Gravitational stress alters metabolism and immune function in *Acheta domesticus* by activating a cold shock response.

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ABSTRACT

An understanding of the deleterious effects of abnormal gravity on the human immune system is necessary for the success of long-term space missions. Insects such as *Drosophila melanogaster* have been used to study gravitational effects since the early 1990s. However, no study has yet addressed the underlying mechanism of immune response to gravitational extremes. In this study, house crickets (*Acheta domesticus*) were used to identify the stress response pathway activated by gravitational extremes. Crickets were exposed to either gravitational stress (1 hour of microgravity-2G cycles at ambient temperature aboard NASA's C-9 aircraft) or common stress (heat shock, cold shock, or ischemia.) Using a battery of immune and metabolic functional assays, we found that gravitational stress significantly decreased immune function and metabolism. These changes were most consistent with those observed in crickets exposed to cold shock or ischemia. Since ischemia activates the cold shock pathway in animals, we hypothesized that gravitational stress also activates this pathway. To confirm this, we showed upregulation of chaperonin-containing *t*-complex alpha (CCTα), a cold shock protein, at 0 hrs and 24 hrs post-gravitational stress. Our results indicate that crickets respond to gravitational stress by activating some or all of the cold shock response pathway. Inhibition of this pathway in crickets (or the homologous pathway in humans) may ameliorate many of the harmful effects that abnormal gravity has on the immune system.

MATERIALS AND METHODS

Materials. Capillary tubes were purchased from Health Management Systems, Corp., TX. Anti-CCTα antibody was purchased from AbD Serotec, and all other chemicals were purchased from VWR.

Insects. Six week old 1 inch female crickets were purchased from Fluker Farms, LA. They were maintained in covered plastic containers at ambient temperature with *ad libitum* water and food (Orange Cube Complete Diet, Fluker Farms). The mean fresh body mass of crickets was 0.65 g. For each hemolymph sample, an cricket was beheaded with sterile scissors, and the hemolymph welling up into the thoracic cavity was collected using acapillary tube. Samples were immediately frozen in a dry ice/ethanol bath and stored at -80°C.

Gravational protocol. Crickets were exposed to 1 hour of microgravity-2G cycles at ambient temperature aboard NASA C-9B, a Boeing aircraft used to train astronaut pilots. The C-9B flew this experiment on August 8, 2006 operating from Johnson Space Center in Houston, TX. NASA sponsored the flight through the 2006 Summer College Campaign of Microgravity University.

Stress protocols. Crickets were randomly selected and divided into four groups: control, heat shock (37°C for 1hour), cold shock (4°C for 1 hour), and hypoxia (5% oxygen for 1 hour). Each group consisted of 35 crickets, 15 of which were implanted with monofilament for the encapsulation assay.

Respiration assay. Closed system respirometry (Hack, 1997) was performed using a Telaire 7001 Carbon Dioxide Monitor from MicroDAQ. The CO2 output of four separate groups of 50 crickets was monitored for periods of 1hr at ambient temperature in flight and under terrestial conditons.

Hemocyte count. A 2 µl sample of fresh hemolymph was diluted 1:20 with a solution of acridine orange (20µg/ml), and hemocytes were counted using a fluorescent microscope.

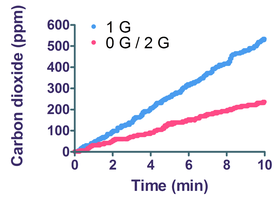
Encapsulation assay. Crickets were chilled on ice for 20 min. A cricket's pleural membrane was punctured between sternites six and seven. A 5 mm long piece of nylon monofilament (Φ = 0.23 mm) was inserted into the hemocoel through the puncture wound. The crickets were kept at room temperature in groups of 15 and exposed to 6 hrs of 1g or 4 hrs of 1g followed by 1 hr of gravitational stress and then 1 hr of terrestrial conditions (See *Gravitational protocol*). After 6 hrs, implants were removed and dried. To help with visualization of deposited protein, the implants were stained with 0.1% (w/v) Ponceau S in 5% acetic acid. They were imaged using 10x air objective on a Olympus 1X71 microscope and optical opacity was measured using ImageJ.

Carbohydrate levels. Glycogen and free carbohydrates were measured using the anthrone method described by Mokrasch (1954) with modifications. Hemolymph was diluted five fold in a 96 well plate to give a final volume of 50 µL. To this 15 µL anthrone reagent (0.13% anthrone in 67% sulfuric acid) and 35 mL of PBS was added. The plate was incubated with agitation at 90°C for 10 min. The samples were allowed to cool to room temperature, and absorbance at 585 nm was measured.

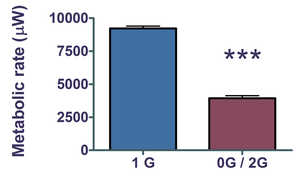
Protein assay. The protein concentration in hemolymph was measured using the BCA Protein Assay Kit (Pierce, IL) following the protocol of the manufacturer.

