Summer July 19, 2018

Investigation of Zebrafish Larvae Behavior as Precursor for Suborbital Flights: Feasibility Study

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Research Article

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ABSTRACT

Suborbital spaceflights, carrying scientific payloads, allow scientists not only to test the feasibility of their payloads, but they also provide the basis for refining scientific hypotheses to be later tested on the International Space Station (ISS). Therefore, it is essential to establish robust pre-flight procedures in order to take advantage of this unique research platform to facilitate payload delivery. In the present study, we assessed zebrafish larvae behavior as a precursor for the future suborbital spaceflight involving research on the musculoskeletal system. Zebrafish larvae were exposed to the same physiological stressors they would encounter during suborbital spaceflight: alterations in light, thermal, and centrifugation conditions. Their behavioral responses were analyzed using the DanioVision (Noldus) behavioral tracking system. Our results showed that zebrafish were most active when kept in a dark environment as measured by swim distance. Also, thermal alterations revealed that zebrafish larvae adapted well to the different temperatures ranging from 25°C to 32°C with the highest levels of locomotor activity observed at 32°C. Finally, the centrifugation tests demonstrated that although zebrafish were exhausted initially, their recovery process was short, lasting for approximately five minutes. Taken together, our findings support the hypothesis that using zebrafish larvae is a feasible model for future suborbital flights. Thus, the lessons learned allow us to propel this research with more refined and realistic procedures as a precursor for orbital flights to the ISS and to cis-lunar space.

Key words: Zebrafish Larvae Locomotor Motion; Suborbital; Physiological Stressors; Muscle Atrophy

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INTRODUCTION

Suborbital spaceflights are becoming an important scientific research platform offering a wide range of new research opportunities and enabling scientists to use microgravity as a unique environment to develop and refine hypotheses to be later tested in orbital space (Wagner et al., 2009; Moro-Aguilar, 2014; Pletser et al., 2016). Suborbital research is currently being undertaken with reusable vehicles such as Blue Origin's New Shepard and Virgin Galactic's SpaceShipTwo. We have flown a scientific payload onboard Blue Origin's New Shepard as part of the Research

Education Mission (REM) M7 in December of 2017. Other emerging suborbital research platforms include the American Vector Space Systems' Vector-R vehicle and New Zealand's Electron launch vehicle. Additionally, Europe's first reusable suborbital rocket, PLD Space's Arion 1, plans to conduct test flights in 2019, with a focus on flying new research science and technology hardware while providing a wide breadth of educational applications for students to send suborbital payloads, such as micro/small satellites and NanoLabs. Given the rising interest in using suborbital spaceflights for scientific purposes, it is essential to test, validate, and optimize the procedures for scientific payload preparation and integration.

The Spaceflight Operations team in the Applied Aviation Sciences (AAS) department at the Embry Riddle Aeronautical University (ERAU) is currently developing several suborbital payloads as part of the Arete STEM (Science, Technology, Engineering, and Math) Project (ARETE) to demonstrate joint commercial spaceflight activities. The AAS department in the College of Aviation (COA) has been allotted a flight opportunity to send a scientific experiment to suborbital space onboard Blue Origin's New Shepard capsule at the end of 2018, early 2019. ERAU is working together with the scientists from the University of Texas Health Science Center at San Antonio and the Medical University of South Carolina on the preparation of a translational science research project aimed at investigating the effect of microgravity on the musculoskeletal system.

It is well established that prolonged space travel has detrimental physiological effects on the human body, particularly the skeletal muscle. As previous studies indicate, the musculoskeletal system is impaired with prolonged duration of weightlessness (Fitts et al., 2000; Fitts et al., 2010; Trappe et al., 2009; Bagley et al., 2012). Long stays in microgravity also severely affect bone density deterioration (Hodkinson et al., 2017), such as bone losses in the spine, femur neck, trochanter, and pelvis. In addition, it is well reported that muscle atrophy is a significant adverse effect from space travel. Furthermore, despite exercising, muscle mass has been shown to decrease dramatically during long duration spaceflights (Gopalakrishnan et al., 2010; LeBlanc et al., 2000). According to NASA, astronauts experience up to a 20 percent loss of muscle mass on spaceflights lasting 5 to 11 days (NASA, n.d.). Moreover, muscle atrophy is also considered a major symptom of many patients with mitochondrial disease, which affects the energy production required for their muscle integrity (DiMauro, 2004). Unfortunately, there is no definitive treatment nor is there a cure for muscle atrophy. Given the upcoming projected long term space travel missions to the Moon, Mars, and beyond, it is essential to use state-of-the-art scientific models to further investigate the impact of microgravity on the musculoskeletal system as well as to identify preventive measures mitigating the severe consequences of extended exposure to weightlessness. In fact, the Space Biology Plan for 2016-2025 released by NASA encourages studies designed to investigate the biology effects of long duration space environment exposure (Tomko et al., 2016).

In this study, we assessed the behavior of zebrafish larvae as a precursor for the future suborbital and orbital flights that will be aimed at investigating the role of space-induced muscle atrophy. To determine the extent to which zebrafish larvae could survive the suborbital flight, we exposed them to the same stressors they would encounter during the actual suborbital flight: alterations in light, thermal, and vibration conditions. Zebrafish make an excellent research model and have many advantages, such as their transparency, fast development, and genetic similarity to humans (Tavares and Santos Lopes, 2013; Chan et al., 2018; Aceto et al., 2016). Furthermore, a recent review suggests that zebrafish mutants and transgenic lines can be used to model human skeletal diseases (Laizé et al., 2014). Specifically, it applies to studying the osteocytic bone, multinucleated osteoclasts, collagen in bone, and various types of cartilages and ossification of the vertebral column with the hope to better understand the underlying mechanisms of diverse musculoskeletal disorders. In addition to using zebrafish as a research model, some researchers (Chatani et al., 2015; Chatani et al., 2016; Ijiri, 1995) have used medaka fish to study microgravity induced effects. These studies revealed that medaka fish exposure to microgravity resulted in impaired physiological function with a change in mechanical force

(Chatani et al., 2015) and altered gene expression in osteoblasts and osteoclasts (Chatani et al., 2016).

Zebrafish behavior is a direct reflection of neural activity and its modulation by external stimuli (Girdhar et al., 2015; McKeown et al., 2009). Today's technology allows the use of high-throughput automated zebrafish tracking systems to generate quantitative results to capture their behavioral responses as readouts of their wellbeing (Ingebretson and Masino, 2013; Liu et al., 2015; Zhou et al., 2014).

MATERIALS AND METHODS

Zebrafish Larvae

Wild-type AB strain zebrafish were crossed according to standard methods and embryos were raised to 6-8 days post-fertilization (dpf) in accordance with Westerfield (2007). Zebrafish were maintained in petri dishes (100 mm diameter) filled with embryo water in a 28.5°C incubator under a 14/10 h light/dark cycle. Zebrafish were exposed to three types of stressors: alterations in light, thermal, and centrifugation conditions. Their locomotor response to these stressors were tracked using the DanioVision® instrument (Noldus) and subsequently assessed using EthoVision® software (Noldus) (Rahn et al., 2014). The behavioral recordings took place by transferring larvae from petri dishes into individual wells of a 48-well plate using 1 mL pipette with a cut tip containing 500 µL of water. This procedure was repeated for each batch of zebrafish that was exposed to a different stressor. Once zebrafish were placed in the 48-well plate, we proceeded to calibrate the DanioVision instrument to track their motion. Given swimming performance is a biological characteristic that has a very important role on fish survival, our main readout was the movement of the zebrafish, also referred to as distance traveled. All animal studies were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee (#180278) and performed in accordance with the guidelines.

Calibration of the DanioVision® Instrument

The calibration process required input information pertinent to our experiment, such as live tracking on subjects (in this case zebrafish larvae) and the arena template: 48 round-well plate at 30 frames per second. Once the reference plate (empty plate) was placed into the instrument, we set the dynamics subtraction and the acquisition settings (e.g., distance traveled). After the instrument was properly calibrated with the empty plate, we proceeded to place the plate containing zebrafish and set up the light conditions using the EthoVision® software. This calibration process was critical for obtaining optimal tracking results, and for maximizing the number of data points tracked by the instrument.

Experimental Procedures

Light condition procedures

To assess the effect of light on zebrafish movement, twenty one zebrafish (6 dpf) were transferred into individual wells in a 48-well plate. In general, zebrafish show good spontaneous motion between six days and four weeks post-fertilization. Thus, better tracking is obtained as the zebrafish gets older. As depicted in Figure 1, we conducted a study where we first placed zebrafish in wells C2 to C6 and D2 to D6. Other zebrafish were placed in wells B2 to B7 and E2 to E6, while the A wells were left empty. To set up this test, we placed the 48-well plate with the zebrafish into the calibrated instrument with a 30 min delay to acclimate the fish to the current condition. The instrument started tracking for the next 10 min, which is a typical timeframe for a suborbital flight on Blue Origin's New Shepard. Video was taken over a 10 min period starting with 5 min of lighted conditions followed by 5 min of dark condition. A switch condition was applied in between lighted and dark conditions. Locomotor activity was measured for 4 min of the lighted portion and for 5 min of the dark condition as displayed in Figures 3-6.

