PEBBLE TO THE METAL: A BOULDER APPROACH TO ENRICHMENT FOR *DANIO RERIO*

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BACKGROUND

- Zebrafish are well-established animal models in research, yet there is no consensus on the use of environmental enrichment for their well-being.
- Zebrafish are social animals that shoal in both the wild[1] and captivity, and zebrafish prefer housing with conspecifics.[2]
- Wild zebrafish environments are vegetated and over gravel or silt substrate[3], while standard laboratory housing racks are barren.
- Commonly raised objections for using in-tank enrichment:
  - Health of the zebrafish
  - Hygiene of the in-tank enrichment
  - Husbandry workload with increased time/cost commitment

AIMS

- To test whether a low-cost, easy to implement outside-of-tank enrichment plan can improve the welfare of zebrafish.
- To compare the physiological and behavioral preferences of zebrafish for housing over a picture of pebbles using the visual and/or physical access to conspecifics as a positive control.

HYPOTHESES

- Zebrafish will show a behavioral preference for the enriched half of the tank.
- Zebrafish housed with conspecifics will have lower cortisol levels than those that are singly housed.
- Zebrafish housed with image of pebbles under the tank will have lower cortisol levels.

PREFERENCE TEST

- **Substrate Image (Enrichment)**: Pictures of pebbles were placed underneath the tanks spanning half the tank floor, with locations of the images alternating between front and back to prevent location bias.
- **Visual Access (Positive Control)**: Blinders were placed on the sides of tanks to exclude visual access to conspecifics in neighboring tanks on either the front half or back half of the tank.
- **Measuring Preference**: Video cameras were attached above the tanks to record the tanks over a two-week period, and the videos were analyzed by a blinded observer for time spent over the enriched or barren half of the tanks (Figure 1).

Figure 1:
Preference test for under-tank pebble picture. Singly housed zebrafish in barren (top) and enriched (bottom) front halves of tank during video analysis.
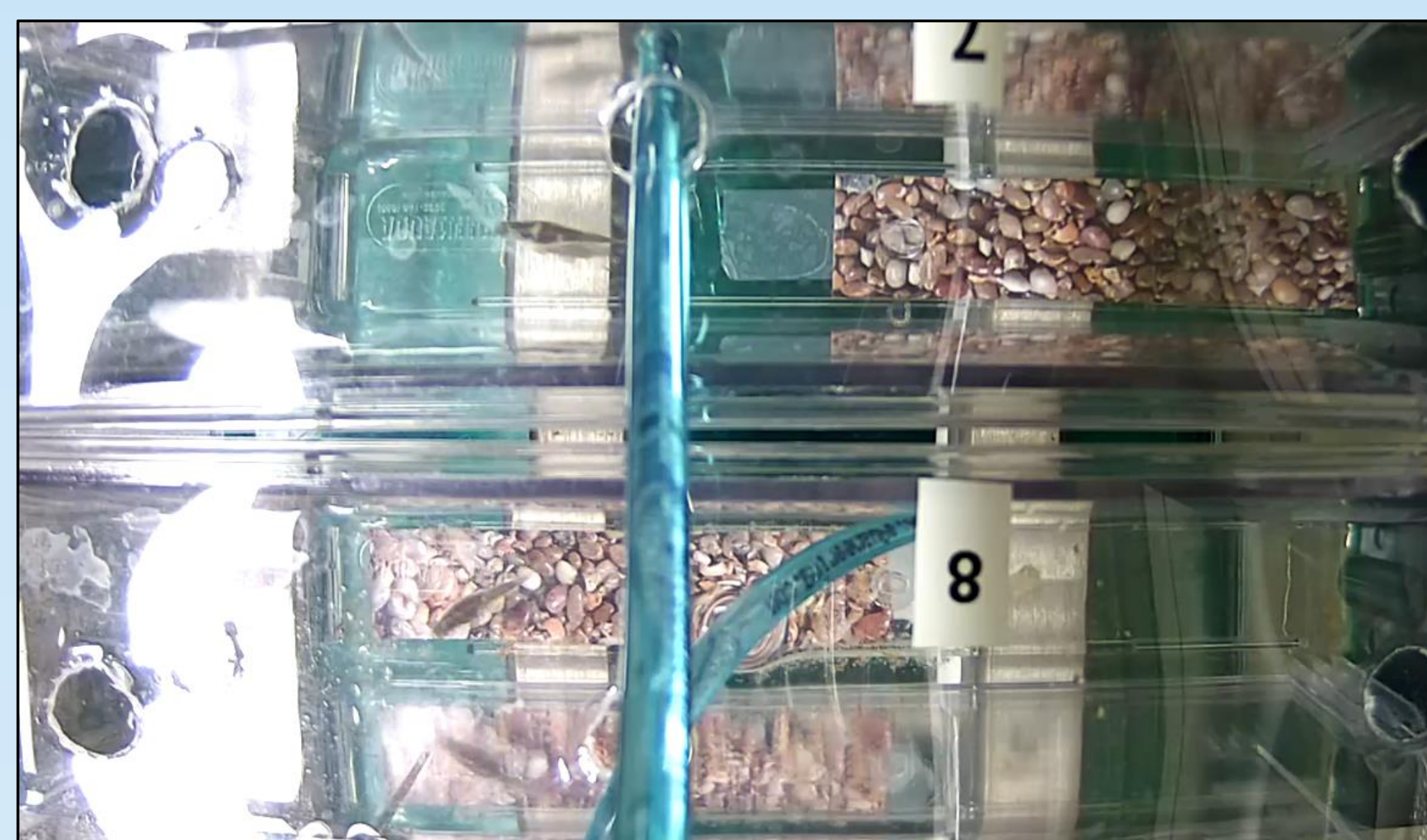
CORTISOL AND OTHER METHODS

