixed with seawater, stirred, then gently poured into spawning dishes and mixed with eggs for ~15 min. In this species, fertilized eggs attach to window screen with chorionic filaments. Hence, within two hours of fertilization, screens with precisely counted 100 attached embryos were randomly distributed into replicated 20-l rearing containers preconditioned for the intended CO2 treatment and placed within temperature-controlled water baths (Aqualogic® thermostats connected to commercial aquarium heaters).

***CO2 manipulation and control***: We applied a target CO2 level of 400 μatm (~8.15 pH) for ambient treatments, a level characteristic of the open surface ocean and of coastal systems at the onset of the silverside spawning season (April - July) [3]. The target level for high CO2 was 2000 μatm (~7.50 pH), a level that is commonly experienced by silverside offspring during summer months, but also represents the maximum prediction of average OA for the next 300 years [[13](#_ENREF_13)] and therefore similar levels have been employed as a common benchmark in many OA studies [[14-17](#_ENREF_14)]. During experiments 1-12 (2012-2014), 14 (2016), 16 and 18 (2017), CO2 levels were manipulated via gas proportioners (ColeParmer®), which mixed ambient air with 100% bone-dry CO2 and delivered gas mixes to the bottom of each rearing container via air stones. Target levels were controlled via daily pH measurements using hand-held pH probes (Orion ROSS Ultra pH/ATC Triode, and Orion Star A121 pH Portable Meter, Thermo Scientific; Hach HQ40d portable meter with a PHC201 standard pH-probe) that were calibrated weekly with 3-point NIST buffers.

Later and in addition, we constructed an automated acidification system designed for larval fish rearing and used for experiments 13, 15, and 17 (2016-2017). Nine identical recirculating-units exist, containing sump (90l), a header tank (40l) and a main tank (240l) that holds up to five replicate rearing-containers (20l) fitted with screened overflow holes (100μm). In these units, seawater continuously circulates from the sump through a UV sterilizer into the header tank, where it is gravity fed to the bottom of each rearing-container, from which it overflows in the main tank and back into the sump. We designed a LabView (National Instruments®) program to fully automate the control of seawater chemistry. The software interfaces with the recirculating-units via a data-acquisition module (NI cDAQ-9184, National Instruments®), which controls nine sampling-pumps (one per tank) and a series of gas and water solenoid valves, while receiving input from a central pH electrode (Hach pHD® digital electrode calibrated weekly using NIST 2-point pH references) and DO probe (Hach LDO® Model 2). The software sequentially assesses the pH conditions in each recirculating-unit (once per hour) by pumping water for ~7.5 min through the housing of the central pH probe, comparing measured pH levels to set-points and then adjusting levels by bubbling standardized amounts of 100% CO2 (bone dry grade, AirGas®) or CO2-stripped air into the sump of each tank. The software also maintains DO saturation (>8 mg/L) by bubbling in CO2-stripped air. LabView logs current pH, temperature, and DO conditions before cycling to the next unit. Temperatures were controlled by thermostats (Aqualogic®) powering submersible heaters.

Two methods were employed to measure treatment seawater carbon chemistry parameters. For experiments 1-10 (2012-2013), at least one discrete water sample per treatment was taken during each experiment (borosilicate bottles, preserved with 200 μl HgCl2) and analyzed for total dissolved inorganic carbon (DIC, mol kg seawater-1) with an EGM-4 Environmental Gas Analyzer (PP Systems®) that quantified total DIC levels after separating the gas phase from seawater using a Liqui-Cel Membrane (Membrana®). This instrument provided a methodological precision of ±3% for replicated measurements of total DIC and provided full recovery (104±5%) of Dr. Andrew Dickson’s (University of California San Diego, Scripps Institution of Oceanography) certified reference material for total inorganic carbon in seawater (Batch 117 = DIC 2009.99 µmol kg-1 seawater), as reported in [[18](#_ENREF_18), [19](#_ENREF_19)].