Thermal procedures

Ten zebrafish larvae (8 dpf) were placed in 15 mL conical tubes containing 14 mL of water and were exposed to the following temperatures: 25°C, 28.5°C (reference temperature), and 32°C for 2 days. Thermal stress was performed at 3.5°C above and below the reference temperature. Afterwards, larvae were transferred into the 48-well plate using the following template: rows A and B had no zebrafish, row C contained larvae

exposed to 32°C, while rows D and E contained zebrafish exposed to 25°C and 28.5°C, respectively.

Centrifugation procedures

For the centrifugation test, zebrafish larvae (n=10) were placed in 15 mL conical tubes containing 14 mL of water and were centrifuged for 5 min at 10 *g*. Centrifugation was performed at 28.5°C, since this is the optimum temperature for zebrafish. Zebrafish exposed to 25°C underwent centrifugation stress, while zebrafish exposed to 32°C were not exposed to centrifugation stress, as zebrafish exposed to temperatures above the reference temperature (control) are more sensitive than those exposed to temperatures below. Previous studies (Scott and Johnston, 2012) support the fact that high cruising speed zebrafish are observed at warmer temperatures. Therefore, we decided to not put additional stress on the zebrafish. However, this will be addressed in future studies. After centrifugation, zebrafish larvae were transferred into a 48-well plate using the following template: wells A1 to B1 had no zebrafish, B2 to C5 contained zebrafish that did not undergo centrifugation, while wells C6 to D3 contained zebrafish that were exposed to centrifugation.

Tracking

After exposing zebrafish to the above conditions, the locomotor activity of the zebrafish was recorded for 11 min (660 s), the same as the total time of a suborbital mission. The motion was sampled at 24.55 Hz generating 16204 positions (X, Y) and velocity data samples per individual. The tracking software provided information at the end of the simulation of missed samples and samples not found (expressed as a percentage). If the fish were not found, then the path of the zebrafish would appear in yellow instead of blue (zebrafish was tracked). In our thermal test, the instrument tracker only missed 0.1% of all the samples obtained in all the wells where the zebrafish were located and 0.1%-0.2% of all the samples. Thus, the overall tracking performance of the instrument was excellent, as the zebrafish tracking efficiency was about 99.8%.

Data Extraction and Analysis

The locomotor activity data for the zebrafish was obtained using the EthoVision XT® software. Each observation was stored in an Excel data file containing the recording time in seconds, and the X center and Y center coordinates of the zebrafish larvae. The motion of the zebrafish was captured by an IR sensor that would take 30 frames of the zebrafish every second in a 48 round-well plate. The sensor detected the 2-dimensional position (X, Y) and velocity of the zebrafish during each frame but did not provide the Z position of the zebrafish. We approximated the Z position as follows: we added a 500 µL volumetric solution pipetted into each of the 13 mm diameter wells. Thus, the height of the cylinder where the zebrafish were swimming was about 1-1.28 cm. We assumed the zebrafish swam within this volume.

We have approximated the turning speed, ω, also known as the angular velocity, of each fish at each step as follows (Zienkiewicz et al., 2015):

$$\omega = \mathrm{sgn}\left[\bar{v}(t+\Delta t)\times\bar{v}(t)\right]_z \frac{1}{\Delta t}\cos^{-1}\left(\frac{\bar{v}(t+\Delta t)\cdot\bar{v}(t)}{\left\|\bar{v}(t+\Delta t)\right\|\left\|\bar{v}(t)\right\|}\right)$$

where $\left[\,\cdot\,\right]_z$ indicates that the sign of the z-component of the cross product provides you the turning direction and a positive sign denotes movement in the anti-clockwise direction. The change in time, Δt, is defined as the inverse of the sampling frequency (fs=30 Hz). This turning speed of the zebrafish is associated with the rate of the change of the orientation of the velocity vector. Our model also computes the radial and tangential velocity of the zebrafish at each time step. In our results section, we will show how the turning speed or angular velocity parameter can be expressed in contour maps having the tangential velocity in the Y-axis and the radial velocity in the X-axis in order to have a better understanding of the swimming behavior of the zebrafish.

RESULTS

Light Conditions Study

In this study phase, we exposed zebrafish to different light conditions for various time durations. First, the zebrafish were exposed for 5 min in the lighted condition (100%), then the light was turned off for 6 min (Figure 1). Data was analyzed during the first 5 min under lighted conditions, 1 min after the light was turned off, and in the last 5 min of dark conditions. Total amount of this exposure (10 min) mimics the duration of the suborbital spaceflight.

During the first 5 min under lighted conditions, the average distance that zebrafish larvae moved from the center-point for all wells was 91.26 mm (9.13 cm). The maximum distance traveled measured was 173.76 mm (17.4 cm) observed in C6 (well number 22 in Figure 1), while the minimum distance was 9.21 mm (approximately 1 cm), observed in C2 (well number 18 in Figure 1). We used the following notation (Figure 1): well numbers 2-8 will be indicated by A; well numbers 9-16 by B; well numbers 17-24 by C; well numbers 25-32 by D; well numbers 33-40 by E; and well numbers 41-48 by F.

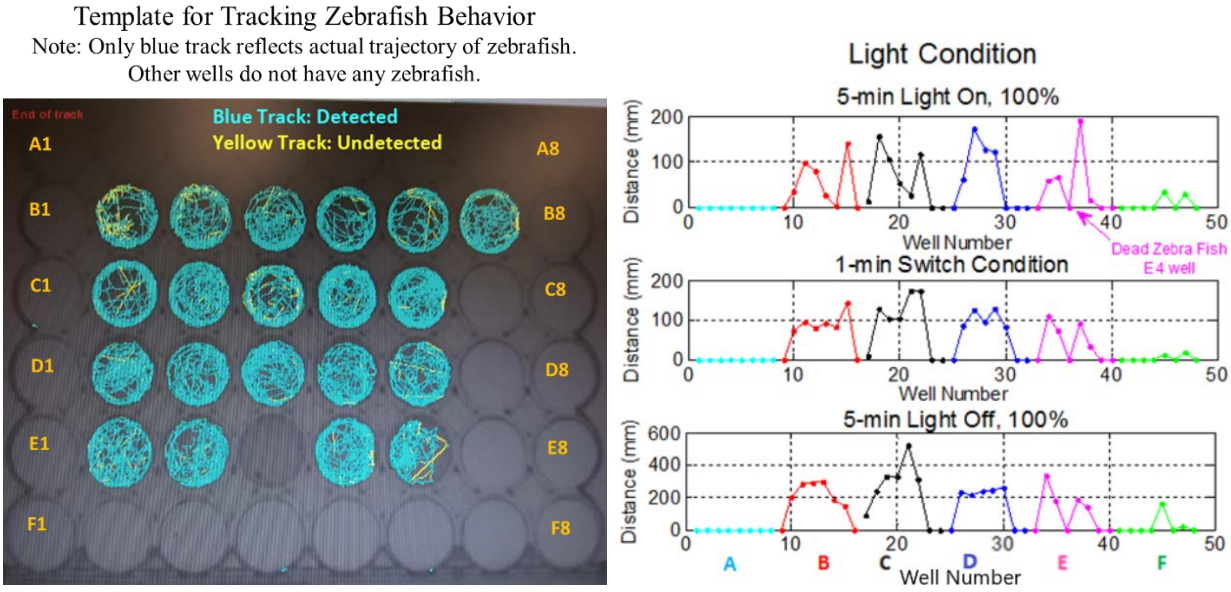

Figure 1. Distance traveled by zebrafish larvae during various time phases: 5 min light on (top), 1 min switch condition off where the light intensity is reduced from 100% light on to 100% light off (middle), 5 min light off (bottom). Each dot represents individual well of the 48-well plate. Colors correspond to the letters of the well: A comprises well numbers 1-8 with no zebrafish; B represents well numbers 9-16 with zebrafish in wells B2-B7; C indicates well numbers 17-24 with zebrafish in wells C2-C6; D corresponds to well numbers 25-32 with zebrafish in wells D2-D6; E comprises well numbers 33-40 with zebrafish in wells E2-E6; and F represents well numbers 41-48 with no zebrafish. Blue dots in wells C1, F4, and F6 are residues and do not reflect any motion.

The average distances moved in the B2-B7 (red), C2-C6 (black), D2-D6 (blue), E2, E3, E5-E6 (magenta), and F4, F6 (green) wells were 9.37 cm, 11.53 cm, 10.23 cm, 7.61 cm, and 1.43 cm, respectively. Second, we turned off the light to measure the startle response to the change in light condition in the next 1 min. The distances moved in the same wells by the zebrafish larvae were 6.23 cm, 7.80 cm, 12.03 cm, 8.16 cm, and 3.06 cm, respectively. The average traveled distance was 74.56 cm with a maximum distance traveled of 18.98 cm observed in E5, and a minimum distance traveled of 0.08 cm in B6. Third, we kept the light off (100%) during the last 5 min and

observed high activity of the zebrafish larvae as seen at the bottom of Figure 1. The average distance moved by the zebrafish in the wells was 23.24 cm, 30.04 cm, 23.64 cm, 20.82 cm, and 8.77 cm, respectively. The maximum distance traveled was 51.79 cm observed in well C5, and the minimum distance traveled was 1.61 cm in well F6.