- **Subjects**: 40 adult wildtype zebrafish split into two cohorts; 8 tanks housed in groups of 4, and 8 tanks singly housed.
- **Housing**: Zebrafish were housed in 1.4 L tanks on a fixed recirculating rack, with water maintained at species appropriate conditions, in a room with a 14:10 light:dark cycle. Feeding occurred 2x daily with brine shrimp/commercial flakes.
- **Measuring Chronic Stress**: Water cortisol was taken from the occupied tanks and measured using Cayman Chemical's Cortisol ELISA Kit (500360).
- **Measuring Acute Stress**: American Veterinarian Medical Association approved method of euthanasia was performed using rapid chilling in a 2-4°C ice bath, and whole-body cortisol measured using Cusabio's Fish Cortisol ELISA Kit (CSB-E08487f).
- **Statistical Analysis**: Analyzed using GLM in JMP 16 Pro. Analysis was controlled by rack and location of tank on rack, and number of fish and treatment crossed as experimental factors. Assumptions were checked and there were no transformations required. Post-hoc tests were performed using Bonferroni-corrected planned contrasts where needed.

PREFERENCE RESULTS

Figure 2:
Zebrafish significantly preferred the enriched half of the tank (P=0.0013), with no difference between visual access and pebbles. Singly housed fish always preferred enrichment; group housed fish only preferred enrichment in the back (P=0.0142).
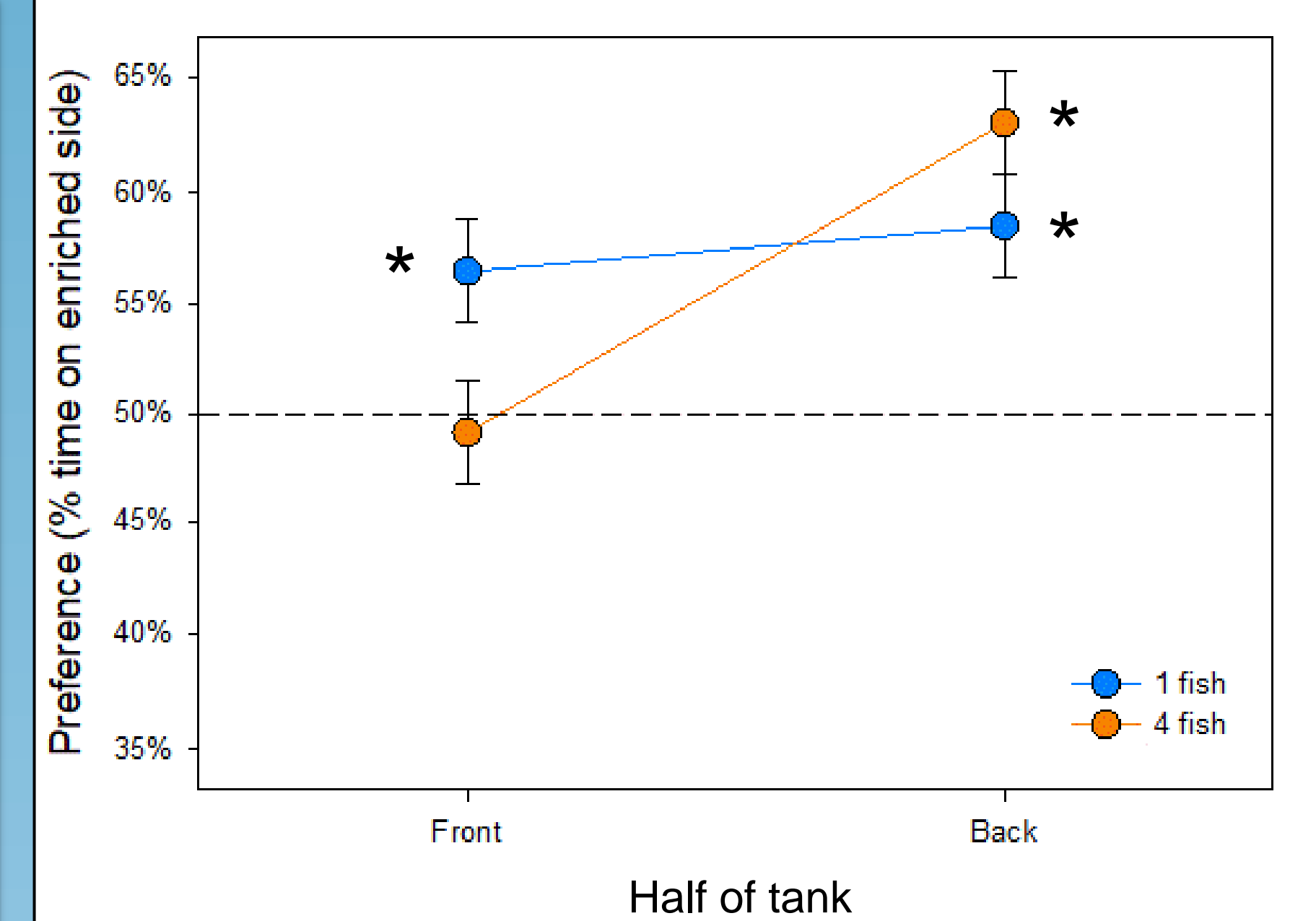
CORTISOL RESULTS