For experiments conducted 2014-2017, water samples were drawn from one randomly selected replicate within each treatment at three distinct time points during each experiment and analyzed for total alkalinity (*AT*, µmol kg seawater-1) via endpoint titration (Mettler Toledo™ G20 Potentiometric Titrator). Assessing *AT* directly is advantageous over the previous method, since it does not rely on HgCl poisoning and is even more precise. The instrument has previously been shown to quantify *AT* in Dr. Andrew Dickson’s reference material (batches 147 *AT*= 2231.39 µmol kg seawater-1) with an average error within ±1% [2]. We used CO2SYS (V2.1, <http://cdiac.ornl.gov/ftp/co2sys>) to calculate unmeasured carbonate parameters (e.g., *AT* or total DIC, partial pressure and fugacity of CO2 (*p*CO2, *f*CO2; µatm), and carbonate ion concentration (CO32-, µmol kg seawater-1)) based on measured *AT* or DIC, pH (NIST), temperature, and salinity using K1 and K2 constants from Mehrbach et al. [[20](#_ENREF_20)] refitted by Dickson and Millero [[21](#_ENREF_21)] and Dickson [[22](#_ENREF_22)] for KHSO4. Refer to source publications for complete carbon chemistry parameters.

***Rearing and sampling procedures***: All included ‘standard’ experiments were conducted at 24°C, which is the thermal growth and survival optimum for *M. menidia* early life stages [[23](#_ENREF_23)]. Hence, this temperature allowed us to quantify CO2 effects in isolation of potential temperature interactions. Experiments 1-12 used seawater of ~28 psu, while experiments 13-20 used seawater of ~32 psu. For comparison, the Atlantic silverside commonly occurs in salinities as low as 10 psu in estuaries, and as high as 40 psu in shallow hypersaline coves, they are very eurohaline. Consistent with long-standing rearing protocols in this species [[1-9](#_ENREF_1), [12](#_ENREF_12)], all experiments used a photoperiod of 15h light:9h dark (approximating the average natural photoperiod during spring in our latitudes).

Larvae hatched 6 days post fertilization and were immediately provided with equal rations of powdered weaning diet (Otohime Marine Fish Diet, size A1, Reed Mariculture® to stimulate feeding and *ad libitum* levels of newly hatched brine shrimp nauplii (*Artemia salina,* San Francisco strain, brineshrimpdirect.com). Larvae were fed daily *ad libitum* rations of newly hatched nauplii for the remainder of the experiment. Rearing vessels (20l) were siphoned of waste and checked for ammonia concentrations regularly (Saltwater Ammonia Test Kit, API®). Water changes were made as necessary to ensure ammonia levels remained uncritical (<0.25 ppm). To quantify survival to hatch, 1dph larvae were counted by gently scooping small groups into replacement rearing-containers. For initial hatch standard length (SL, nearest 0.01 mm) measurements, larvae were randomly sub-sampled (*N* = 10) from each replicate and preserved in 5% formaldehyde/freshwater solution buffered with saturated sodium tetraborate. Similarly, all larval survivors 10dph were sampled, preserved in 5% formaldehyde/freshwater solution and measured for SL. Larval measurements were made using calibrated digital images made with a stereomicroscope (4x, Nikon® SMZ-1000) and analyzed in Image Pro Premier (V9.0, Media Cybernetics®).

**References**

[1] Malvezzi, A., Murray, C.S., Feldheim, K.A., Dibattista, J.D., Garant, D., Gobler, C.J., Chapman, D.D. & Baumann, H. 2015 A quantitative genetic approach to assess the evolutionary potential of a coastal marine fish to ocean acidification. *Evol. Appl.* **8**, 352-362. (doi:10.1111/eva.12248).

[2] Murray, C.S. & Baumann, H. 2018 You better repeat it: complex temperature × CO2 effects in Atlantic silverside offspring revealed by serial experimentation. *Diversity* **10**, 1-19. (doi:10.3390/d10030069).

[3] Murray, C.S., Malvezzi, A.J., Gobler, C.J. & Baumann, H. 2014 Offspring sensitivity to ocean acidification changes seasonally in a coastal marine fish. *Mar. Ecol. Prog. Ser.* **504**, 1-11. (doi: 10.3354/meps10791).

[4] Snyder, J.T., Murray, C.S. & Baumann, H. 2018 Potential for maternal effects on offspring CO 2 sensitivities in the Atlantic silverside (Menidia menidia). *J. Exp. Mar. Biol. Ecol.* **499**, 1-8. (doi:10.1016/j.jembe.2017.11.002).

[5] Conover, D.O. & Kynard, B.E. 1981 Environmental sex determination: interaction of temperature and genotype in a fish. *Science* **213**, 577-579.

[6] Conover, D.O. & Fleisher, M.H. 1986 Temperature-sensitive period of sex determination in the Atlantic Silverside, *Menidia menidia*. *Can. J. Fish. Aquat. Sci.* **43**, 514-520.

