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Hypoxia Induced Suspended Animation And Recovery In *Caenorhabditis Elegance*

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Abstract

Purpose: Hypoxia induced suspended animation and recovery in *Caenorhabditis Elegance.*

Suspended animation is a state is characterized by the none moving, none feeding and they regain their ability to move and feed and all other functions on recovery from suspended animation. Suspended animation can be induced in invertebrates by exposure to CO2, O2, N2, and H2S and upon stressed condition to young ones at L1 stage. In the present study the worms were exposed in the custom designed Glove box with the supply of Nitrogen N2 till 98.5% of the volume. Inside the glove box worms were recorded using Lumaascope-620 with laptop attached for imaging. Images were taken at 7fps and utilized for the analysis of speed, peed, peristaltic movement, track length, egg hatching and recovery by using N2 gas as an inducer of suspended animation to create an anoxic environment. The time-lapse images were recorded and combined to create the movie for the analysis in WORMLAB software to examine them for movement, speed, track length and peristaltic pumping. Analyses are conducted on the track length of C elegans before, during, and after the period of recovery from suspended animation.

Method: In this study, the worms were exposed in glove box at 99.8% of nitrogen to induce suspended animation and the movement was recorded with the lumascope-620 attached to the microscope on exposure. The revival was assessed by K-medium, M9 and blue light exposure or their effect on suspended animation on worm movement in terms of speed, peristaltic movement, track length, egg hatching and recovery. Time lapsed images were recorded for further analysis by WORMLAB software. Analyses are conducted on the track length of C. elegans before, during and after the period of recovery from suspended animation.

Results: The track length was recorded at 157.328 m/sec prior to hypoxia exposure and 50.389 m/sec during suspended animation. The worms resumed movement and reached a speed of 1177.621 m/sec after three hours of the N2 gas supply being removed or stopped.

Conclusions: The observed speed data indicates that the measured peristaltic track length reduced by 67.98%, while the worm's peristaltic length track increased by 54% following the recovery. The track length recorded was 1227.476m/sec prior to hypoxia exposure it was 74.090m/sec during suspended animation during recovery period the worms resumed movement and reached and the track length was 1083.136 m/sec. The speed as measured by the worm lab based on analysis was a speed of 610.707 m/sec during suspended animation was 74.079 and during recovery period was 1177.621. Similarly peristaltic track lengths before suspended animation was157.328m/sec to hypoxia exposure it was 50.389m/sec during suspended animation and during recovery period it was 242.416 m/sec After three hours of the N2 gas supply being removed or stopped. The observed speed data indicates that the measured speed has been significantly increased during recovery and peristaltic track length reduced by 67.98% during suspended animation, while the worm's peristaltic length track increased by 54% during recovery. The worms reached the state of suspended animation by at 98.5% and above exposure to nitrogen gas and were able to recover after 72h of suspended animation to normalcy.

Key words: Hypoxia, suspended animation, *Caenorhabditis Elegance*

Introduction

Hypoxia has been known to induce suspended animation in various invertebrates and organisms *viz., C.elegans*, Zebrafish, embryos. In zebrafish embryos complete arrest of heart beat, movement, cell cycle progression and developmental progression has been well established. Similarly in *C.elegans* the worms enter into suspended animation by arresting its movement, cell division and developmental stages. It has been observed that *C.elegans* can survive two or more upon reoxygenation or return to normoxia. (Nystul and Roth 2004). In the hypoxia conditions organisms require minimum amount of oxygen to generate ATP power the cellular reaction and *C. elegans* adopts itself moderate to survive anoxia stress survival mechanisms and their mechanisms are conserved these mechanisms compensate to the loss of aerobic energy production or decrease in demand. The extreme hypoxia condition leads to the anoxia and it defined as <0.001 kPa of oxygen. At this level of oxygen the oxidative phosphorylation decreases and capacity to generate energy is drastically reduced. In this condition the cell needs to decrease energy demand and must reduce the cellular activity. But in case of mild hypoxia partial cell activity is active for cell survival. (Nystul and Roth 2004). In hypoxia conditions animals adapt to the