Figure 3:
Enriched (P=0.0232) and group housed (P=0.0106) tanks had significantly reduced tank water cortisol levels. Singly housed enriched tanks did not differ significantly from the barren group housed zebrafish.
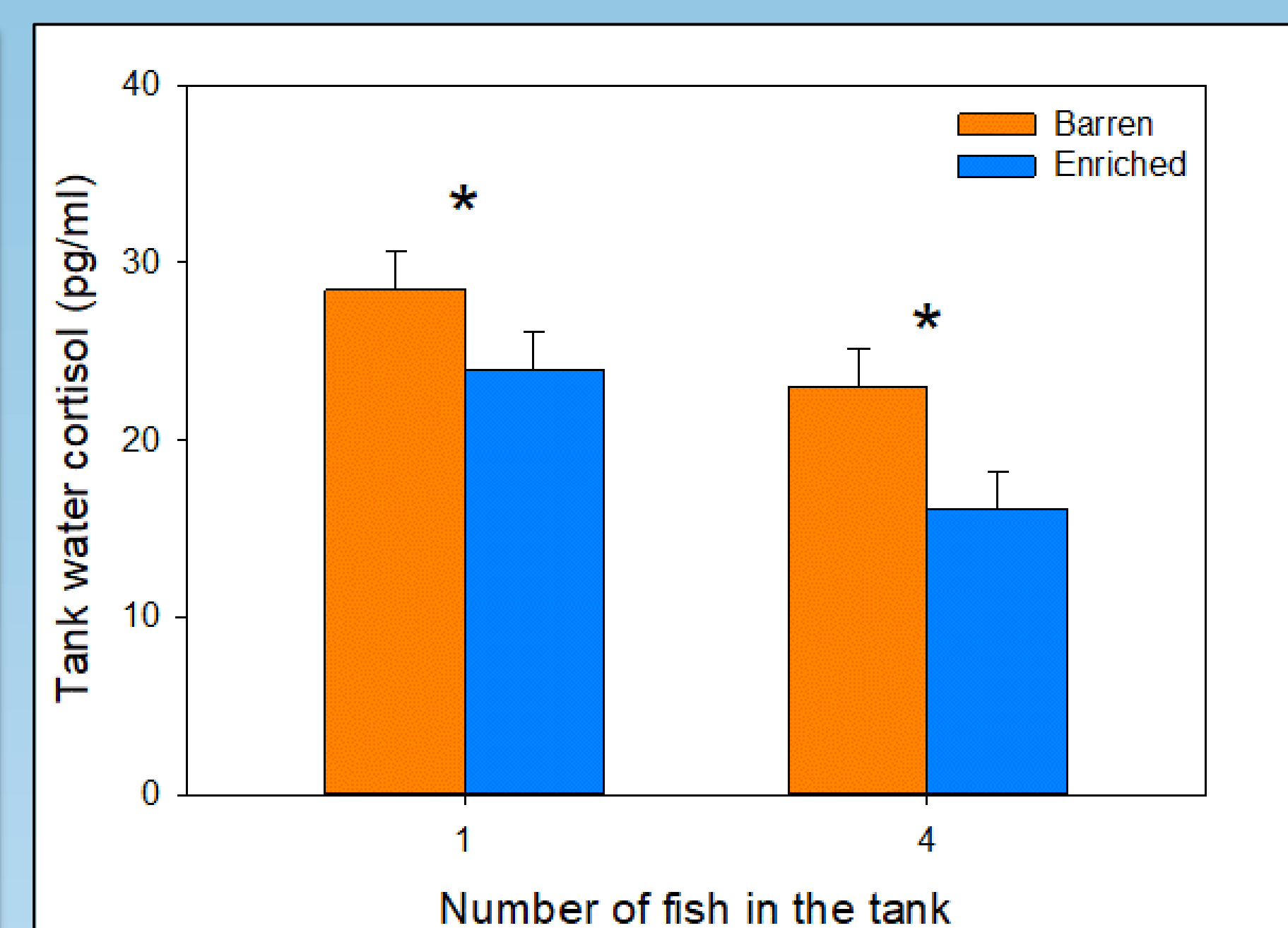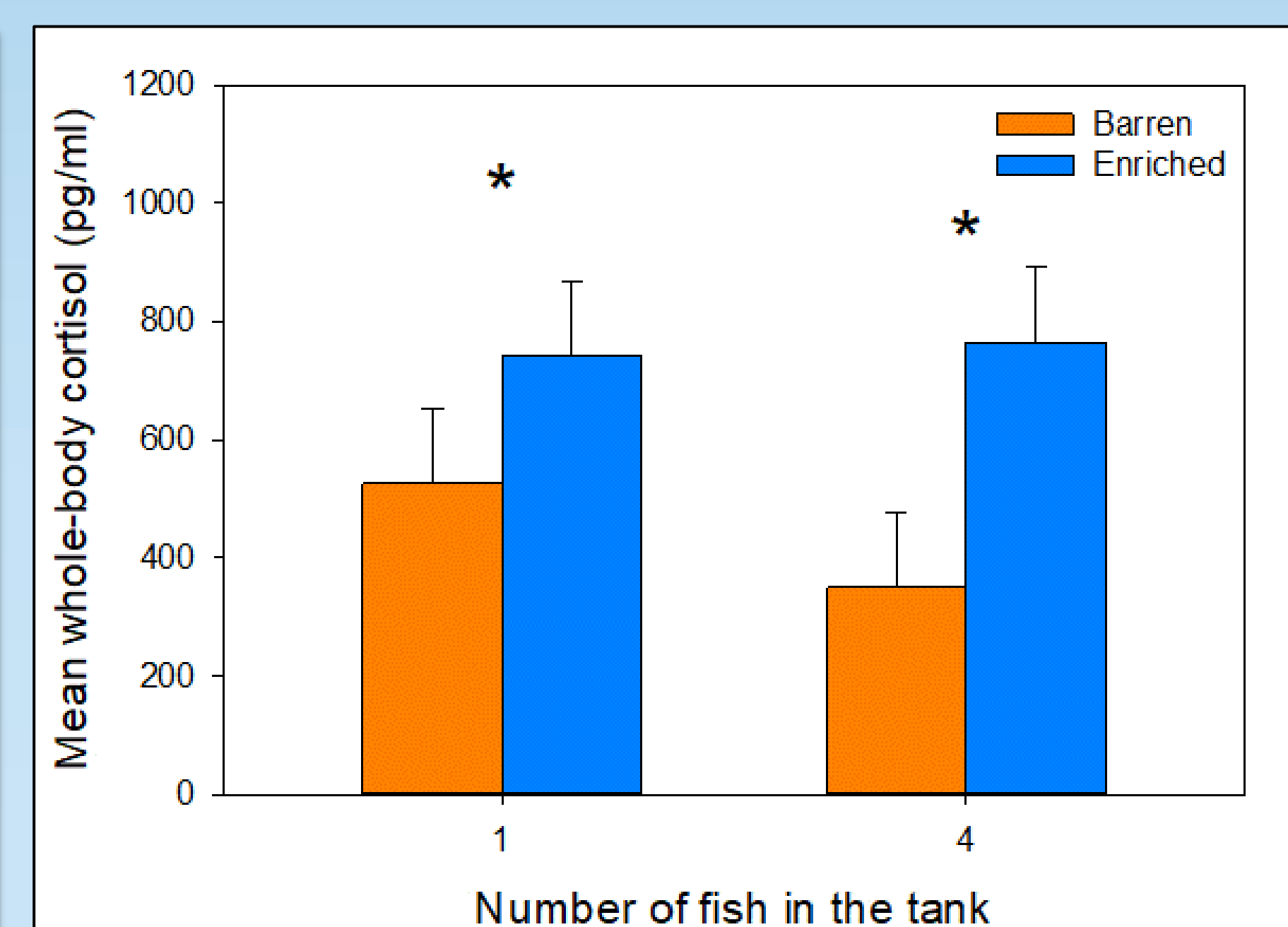