In Figure 2, we compared the distance traveled (in mm) during the first 5 min period when the light was on (dashed lines) and during the last 5 min period when the light was off (solid lines). Our results indicate that zebrafish were more dynamic when the light was off. The maximum distance moved by zebrafish was about three times higher for dark conditions as compared to the light conditions.

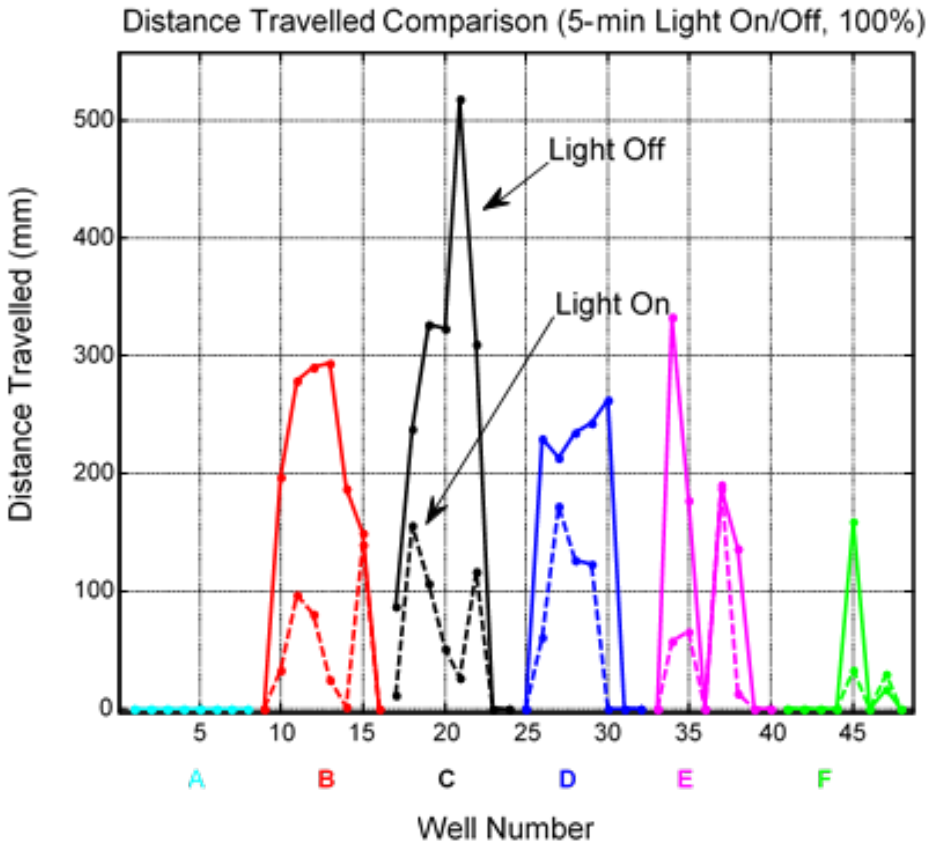

Figure 2. Comparison of total distance traveled of zebrafish larvae during various light phases. 5 min light on is represented by the dashed line and 5 min light off is represented by the solid line. Color representations are as in Figure 1.

To further demonstrate the effect of light on zebrafish movement, we analyzed zebrafish behavior derived from the individualized wells of the 48-well plate (Figures 3-6). Our data demonstrate that in B wells zebrafish are the most active when exposed to the dark environment. This finding was consistent throughout the rest of the wells with a few exceptions observed in wells E5 and C2 where the difference in distance traveled was not significant between light and dark conditions.

Statistical evaluation in Figures 3-6 for the average motion was analyzed for each of the three conditions: 4 min with light on, 1 min switch condition, and 5 min light off at each of the wells B, E, C, and D (Figure 1). This was the order chosen so that control zebrafish were in B wells, zebrafish at 28.5ºC were in E wells, zebrafish at 32ºC were in C wells, and zebrafish at 25ºC were in E wells.

Wells B2-B7 showed an average motion during each of these conditions of 31.15%,

26.73%, and 78.23%, respectively. Wells E2-E7 (zebrafish in E4 well was dead) corresponded to an average motion of 50.73%, 40.82%, and 75.18%, respectively. Wells C2-C6 displayed an average motion of 48.78%, 51.68%, and 88.20%, respectively. Well D2-D6 had zebrafish with an average motion of 51.94%, 40.00%, and 81.14%, respectively. Taking the mean of the means for each condition, we observed that the average motions across all the wells for each of the three conditions were 45.90%, 39.81%, and 80.69%, respectively. This statistical analysis showed that zebrafish were about 1.8 times more active during the 5 min light off condition than during the 4 min light on condition, and 2.0 times more active than during the switch condition.

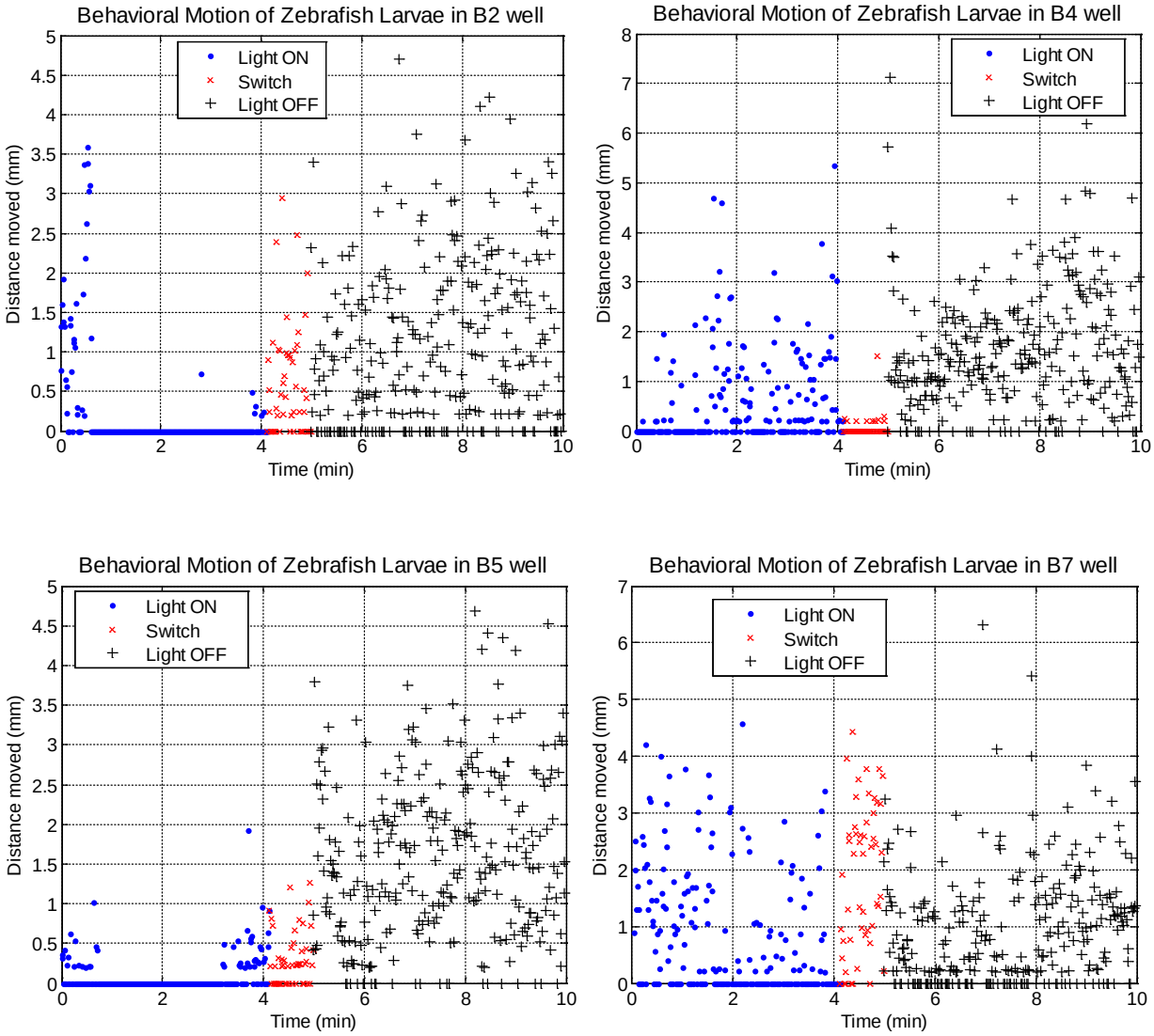

Figure 3. Behavioral motion of zebrafish larvae in B wells during different light conditions. The blue dots represent the distance traveled by larvae during the 5 min lighted condition. The red crosses denote the distance traveled by larvae during the switch condition. The black crosses correspond to the distance traveled by larvae under the 5 min dark condition.

We also studied the average motion of zebrafish between each condition for each well. For B wells (control), the zebrafish average motion increased about 7.6% from the condition at 4 min with light on to the 1 min switch condition, and the average motion increased 38.5% from the switch condition to the condition at 5 min with light off. For E wells, the average motion was decreased about 9.9% and increased 26.2% between conditions, respectively. For C wells, the average motions between conditions increased by 2.9% and by 36.5%, respectively. Finally, for D wells the average motions between conditions were 4% decrease and 39.1% increase, respectively. These results show an increase of 35.1% in the average motion going from the 1 min switch condition to the 5 min with light off condition.

