Ocean acidification effects on fish hearing

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Detailed Methods

*Aquarium set-up and Husbandry*

A detailed description of the aquarium setup and experimental design can be found in MacMahon et al. (2019, in press). Briefly, this study was conducted at the National Institute of Water and Atmospheric Research Northland Marine Research Centre (NMRC), in Ruakaka, New Zealand. Brood stock fish (*n* = 39) were captured from the wild population of *C. auratus* by longline fishing in Bream Bay, adjacent to the NMRC, during September of 2017. The brood stock were split between two 20 m3 circular tanks at the ambient temperature when they were collected (~ 16 °C). Each tank was supplied with 130 L min−1 ambient seawater, filtered to 10 μm, and followed the natural temperature increase up to the summer average (18 °C), at which point it was maintained for spawning (Table 1). Spawning occurred naturally within brood stock tanks during January 2018. To maximize genetic variation in the experiment, eggs were collected from both broodstock tanks in even proportions. Eggs were collected using an external egg collector as described by Moran et al. (2007). An equal proportion of floating eggs from both contributing tanks were mixed, rinsed with oxygenated seawater for 5 min, and disinfected with Tosylchloramide (chloramine-T) at 50 ppm for 15 min. Eggs were then rinsed with seawater and evenly distributed between two 400 L conical hatching tanks. Each tank was stocked with approximately 100,000 fertilized eggs and received flow-through seawater at ambient temperature (18 °C) at a flow rate of 4 L min-1. Photoperiod was maintained at 14 h light 10 h dark. Snapper eggs hatch in 24–48 h at ambient summer temperatures at Bream Bay. Newly hatched larvae remained in the conical rearing tanks until 2 days post-hatching (dph). Larvae were not fed during this period as they rely on their endogenous reserves (Battaglene and Talbot 1992). Any dead eggs, larvae, and egg shells were removed daily by draining from an outlet at the bottom of the rearing tank. At 2 dph larvae were transferred to two 1500 L tanks located in the same facility for grow-out. These tanks received flowthrough ambient seawater (18 °C, ∼ 400 μatm CO2) at a maximum flow of 20 L min−1 per tank. Larvae were grown out at ambient conditions until 21 dph, at which point they were transferred to the experimental treatments. Larvae were fed enriched rotifers (INVE, Selco S.Presso) three times a day (0800, 1200, and 1600) from 2dph. Larvae were transitioned onto enriched artemia between 20 and 26dph with feeding twice a day (0800 and 1600) until 42dph.

*Experimental design*

At 21 dph larval fish were transferred into a fully crossed experimental design with 2× temperatures and 2 × CO2 levels. For the purposes of the present experiment, however, only fish reared at 22 °C were used. This was because the fish from the other temperature treatment (18 °C) were too small to perform auditory manipulations. The rearing temperature of 22 °C is close to the maximum temperature recorded in the region (Evans and Atkins 2013) and matches heatwave conditions in 2017/2018 (NIWA 2018). For fish reared at 22 °C the two CO2 treatments that we compared auditory ability and otolith morphology had ambient (~ 400 μatm) and elevated (~ 1000 μatm) levels of CO2, which is within the current range of pH fluctuations in habitats used by juvenile *C. auratus* in New Zealand (Law et al. 2018). Treatments were duplicated (four treatments each with two replicate rearing tanks) and each rearing tank had independent temperature and CO2 control as per best practice (Cornwall and Hurd 2015). Temperature was controlled by 1 Kw bar heaters in 200 L sumps tanks, mixed with recirculating submersible pumps, and maintained to ± 0.1 °C. The elevated ~ 1000 μatm treatment was achieved by dosing CO2 to the appropriate pH set point in the same 200 L sump tanks. CO2 dosing was regulated by a pH computer (Aquamedic) connected to a pH probe and a solenoid valve, which maintained the desired pH by slowly dosing CO2 when pH deviated above the set point. Water was delivered at 4 L min−1 from the sump tank to the respective rearing tank.

Approximately 1000 larval *C. auratus* (21 dph) were stocked to each rearing tank at ambient conditions, with the required temperature and CO2 adjustments turned on to produce a gradual change over a 24 h period. Larvae were held under these treatments for a further 21 days to 42 dph, during which they metamorphose into juveniles.

*Seawater chemistry*

Seawater chemistry is the same as that reported in McMahon et al. (2020). The pHtotal of each rearing tank was measured daily by spectrophotometry (Hach, DR3900) with cresol purple dye (Clayton and Bryne, 1993). Temperature was measured daily with a digital thermometer (Comark C22). Water samples were taken from each tank at the start of the experiment and then every seven days throughout the experiment (21, 28, 35, 42dph) for total alkalinity (TA) analysis. Water samples were immediately poisoned with a saturated solution of mercuric chloride (0.05 % of the sample volume) and later analysed at the University of Otago Research Centre for Oceanography, Dunedin, New Zealand. Alkalinity was determined by potentiometric titration in a closed cell using a Metrohm Dosimat burette (model 765, Metrohm, Switzerland), a Fluke model 8846A voltmeter, and with 0.2M HCl (nominal concentration, fortified with NaCl to the ionic strength of seawater) added in 0.1 mL steps. Samples were water-jacketed at 25 °C. TA was determined from the titration data using a least squares minimisation technique and calibrated with certified reference material (Prof. A.G. Dickson, Scripps Institution of Oceanography, U.S.). The salinity of each sample was measured with a YSI Pro30 salinity probe. The daily pCO2 of each rearing tank was then calculated in CO2SYS (Pierrot et al. 2006) from the measured values of pHtotal, temperature, TA and salinity and using the constants of Mehrbach et al. (1973), refit by Dickson and Millero (1987) (Table S1).

Table S1. Mean (± SD) of experimental seawater chemistry parameters for *Chrysophrys aurtus* broodstock tanks and juvenile treatment tanks. Broodstock tanks were measured during the week of spawning at the start, middle, and end of the week. Temperature and pHtotal were measured daily in each juvenile rearing tank over the 21 day experiments (21-42 dph). Total alkalinity and salinity were measured at the start of the experiment and then every 7 days. *p*CO2 was estimated from these parameters in CO2SYS.

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| **Treatment** | **Salinity** **(ppt)** | **Temperature (oC)** | **Total Alkalinity (mmol/kgSW)** | **pH** **(Total)** | **pCO2 (µatm)** |
| **Brood stock**  | 35.60 ±0.05 | 18.03 ±0.04 | 2154 ±6 | 7.88 ±0.01 | 583 ±14 |
| **Juveniles tanks Control CO2** | 35.34±0.21 | 22.03 ±0.07 | 2313 ±9 | 7.99 ±0.02 | 465 ±26 |
| **Juvenile tanks Elevated CO2** | 35.29 ±0.16 | 21.96 ±0.20 | 2316 ±10 | 7.69 ±0.01 | 1027 ±32 |

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