**Molecular basis of parental contributions to the behavioral tolerance of elevated pCO2 in a coral reef fish**

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**Supplementary information:**

***Response to chemical alarm cue (CAC)***

Behavioral phenotypes of adult *Acanthochromis polyacanthus* that had been exposed to elevated CO2 were determined as previously described (1,2). A 1:1 ratio of test fish to donor fish was used to produce CAC. Donor fish were the same size and age as test fish, and were kept in control conditions. CAC was produced by euthanizing donor fish with a blow to the head. They were then cut along the side multiple times, and rinsed with 60ml of control water, which was then mixed with 10L of treatment water. CAC water was fed into the flume at a constant rate of 450 ml per minute. *CAC was made immediately before every trial. Trials lasted 9 minutes, thus CAC water was used within 10 minutes accounting for transfer time. CAC was made fresh for every fish to reduce any possibility of degradation.* Each 9 minute trial was broken down into 2 minutes habituation, 2 minutes recording, followed by a 1 minute switch for water sides where the fish was recentered at the end of this minute, followed by a repeat of the 2 minutes habituation and 2 minutes recording. The location of the fish was recorded every 5 seconds during each 2 minute recording period.

Substantial variation in behavioral response to CAC was observed among the adult fish that had been exposed to elevated pCO2 (754 µatm) for seven days; 38.02% of collected adults fell into the tolerant category of less than 30% time spent in the CAC water stream and 38.84% fell into the sensitive category of spending more than 50% of their time in the CAC water stream. These fish were sexed based on the shape of their genital papillae, measured (length) and then sorted into breeding pairs containing the required mix of tolerant and sensitive adults for the experiment.

***Water Quality***

The experimental system follows that previously described (1,2). Two 10,000L recirculating systems were set to different pH levels corresponding to current day control pCO2 (414μatm) and end of century elevated pCO2 (754μatm). An Aqua Medic AT Control System (Aqua Medic, Germany) was used to dose a 3,000L sump tank to maintain desired pCO2 levels in the elevated CO2 treatment tanks. The other 3,000L sump attached to the control tanks was not dosed. Water from the respective sumps was delivered to the holding aquaria at a rate of 1.5 L/min. A pH electrode (SevenGo Pro, Mettler Toledo, Switzerland) and temperature probe (Cormark C26, Norfolk, UK) were used to take daily measurements. Salinity was measured with a conductivity sensor (HQ15d; Hach, Loveland, CO, USA), while alkalinity was estimated using Gran titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) and using certified reference material from Dr. A.G. Dickson (Scripps Institution of Oceanography), both weekly. pCO2 was calculated from measured values of pH, temperature, salinity and total alkalinity using CO2SYS. Calculation constants are listed in Welch and Munday (1). Mean seawater parameters for the duration of the experiment are shown in Supplementary Table 1, below.

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| **System** | **pHNBS** | **Temperature (°C)** | **Salinity** | **TA (μmol kg−1 SW)** | ***p*CO2 (μatm)** |
| Control | 8.15 (±0.04) | 28.5 (±0.2) | 35.0 (±1.2) | 2,146 (±125) | 414 (±46) |
| High CO2 | 7.94 (±0.04) | 28.5 (±0.3) | 35.1 (±1.2) | 2,223 (±152) | 754 (±92) |

Supplementary Table 1. Mean (±SD) seawater parameters in the experimental systems. Seawater chemistry was consistent for the breeding and experimental components of the study.

***Offspring growth***

Total body weight to the nearest 0.01g was measured for all offspring at the same age following euthanasia. A two-way ANOVA followed by a Tukey’s post-hoc comparison was used to analyze weight differences between experimental treatments and parental pairs in R v4.0.3 (3). Graphs were created using the R package ggplot2 (4).

Offspring ranged from 0.89g to 4.63g (Supplementary Table 3). A significant difference (ANOVA, df=92, F=7.66, p=0.007) was found between offspring of different parental pairs within the transgenerational treatment. Offspring from tolerant fathers were significantly larger than those of tolerant mothers. This appears to be a trend throughout all four treatments (Supplementary Figure 1), however no statistical significance was found in any other comparison.

In several transcriptomic comparisons we identified downregulation of growth and development genes (*TNNI2, TNNC2, MYSS, ACTA1, ACTN3)* in offspring of tolerant mothers. This was most apparent in the acute exposure conditions, but we also identified them when comparing control to transgenerational treatments for tolerant mother offspring (Figure 5). At the same time, we identified many upregulated genes linked to neural plasticity suggesting a possible trade-off with body size when compensating for changes in seawater chemistry.



Supplementary Figure 1. Boxplot of offspring weight across different pCO2 treatments. Colors represent parental pairs; blue indicates tolerant mothers while green are offspring from tolerant fathers.

**References**

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