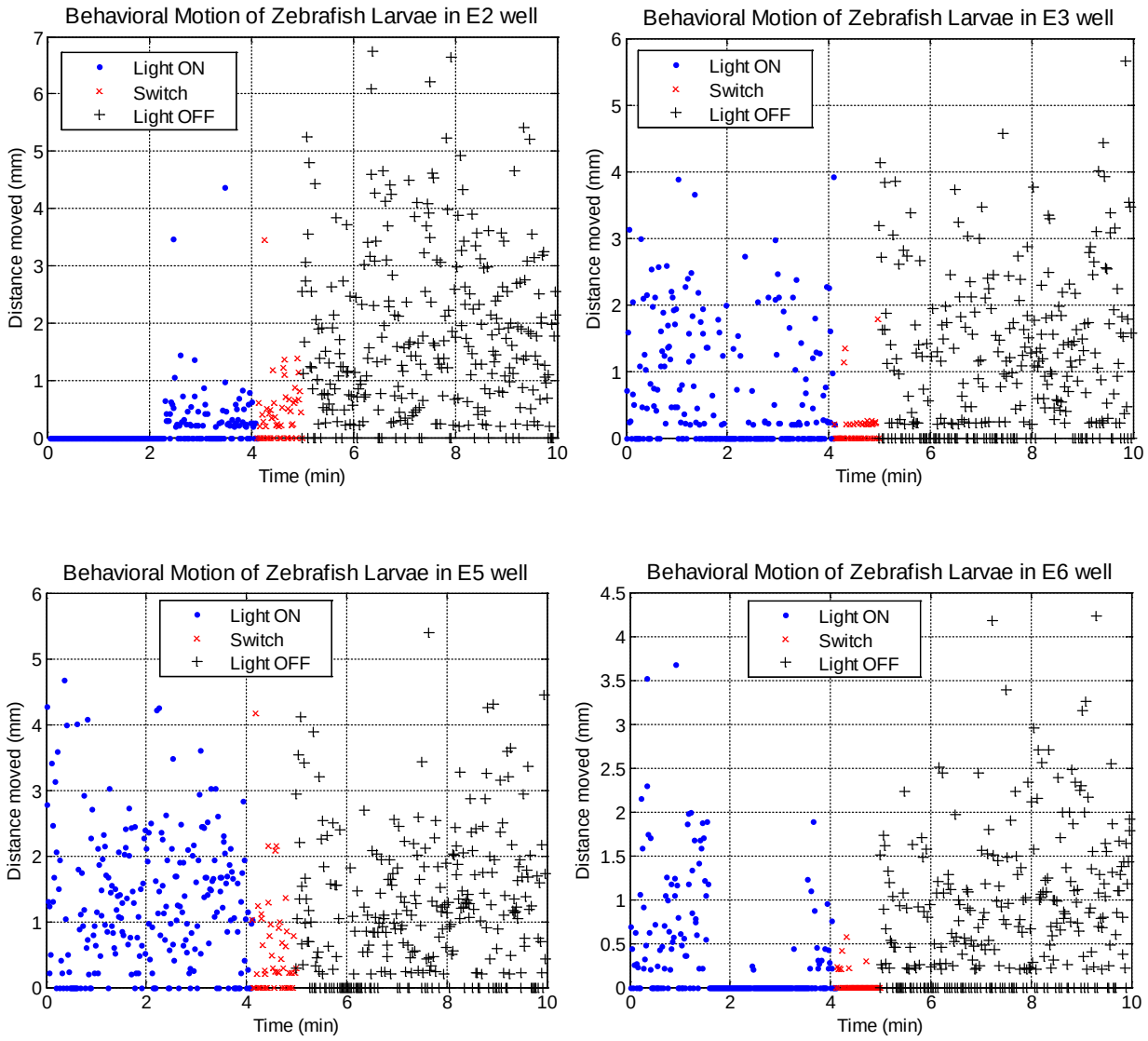

Figure 4. Behavioral motion of zebrafish larvae in E wells during different light conditions. The blue dots represent the distance traveled by larvae during the 5 min lighted condition. The red crosses denote the distance traveled by larvae during the switch condition. The black crosses correspond to the distance traveled by larvae under the 5 min dark condition.

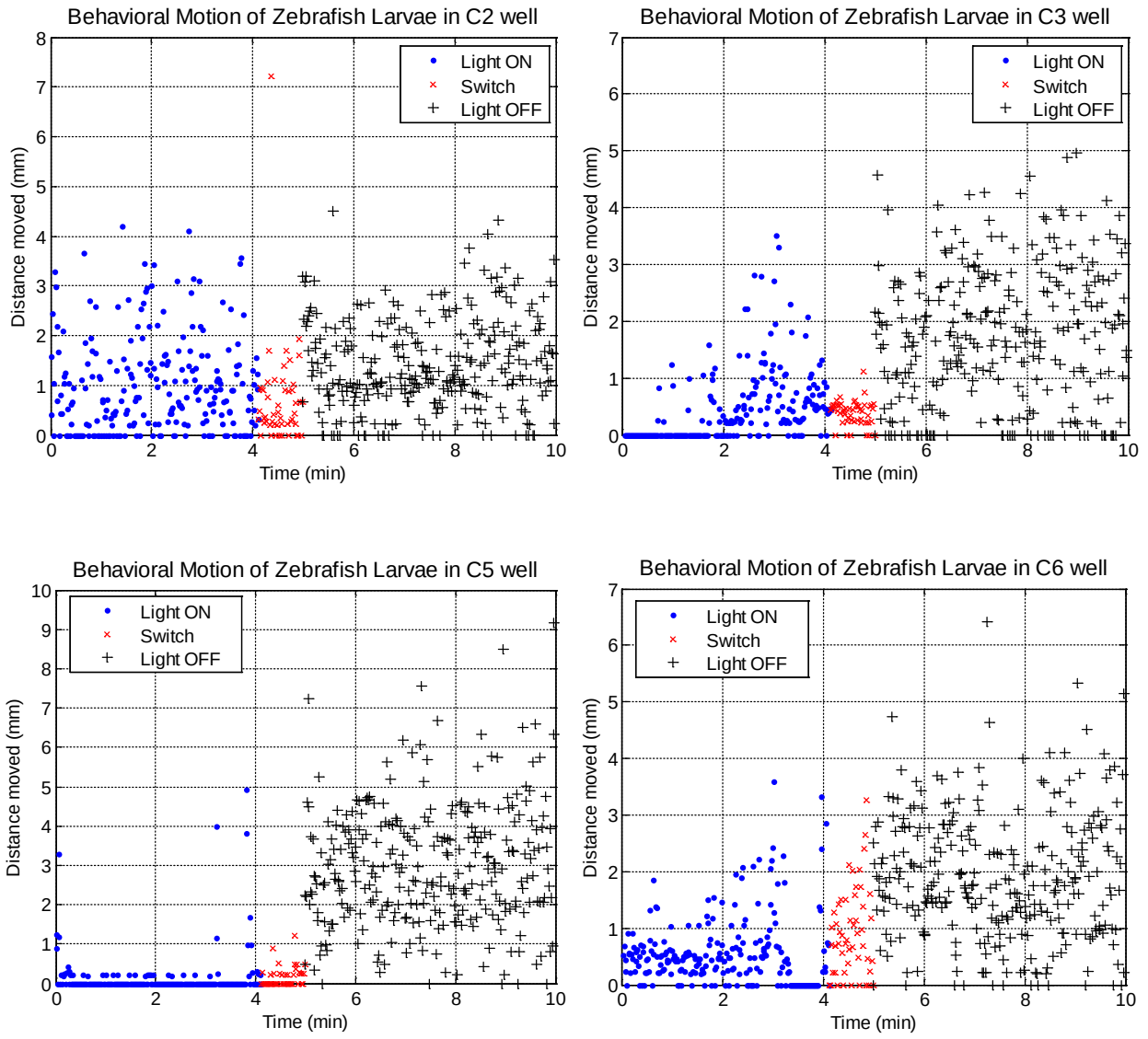

Figure 5. Behavioral motion of zebrafish larvae in C wells during different light conditions. The blue dots represent the distance traveled by larvae during the 5 min lighted condition. The red crosses denote the distance traveled by larvae during the switch condition. The black crosses correspond to the distance traveled by larvae under the 5 min dark condition.

Thermal Conditions Study

Next, we conducted a feasibility study of the zebrafish larvae under various temperatures: 28.5°C (reference temperature), 32°C, and 25°C. This zebrafish testing profile was based on a more realistic light conditions scenario based on Blue Origin's New Shepard Crew Capsule flight profile, which lasts about 10 min. Thus, we initially exposed the zebrafish larvae during 3 min of light conditions (mimicking ascent), followed by 1 min transition from dark to light, followed by 3 min in dark light conditions (mimicking darkness in microgravity), followed by 1 min transition from dark to light, followed by 3 min in light conditions (mimicking descent).

