INTRODUCTION
The physiology of both vertebrates and invertebrates follows internal rhythms coordinated in phase with the 24-hour daily light cycle. This circadian clock is governed by a central pacemaker, the suprachiasmatic nucleus (SCN) in the brain. However, peripheral circadian clocks or oscillators have been identified in most tissues. How the central and peripheral oscillators are synchronized is still being elucidated.

Light is the main environmental cue that entrains the circadian clock. Under the absence of a light stimulus, the clock continues its oscillation in a free-running condition. In general, three functional compartments of the circadian clock are defined:

- Molecular clock: governed by a central pacemaker, the suprachiasmatic nucleus (SCN) in the brain.
- Peripheral clock: oscillators have been identified in most tissues.
- Input: light input

The vertebrate retina contains endogenous clocks that control many aspects of retinal physiology, including retinal sensitivity to light, neurohormone synthesis1 (melatonin and dopamine), rod disk shedding, signalling pathways and gene expression.

Neurons with putative local circadian rhythm generation are found among the major neuron populations in the mammalian retina. In the mouse, clock genes and function are more localized to the inner retinal and ganglion cell layers2. The photoreceptor, however, secretes melatonin which may still serve an important circadian signal.

The reception and transduction of the non-visual photic stimulus resides in a small subpopulation (1-3%) or retinal ganglion cells (RGC) that express the pigment melanopsin (Opn4) and are called intrinsically photoreceptive RGC (ipRGC). Melanopsin peak absorption is at 420 nm and all the axons of the ipRGC reach the SCN3.

A common countermeasure for circadian re-entrainment utilizes blue-green light to entrain the circadian clock4,5 and mitigate the risk of fatigue and light damage. Mitigation of fatigue and health and performance decrement due to circadian rhythm disruption. However, an effective countermeasure targeting the photoreceptor system requires that the basic circadian molecular machinery remains intact during spaceflight.

We hypothesize that spaceflight may affect ipRGC and melanopsin expression, which may be a contributing cause of circadian disruption during spaceflight.

OBJECTIVE
To compare the ipRGC population and melanopsin expression in retinas from mice flown aboard STS-133 with ground controls.

MATERIALS AND METHODS
The STS-133 flight animal experiment consisted of two albino BALB mice per group (and their ground controls, n=3) whose retinas were collected at R+1, R+5 and R+7. Ground controls consisted in vivarium and animal enclosure module (AEM) animals. All mice were maintained in a 12/12 hour light/darkness cycle; standard illumination in the vivarium is approximately 10-fold the illumination in the AEM. After enucleation, one eye was fixed for paraffin embedding and immunohistochemistry; the contralateral eye was stored in RNA later until processed for RNA isolation.

Immunohistochemistry and immunofluorescence

The oxidative damage marker 8-hydroxydeoxyguanosine (8-OHdG), was detected with a polyclonal antibody to 8OHdG (ab10802, Abcam), and a secondary antibody conjugated with peroxidase and AEC (3-Amino-9-ethylcarbazole) as a substrate to develop color. Light microscopy images were analyzed using the open access software Image J, processing all images identically.

Melanopsin was detected with a rabbit polyclonal antibody that reacts with mouse and rat melanopsin (PA-1-780, Thermo Scientific). Secondary antibody was a goat anti-rabbit IgG conjugated with Alexa 488 (Invitrogen). Images were obtained with a Leica confocal microscope.

Real Time qPCR
Total RNA and DNA were isolated from whole retina using the AllPrep DNA/RNA Micro isolation kit (Qiagen). cDNA was synthesized using the Quantitect Whole Transcribe kit (Qiagen) and PCR amplifications were performed using specific primers (Quantitect primers, Qiagen) and SYBR Green as a fluorescent probe (IQ SYBR Green Supermix, BioRad).

RESULTS
Our immunofluorescence results are in agreement with the description of the distribution of ipRGC. RGC positive for melanopsin were found uniformly distributed in the RGC layer throughout the retina, with occasional crowding along the periphery. Virtually no immunoreactive cells were found in retina samples from mice aboard STS133 after one day upon return; however several positive cells were seen in samples from mice after flight on R+7. Likewise, both vivarium and AEM ground controls showed evidence of ipRGC.

Analysis of melanopsin mRNA levels by real time PCR revealed a drop in melanopsin gene expression in flight samples upon return to Earth, compared to ground controls, but control levels are recovered after 7 days after landing.

Melanopsin (Opn4) gene expression levels in retina samples from BALB mice in the STS133 experiment, measured by real time qPCR. Y axis: logarithmic scale of comparative gene expression level normalized to housekeeping genes. The numbers in green indicate the mouse # in the experiment. For #13, the expression levels were closest to zero, as pointed by the arrow (qPCR amplification curved shifted to the right, below threshold, so the value that appears in the graph is actually an artificial value close to zero that was necessary to be added in the calculations process).

In order to investigate the levels of oxidative stress in the samples, the DNA damage oxidative stress marker 8OHdG was qualitatively measured in each immunostained retinal section. The figure below shows representative fields of retinal sections comparing DNA damage due to oxidative stress. RGC appear to be a target of damage in flight samples, whereas photoreceptors are more affected in vivarium samples. AEM ground controls showed the lowest incidence of oxidative stress.

CONCLUSIONS
In conclusion, the number of melanopsin-immunoreactive RGC as well as melanopsin gene expression were decreased in flight samples immediately after flight but this change was attenuated in flight sample 7 days after return. Retinal ganglion cells are a target of the effects of oxidative stress induced by spaceflight, based on immunohistochemistry of 8OHdG in eye samples. We propose that oxidative stress can lead to a decrease in melanopsin expression, likely via ipRGC loss or impairment, and thus, it can be a contributing factor to circadian disruption during spaceflight.

Future questions to be answer include: a) is the decrease in melanopsin expression observed after spaceflight due to RGC loss or to RGC impaired gene expression?; b) are other clock genes also affected?; c) is the local retinal clock output affected?; d) does the decrease in melanopsin translate into a significant alterations in the signaling to the SCN to contribute to circadian rhythm disruption?; e) which retina-specific cellular rhythms might be affected by a local circadian clock disruption?

BIBLIOGRAPHY

ACKNOWLEDGEMENTS
This work was funded by NASA HRP (MTL707). We thank the assistance of Audrey Nguyen, JSC SLSD Intern, Clear Lake High School, in the preparation of immunostained slides and image analysis, and James Fiedler, PhD, for help with graphing the gene expression data.