[7] Conover, D.O. & Present, T.M.C. 1990 Countergradient variation in growth rate: compensation for length of the growing season among Atlantic silversides from different latitudes. *Oecologia* **83**, 316-324.

[8] Billerbeck, J.M., Ortí, G. & Conover, D.O. 1997 Latitudinal variation in vertebral number has a genetic basis in the Atlantic silverside, *Menidia menidia*. *Can. J. Fish. Aquat. Sci.* **54**, 1796-1801.

[9] Yamahira, K. & Conover, D.O. 2002 Intra- vs. interspecific latitudinal variation in growth: adaptation to temperature or seasonality? *Ecology* **83**, 1252-1262.

[10] Baumann, H. & Conover, D.O. 2011 Adaptation to climate change: contrasting patterns of thermal-reaction-norm evolution in Pacific versus Atlantic silversides. *P. Roy. Soc. B-Biol. Sci.* **278**, 2265-2273. (doi:10.1098/rspb.2010.2479).

[11] Baumann, H., Rosales-Casian, J.A. & Conover, D.O. 2012 Contrasting latitudinal variations in vertebral number and sex determination in Pacific vs. Atlantic silverside fishes. *Copeia* **2012**, 341-350.

[12] Hice, L.A., Duffy, T.A., Munch, S.B. & Conover, D.O. 2012 Spatial scale and divergent patterns of variation in adapted traits in the ocean. *Ecol. Lett.* **15**, 568-575. (doi:10.1111/j.1461-0248.2012.01769.x).

[13] Caldeira, K. & Wickett, M.E. 2003 Anthropogenic carbon and ocean pH. *Nature* **425**, 365-365.

[14] Bignami, S., Enochs, I.C., Manzello, D.P., Sponaugle, S. & Cowen, R.K. 2013 Ocean acidification alters the otoliths of a pantropical fish species with implications for sensory function. *Proc. Natl. Acad. Sci.* **110**, 7366-7370.

[15] Chambers, R., Candelmo, A., Habeck, E., Poach, M., Wieczorek, D., Cooper, K., Greenfield, C. & Phelan, B. 2014 Ocean acidification effects in the early life-stages of summer flounder, Paralichthys dentatus. *Biogeosciences* **10**.

[16] Hurst, T.P., Fernandez, E.R. & Mathis, J.T. 2013 Effects of ocean acidification on hatch size and larval growth of walleye pollock (*Theragra chalcogramma*). *ICES J. Mar. Sci.* **70**, 812-822. (doi:10.1093/icesjms/fst053).

[17] Heuer, R.M. & Grosell, M. 2016 Elevated CO2 increases energetic cost and ion movement in the marine fish intestine. *Sci. Rep.* **6**, 34480. (doi:10.1038/srep34480).

[18] Gobler, C.J., Depasquale, E., Griffith, A. & Baumann, H. 2014 Hypoxia and acidification have additive and synergistic negative effects on the growth, survival, and metamorphosis of early life stage bivalves. *PLoS ONE* **9**, e83648.

[19] Depasquale, E., Baumann, H. & Gobler, C.J. 2015 Variation in early life stage vulnerability among Northwest Atlantic estuarine forage fish to ocean acidification and low oxygen. *Mar. Ecol. Prog. Ser.* **523**, 145-156.

[20] Mehrbach, C., Culberson, C., Hawley, J. & Pytkowicx, R. 1973 Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol. Oceanogr.* **18**, 897-907.

[21] Dickson, A. & Millero, F. 1987 A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep-Sea Res. I* **34**, 1733-1743.

[22] Dickson, A.G. 1990 Standard potential of the reaction: AgCl (s)+ 12H2 (g)= Ag (s)+ HCl (aq), and and the standard acidity constant of the ion HSO4− in synthetic sea water from 273.15 to 318.15 K. *J. Chem. Thermodyn.* **22**, 113-127.

[23] Middaugh, D.P., Hemmer, M.J. & Goodman, L.R. 1987 Methods for spawning, culturing and conducting toxicity-tests with early life stages of four atherinid fishes: the inland silverside, *Menidia beryllina*, Atlantic silverside, *M. menidia*, tidewater silverside, *M. peninsulae* and California grunion, *Leuresthes tenuis*. (ed. O.o.R.a. Development). Washington, DC, U.S. Environmental Protection Agency.