Figure 7 displays the trajectory of these zebrafish larvae when exposed during the above

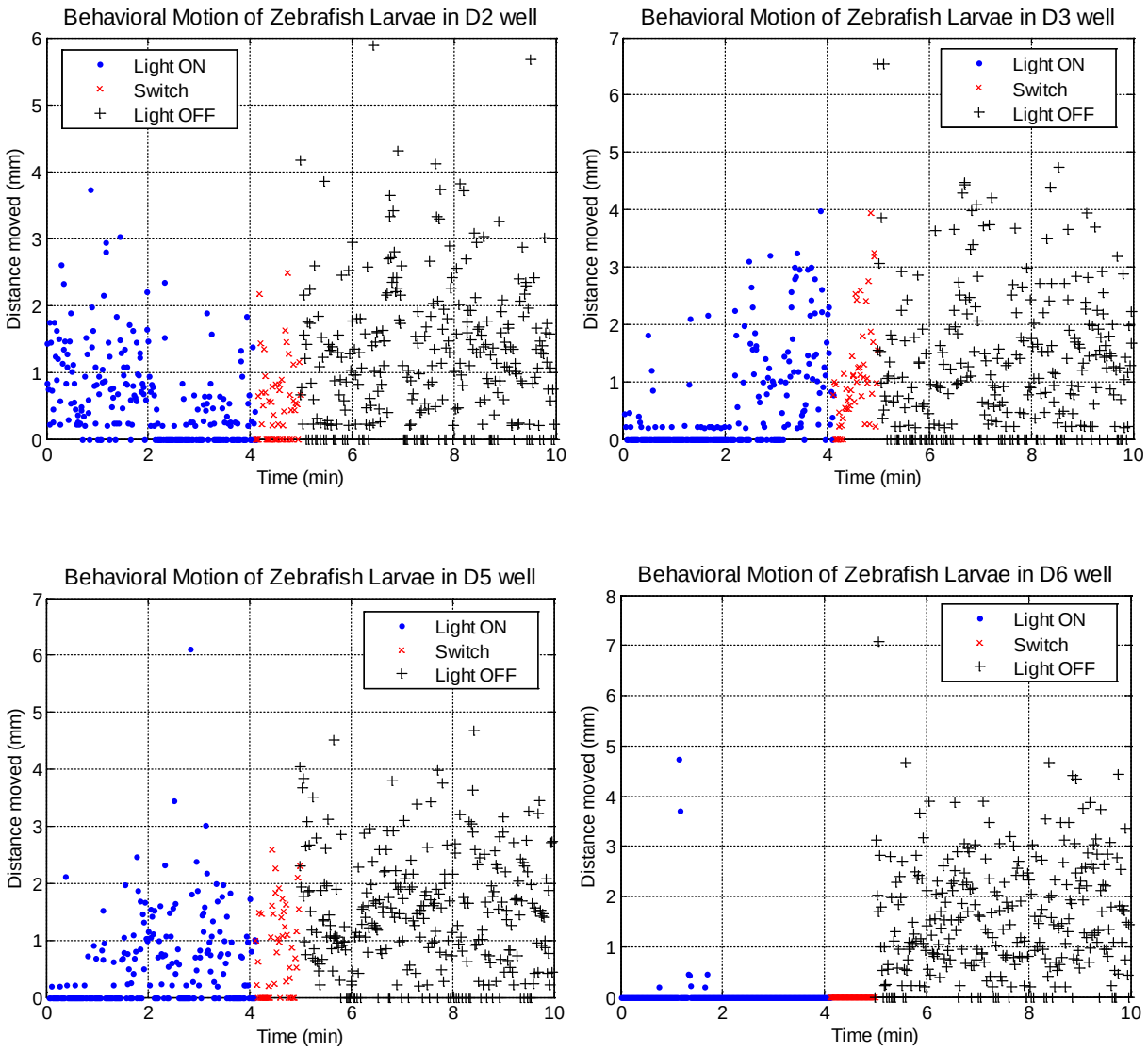

Figure 6. Behavioral motion of zebrafish larvae in D wells during different light conditions. The blue dots represent the distance traveled by larvae during the 5 min lighted condition. The red crosses denote the distance traveled by larvae during the switch condition. The black crosses correspond to the distance traveled by larvae under the 5 min dark condition.

sequence of light conditions at various temperatures. It was observed that the larvae followed more defined circular paths when exposed to 32°C as seen in the two top plots of Figure 7. The larvae followed a similar circular path, although not as well-defined, when exposed to the reference temperature of 28.5°C with various passages through the center of the well as shown in the center two pictures of Figure 7. These zebrafish larvae showed more irregular paths when exposed to 25°C as observed in the bottom two plots of Figure 7. Because zebrafish larvae locomotor activity is affected by temperature variations, an active thermal system may be required to have a sustainable life support system to increase the life of these zebrafish larvae to ensure survivability, especially if the launch is slipped or scrubbed as occurred with the

Blue Origin launch on December 12, 2017. Our experience during the last Blue Origin New Shepard M7 revealed drastic temperature variations during the pre-flight, in-flight, and post-flight operations. The payload, which is integrated at the Payload Processing Facility (PPF) at the West Texas Launch Site (WTLS), must be handed off to NanoRacks and Blue Origin teams before integration into the New Shepard's Crew Capsule at least 7 hours before the launch. Because of this, standard care of these larvae will require an appropriate life support system, proper handling, care, and pain mitigation protocols in order to be airworthy for the suborbital launch.

Interestingly, the zebrafish larvae activity displayed in C4 and C8 wells that were exposed to 32°C had the highest distance traveled and the highest mean velocity (Figure 7). Specifically, zebrafish larvae in C8 well moved a total distance of 78.46 cm over the time period of about 10 min with a mean velocity from the center point of 1.46 mm/s, while larvae in C4 well moved a total distance of 68.04 cm with a mean velocity of 1.26 mm/s. Larvae activity depicted in E1 and E5 wells (exposed to 28.5ºC) moved a total distance of 44.81 cm and 35.75 cm with mean velocities of 0.83 mm/s and 0.66 mm/s, respectively. Finally, larvae activity shown in wells D3 and D6 (exposed to 25°C) traveled total distances of 27.42 cm and 34.29 cm with mean velocities of 0.51 mm/s and 0.64 mm/s, respectively. Next, we also compared the locomotor activity of total distance for each configuration when we combined two stressors: temperature and centrifugation. Wells C1-C8 (only showing C4 and C8 wells) correspond to larvae exposed at 32°C with no centrifugation; wells D1-D8 correspond to larvae exposed at 25ºC (centrifugation); wells E1-E7 correspond to larvae exposed at 28.5ºC (centrifugation); and wells B1-B8 correspond to control larvae (no centrifugation). Control larvae moved an average total distance of 48.19 cm, larvae exposed at 32ºC move an average total distance of 36.43 cm, larvae exposed to the reference temperature moved an average of 63.80 cm, and larvae exposed to 25ºC moved an average distance of 37.82 cm.

Figure 8 depicts the distance traveled by zebrafish in their respective wells when exposed to various subsequent stressors. First, the *Danio rerio* were exposed to various temperature changes (25ºC is shown in red, 28.5ºC is shown in black, 32ºC is shown in green, and control is represented by blue). After completion of this test, they were placed in the 48 well plate and exposed under different light conditions similar to what they would experience during about 10 min suborbital flight: 3 min in light condition representing the part of the ascent, 1 min going from light to dark condition, 3 min in dark representing suborbital altitudes, 1 min transitioning from dark to light, and 3 min in light condition again representing descent. Figure 8A shows the effect of centrifugation on 20 larvae. 80% of these showed distance displacements less than 6 cm and 20% of these showed distances traveled between 8 cm and nearly 13 cm. This indicates high fatigue levels on the zebrafish larvae. In the same graph we also observe that larvae traveled longer average distances when exposed to temperature of 32ºC than in any other case. This observation is also noticed in the other plots in Figure 8. Larvae exposed to various thermal changes traveled longer distances than when exposed to centrifugation which confirms our observation of zebrafish being less active when they undergo spinning effects.

In general, Figure 8B to Figure 8E show that the larvae traveled longer distances at warmer temperatures (32ºC) than at colder temperatures (25ºC) with respect to the reference temperature (28.5ºC), as indicated by the green and red lines, respectively. Another important observation in Figure 8 is that zebrafish larvae exposed to dark conditions traveled much longer distances than when they were exposed to light conditions. For example Figure 8C shows that 88% of all the larvae traveled distance larger than 10 cm and up to 50 cm is some cases while only 12% traveled less than 10 cm. If we compare this with Figure 8A, we observe that about 66% of larvae traveled less than 10 cm, and 44% traveled between 10 cm and 50 cm. Note that these larvae were exposed to 3 min light conditions at the start and end of the light cycle, as indicated in Figure 8A and Figure 8E, respectively. About 90% of these larvae traveled shorter distances during the last 3 min light cycle than during the first 3 min light cycle.