Lysozyme assay. Lysozyme was assayed using methods similar to Rantala et al., 2004. Briefly, 150 µL of 0.35 mg/mL solution of *Micrococcus lysodeikticus* in Tris-HCl (pH 6.5) was combined with 10 µL of hemolymph in a 96 well plate. The plate was incubated at 25°C, and the optical density of wells was measured every minute for 30 minutes. Lysozyme activity was calculated by the change in optical density per minute and compared to a standard curve.

Figure 1. Exposure to gravitational stress alters markers of cricket metabolism

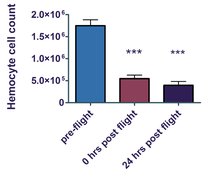


Carbon dioxide (ppm) accumulation was measured under microgravity and terrestial conditions. Crickets in microgravity respire at a rate (24.32 ± 0.75 ppm/min) half that of crickets under terrestial conditions (54.55 ± 0.32 ppm/min).

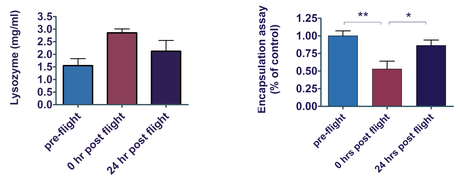


Metabolic rates of crickets under microgravity are decreased by 53.1%. *** p < .001

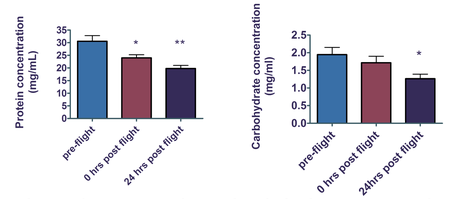
Figure 2. Exposure to gravitational stress decreases markers of cricket immunity



Total hemocytes (cricket immune cells) are decreased 62% post exposure to microgravity and 68% 24 hours post exposure to microgravity.

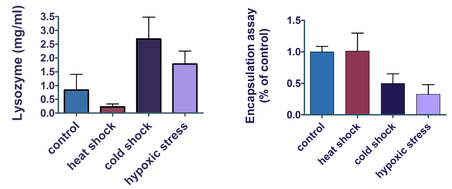


Measurement of the total lymph lysozyme (mg/mL) and encapsulation of foreign body assay show decreases in immune response function immediately post exposure to microgravity and recovery 24 hours post flight. * p < 0.05, ** p < 0.01

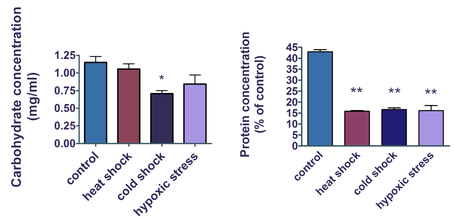


Lymph protein concentration and carbohydrate concentration, markers of metabolic activity, show decreases post exposure to microgravity. * p < 0.05, ** p < 0.01

Figure 3. Altered metabolism and immune markers found in the lymph can be reproduced by inducing cold shock

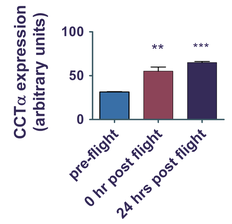


Both cold shock and hypoxic stress show trends of decreased immune function.



All three stresses cause significant decreases in lymph protein concentration while only cold shock caused a decrease in lymph carbohydrate concentration. * p<0.05, ** p <0.01

Figure 4. Microgravity induces expression of cold shock protein CTTα.



Expression of cold shock protein CCTα was increased immediately after exposure to microgravity and 24 hours post exposure. Levels were quantized from western blots (3 crickets per group per western blot, average of three western blots) using Image J software.

CONCLUSION

•Our results show that gravitational stress suppresses respiration, decreases amounts of circulating protein, sugar, and hemocytes, and inhibits encapsulation and lysozyme activity. These same physiological effects occur during cold shock and ischemia in crickets.

•On a molecular level, gravitational stress appears to act via the stress response pathway common to cold shock and ischemia. Both cold shock and gravitational stress lead to upregulation of cold shock protein CCTα.

•Further work on the relationship between cold shock and gravitational stress should increase our knowledge of sensation and adaptation to gravitational extremes.

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REFERENCES

Hack, M.A., 1997. The effects of mass and age on standard metabolic rate in house crickets. Physiological Entomology 22, 325-331.

Mokrasch L.C., 1954. Analysis of hexose phosphates and sugar mixtures with the anthrone reagent. J. Biol. Chem. 208, 55-59.

Rantala, M.J. et al., 2004. Fluctuating asymmetry and immune function in a field cricket. Oikos 107, 479-484.