Figure 4:
The zebrafish in enriched tanks showed significantly higher levels whole-body cortisol (P=0.0320). The number of zebrafish in the tank did not have an effect on the zebrafish's whole-body cortisol levels (P=0.5575).

LEAVING NO STONE UNTURNED

- Zebrafish preferred the half of the tank that was enriched. The preference for enrichment in singly housed fish counteracted the aversion to the front of the tank shown by group housed fish.
  - Pebble enrichment is equivalent to the positive control (visual access).
  - Back of tank may feel safer, away from humans.
- Water cortisol represents a zebrafish's chronic stress levels, as it is a measurement of the cortisol that naturally leeches from the body over a longer period, and enriched fish experienced less chronic stress overall.
  - The benefit of pebble enrichment was equivalent to the positive control of the group housing, which compensated for single housing.
- Whole-body cortisol is a representation of zebrafish's acute stress levels at the time of death.
  - Higher acute stress responses in zebrafish enriched with a picture of pebbles could possibly show a ceiling affect, where zebrafish with higher overall chronic stress levels have a less significant response to acute stress events such as euthanasia.

FUTURE DIRECTIONS

- Testing for the impact of different types of substrate images underneath zebrafish tanks.
- Testing for the impact of under-tank images in other laboratory fish species, such as killifish or sticklebacks.
- Implementing the use of pebble pictures under every zebrafish tank at Stanford University!

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