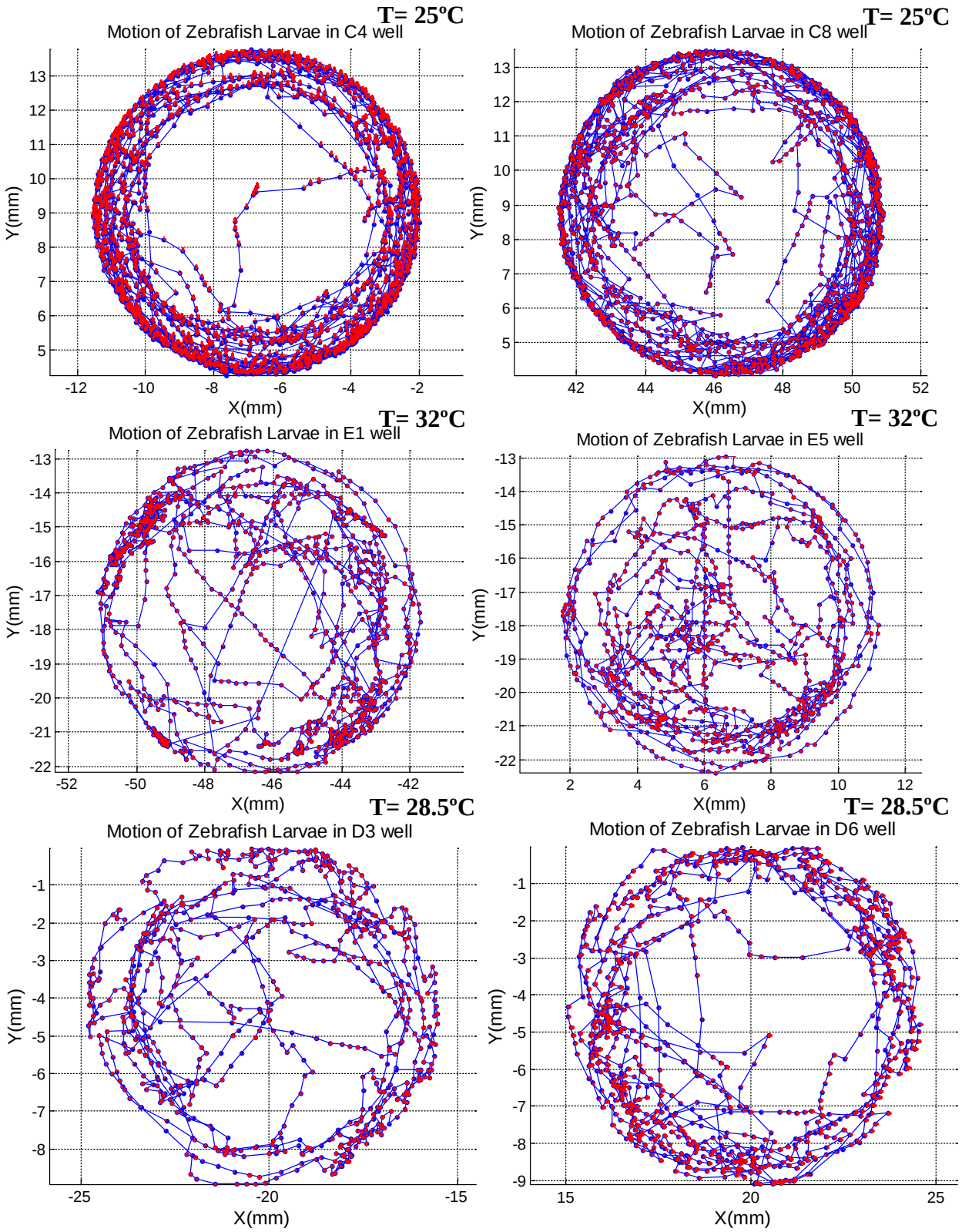

Figure 7. Behavioral motion of zebrafish larvae exposed to different temperature variations: 32°C (top), 28.5°C (middle), 25°C (bottom). Each red dot represents the position for each discrete data point tracked by Ethovision®. The blue line indicates the approximated path of the zebrafish between data points.

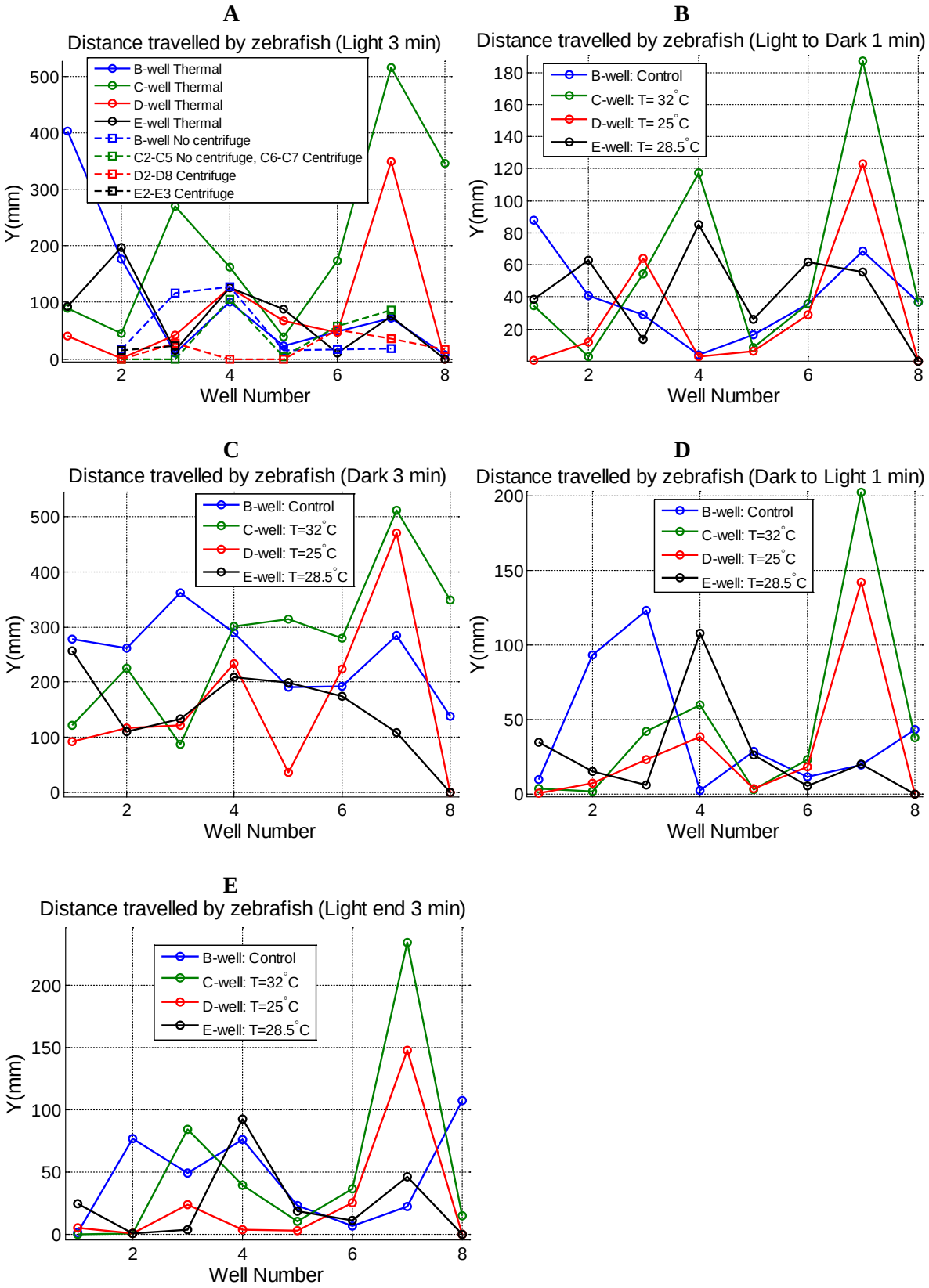

Figure 8. Distance traveled by zebrafish larvae when exposed to an 11 min varying light cycle. (A) 3 min in light. (B) 1 min light to dark. (C) 3 min in dark. (D) 1 min dark to light. (E) 3 min in light.

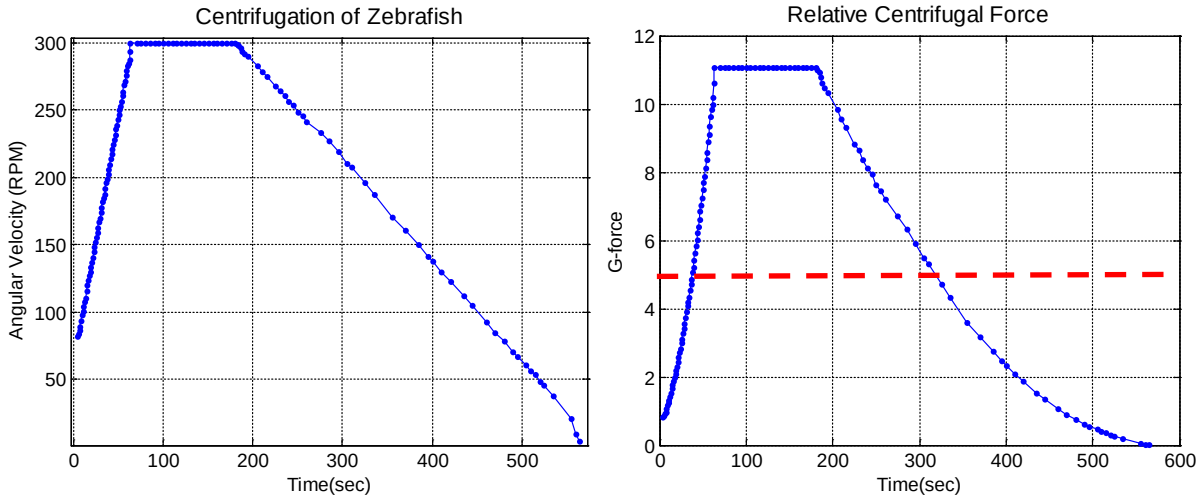

Figure 9. Graphical representation of the zebrafish exposure to centrifugation profile. Left: Angular velocity as function of time. Right: G-force as function of time.

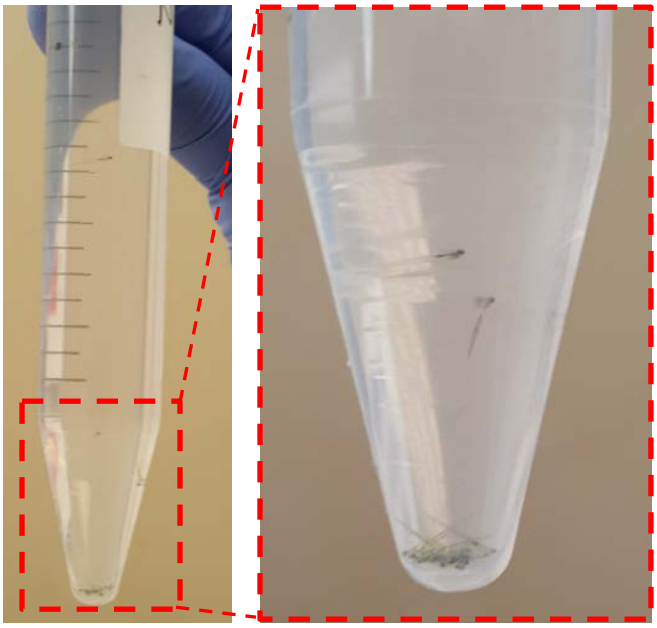

Figure 10. Representation of zebrafish after centrifugation. Left: Majority of zebrafish larvae localized in the bottom of the conical tube, whereas very few were swimming in the mid-top sections of the tube. Right: Magnification of the picture on the left showing some zebrafish swimming with their tails down or upside down.

Centrifugation Study

In our final test, we performed the centrifugation phase as a final test in our study. Figure 9 depicts the centrifugation profile: maximum G-sensed acceleration in New Shepard is 4.7 *g* (red dashed line) for a few seconds during reentry (Blue Origin, 2017). Our study showed that zebrafish underwent angular velocities up to 300 rpm for 2 min, with a safety margin of about 2.3 or about 11 *g*. It is important to allow for a safety margin as an effective test to diagnose any forms of dysfunctional zebrafish activity that could be exacerbated by unexpected flight forces or flight anomalies.

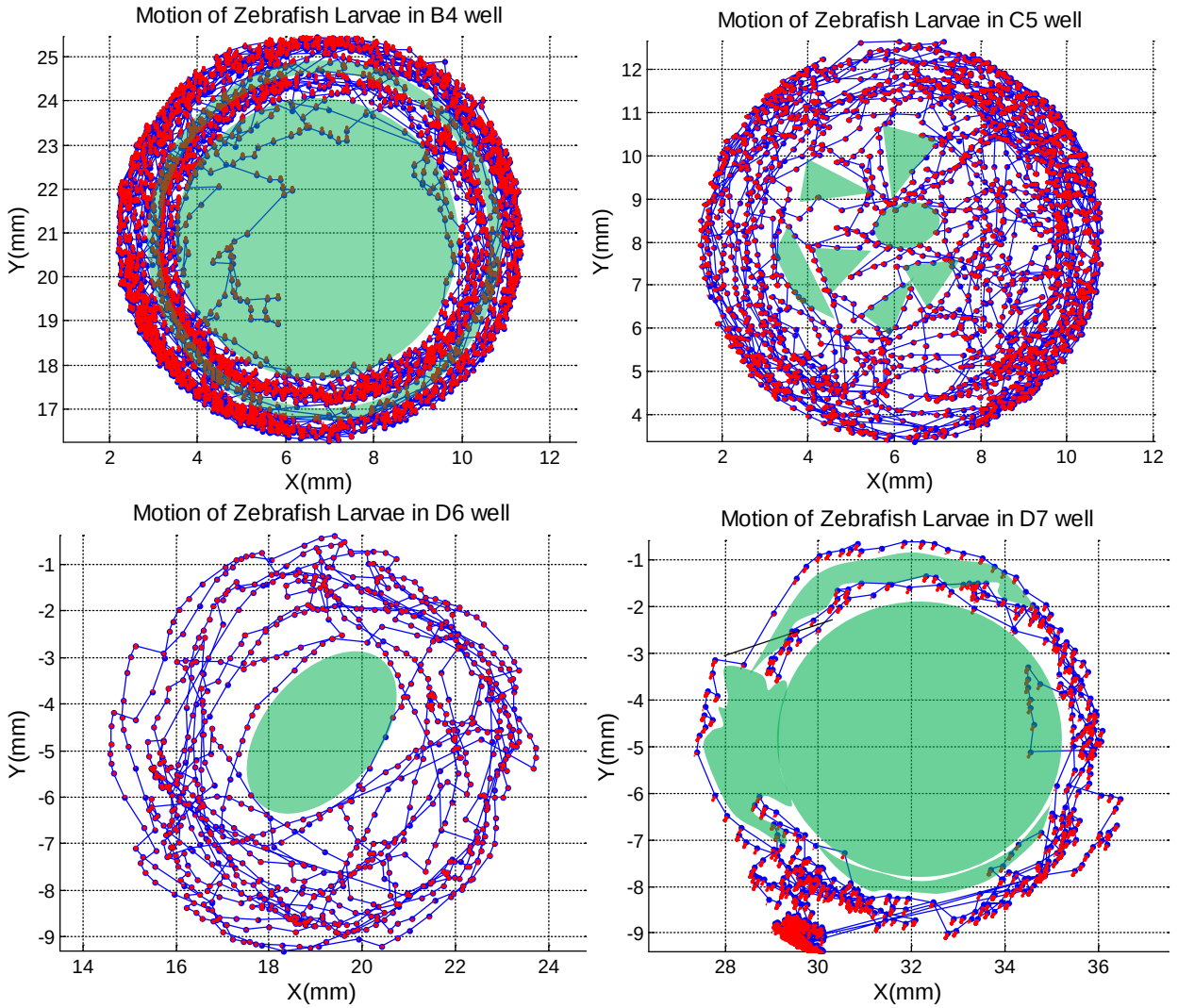

Figure 11. Motion of zebrafish after exposure to centrifugation: no centrifugation (above), centrifugation (below). Green regions depict areas where the larvae did not swim (forbidden regions). X and Y represent the coordinates of each well on the 48-well plate.

This research will be further expanded later with various organisms so we could fly them on other national and international research platforms besides the Blue Origin's New Shepard vehicle. In our future work, we will examine the effects of various hypergravity levels on these organisms (i.e., zebrafish) using various known simulation techniques, such as clinostats and rotating wall vessels, to better assess the performance of these organisms in space. As an example, previous research (Remus and Wiens, 2008) has examined the effects of hypergravity level on xenopus embryos that underwent centrifugation levels from 7 *g* to 10 *g*.

Post-swimming recovery of zebrafish took about 3-5 min. Their physiology was notably impacted with most zebrafish being upside down in the conical tube as illustrated in Figure 10 (right). Very few zebrafish (3-4 zebrafish out of 10) were observed to have aberrant swimming activity after centrifugation stress since most of them were at the bottom of the tube as shown in Figure 10 (left). In addition, zebrafish showed very slow responses when they were tapped at the tip of the tube indicating they had a reduced startle response, while the more active zebrafish showed a higher response to these stimuli. Some zebrafish swam at an angle (tilting swimming),

exhibiting disorientation signs as shown by their droopy tail (right Figure 10). This lasted for about 4 min. All zebrafish were active again after 5 min. It is important to note that these effects were induced by the centrifugation process and not by using any of the neuroactive or neurotoxic substances that commonly provoke this type of behavior (Weichert et al., 2017; Lee and Freeman, 2014).

Furthermore, as illustrated in Figure 11, the exposure of zebrafish larvae to centrifugation revealed that this stressor led to the formation of more chaotic patterns of zebrafish movement as compared to the well-defined trajectories of fish not exposed to centrifugation as observed during 30 min data collection process with the DanioVision® (Noldus) behavioral tracking system.

Radial and Tangential Angular Velocity Maps

Figure 12 depicts various contour map representations of the radial and tangential velocities in mm/s for representative wells for a given condition. Figure 12A and Figure 12B show the radial and tangential velocities for wells C4 and C8, respectively. The maximum radial velocities were 80.58 mm/s and 87.28 mm/s, and the maximum tangential velocities recorded were 77.99 mm/s and 118.4 mm/s, respectively for these wells. Similarly, for wells E1 and E5 (Figure 12C and Figure 12D) the radial velocities were 105.6 mm/s and 43.2 mm/s, while the tangential velocities were 156.3 mm/s and 49.85 mm/s. The radial velocities for D3 and D6 wells (Figure 12E and Figure 12F) were 56.29 mm/s and 52.24 mm/s, and the tangential velocities were 35.38 mm/s and 44.91 mm/s, respectively. The means of the radial velocities for each well (C4, C8, E1, E5, D3, and D6) were 0.86 mm/s, 1.81 mm/s, 0.52 mm/s, 0.46 mm/s, 0.34 mm/s, and 0.44 mm/s, respectively. The means of the tangential velocities for these wells were 3.63 mm/s, 30.54 mm/s, 3.43 mm/s, 3.30 mm/s, 3.27 mm/s, and 3.30 mm/s, respectively. Note that both radial and tangential velocities are notably smaller for the larvae exposed to 25ºC if compared with those velocities for larvae exposed at reference or higher than reference temperatures. The sum of all the radial and tangential velocities for each condition was analyzed. For instance, the sum of the radial velocities for larvae exposed to 32ºC is greater than that for larvae at reference temperature and for larvae at 25ºC. A smaller difference is observed between 32ºC and 28.5ºC conditions, which may explain better adaptability of the larvae at slightly larger temperatures than at lower temperatures. This behavior is consistent for other wells.

DISCUSSION

There is a significant interest in using ISS for both conducting research and habitation for the next decades. However, such missions are currently burdened by the severe consequences space has on human health. Therefore, there is a great need for effective multidisciplinary studies comprised of both basic and applied science aimed at producing effective countermeasures against the deleterious influences of spaceflight on the human body (Alwood et al., 2017).

In this study, we have shown that exposing zebrafish larvae to the same physiological stressors they would encounter during the actual suborbital flight leads to alteration of their behavior, but does not affect their survival. Furthermore, this study provided an insightful contribution to various stressor-based effects on the zebrafish larvae to establish a risk assessment of the model organism *Danio rerio* when designing future suborbital and orbital spaceflights. Our study is currently being extended by using clinostats as a ground lab research platform that will give us further understanding on the behavior of these organisms under different microgravity levels.

Although zebrafish is a diurnal animal that is active during the light phase of the light-dark circadian cycle (Facciol et al., 2017), our light study indicated that zebrafish were more active in the dark. Some literature suggests (Serra et al., 1999; Maximino et al., 2010) that zebrafish have a natural preference for a dark environment. However, there is a contradiction to this observation in the field as some studies report zebrafish preference for brighter light environments (Champagne et al., 2010; Gerlai et al., 2000). Despite the observed differences among investigators, it was important to us to confirm that zebrafish are capable of adapting to alterations in light conditions and also to determine light preferences so that proper future housing cubes can be made.

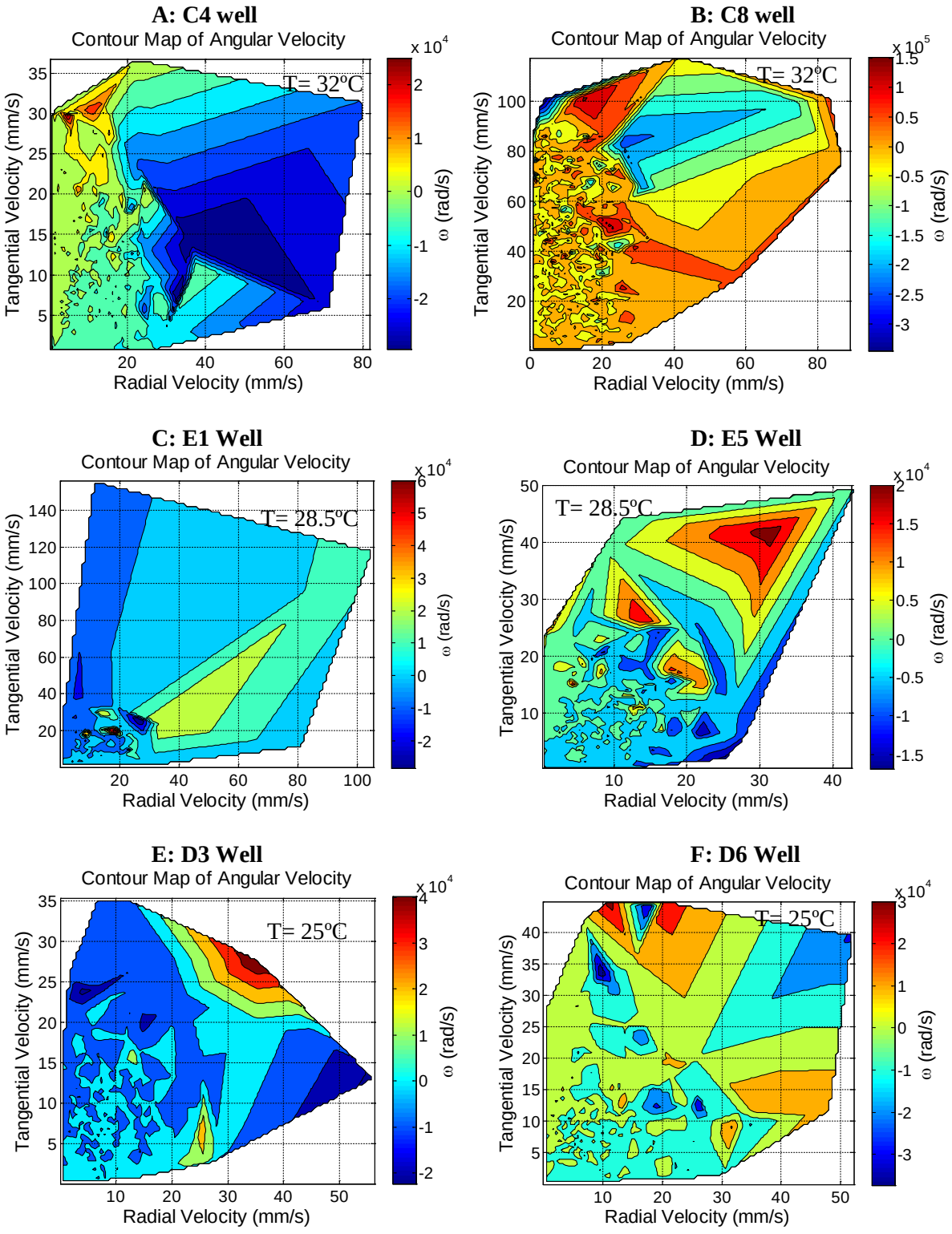

Figure 12. Contour maps for radial and tangential velocities at various temperatures in different wells: (A) Well C4 at 32ºC. (B) Well C8 at 32ºC. (C) Well E1 at 28.5 ºC. (D) Well E5 at 28.5ºC. (E) Well D3 at 25ºC. (F) Well D6 at 25ºC

It is well established that water temperature affects the swimming performance of the fish. Our thermal study revealed the differences in zebrafish movement patterns when they were exposed to various temperatures at various time durations. More specifically, zebrafish exposed to 32°C formed well-defined trajectories as well as were the most active as compared to those exposed to 28.5°C and 25°C. Our finding is consistent with the literature suggesting that zebrafish exhibit the highest swimming capacity when they are raised at 31°C, as compared to the worst performance at 22°C, and moderate activity at 28°C (Sfakianakis et al., 2011).

Next, we assessed zebrafish behavior after administering centrifugation test, the critical phase of the suborbital spaceflight. Our results demonstrate that zebrafish are exhausted initially as observed by their upside down swimming. Our observation of zebrafish avoiding swimming into certain areas could be explained by the gravitaxis (bottom dwelling and diving to the "safer" lower regions) as an indicator of physiological reaction to stress (Blaser et al., 2010; Stewart et al., 2010). Importantly, the recovery processes took only approximately 5 min until larvae were active again. Previous studies (Ijiri, 1995) on medaka fish showed that exposure to microgravity for about 15 days made the fish forget how to swim under normal gravity conditions on Earth, and the fish took 3 days to readapt. During this study, the fish were studied by relying on visual rather than on vestibular cues without being forced to swim aberrantly due to microgravity.

Finally, we computed the radial and tangential velocities of zebrafish exposed to various thermal phases. Our data suggests that lower radial and tangential velocities were observed for zebrafish exposed at 25°C as compared to higher temperatures with a slight difference between 32°C and 28.5°C conditions. Based on our previous observation of zebrafish being more active at higher temperatures, they tend to move in more defined and predictable patterns with higher tangential velocities.

Given zebrafish are very sensitive to various stressors, our team is currently designing a life support system that will be tested and integrated in our next suborbital payload. Some of the hardware will include state-of-the-art sensors to measure the environmental conditions and a microcontroller to regulate our desired conditions. The technology development will be discussed in a subsequent manuscript.

Altogether, our study confirms the likelihood of zebrafish larvae surviving the suborbital flight. Subsequent research efforts will be devoted to further investigate the responses of zebrafish in various microgravity environments (e.g., Moon, Mars, ISS), and from hypergravity to these microgravity levels using various simulation systems (Van Loon, 2016). Our long term goal is to continue research efforts in achieving advancements in human health exposed to space. Given the similarity of the zebrafish genome to humans, our goal is to use zebrafish larvae as a stepping stone for our future suborbital space experiments. Results from our studies and similar studies focusing on space-induced alterations on muscle atrophy could identify the mechanisms mediating these changes, which in turn could lead to the synthesis of new drugs or treatments benefiting not only the space travelers, but also patients on Earth with musculoskeletal disorders.

ACKNOWLEDGEMENTS

This work was partially supported by the Applied Aviation Sciences department at Embry-Riddle Aeronautical University, and by the Department of Drug Discovery and Biomedical Sciences at Medical University of South Carolina. We would like to thank our colleague Jenny Rahn for her valuable research advice, insights, and encouragements on this zebrafish study.

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