moderate survival for this hypoxia tolerance the hypoxia inducile factors (HIF-1) transcription factor needs to be up regulated. The *C. elegans* survives complete lack of oxygen for about a day in normal culture conditions by entering in to reversible state of suspended animation (Shen *et al.,* 2003). Adverse environmental conditions, live starvation, overcrowding, anoxia exposure leads to quiescent states (Chan *et al*., 2010, Angstman *et al*.,2016 and Hajeri *et al.,*2005).

C. elegans also modifies its behavior in response to environmental cues by avoiding or attraction of volatile compounds. carbon monoxide (Nystul and Roth 2004), H_2S by Budde and Roth 2011 and Miller) have studied like arrest of movement. The arrest of embryo hatching, feeding. Previously the arrest of egg development (Miller and Roth 2009), and arrest at blastomeric stage (Hajeri *et al.,*2005) have been reported. Hypoxia induces major effects on cell cyclekinetics and protein expression in *Drosophila melanogaster* embryos (Douglas *et al.,*2005). Mitotic arrest in response to hypoxia and a polar bodies during early embryogenesis (Fischer *et al.,*2004) has been reported. The worm lab is a platform where several experiment has been conducted to study the movement of worms (speed) under various treatments like heavy metals exposure (Wang and Xing 2008), antibiotic (sulfmethoxazole) treatment(Yu *et al.,*2011), sodium azide (Massie *et al.,* 2003) and toxins (McCarter *et al.,* 1997).

Track length and speed has been measured under vitamin B12 exposure (Teggene *et al.,*2018). Speed of worms are calculated from position to mid-point (Angstman *et al.,*2016) with presence of food causes subtle changes in *C.elegans.* Nitrogen is an inert gas which is colourless, odourless and non-irritant, but it has received little attention as a potential euthanasia agent compared to other inert gases. The proposed mechanism of N_2-induced loss of consciousness and death is also by hypoxia was proposed by (Biovin G.P *et al*., 2017). (Sharp J *et al,.*in 2006) suggested that N_2, may be less aversive than argon (Ar) since it does not increase heart rate and mean arterial blood pressure. Additionally, N_2 gas is relatively cheap, abundant and non-polluting in the environment and therefore safe to the operators, in contrast to CO_2, which has a significant environmental impact (Dryden R *et al.,*2018). As *C. elegans* has the short life cycle of three days at 20^{o}C and life span of 20 days. Its genome and nervous system is well characterized having 302 neurons. It can be grown in petriplates seeded with *E. coli*. Hence the experiment was designed to study the response of *Caenorhabditis elegans* to anoxic conditions by exposing worms to the nitrogen gas to induce suspended animation.

Materials And Methods

In the present study four nematode strains [*C. elegans* wild type strain (Bristol N2) and transgenic strain, CX2205, N2, AML 01] were used. These strains were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota USA.

MEDIA AND GROWTH CONDITION

All chemicals required for experiment were procured from Himedia and Sigma Aldrich. All developmental stages of *Caenarhabditis elegans* were maintained in petriplates on nematode growth medium (NGM) seeded with *Escherichia coli* (*E.coli*) at 20ºC.

Nematode Growth Medium (NGM)

The NGM was poured on petridishes, allowed to solidify and stored at 4ºCtill use. NGM agar (0.032M KCl, 0.051M NaCl, 2.5% Bactopeptone, 0.17% Bacto-agar, in distilled water) was autoclaved and cooled to 50 - 60ºC. To the warm medium was supplemented with 0.01% cholesterol, 0.1M $CaCl_2$, 0.1M $MgSO_4$.

Glove box: Custom designed environmental chamber for the long-term exposure was built by IMSET BOMBAY with the automated oxygen inflow sensors, carbon dioxide sensors and with pressure measurements inside the chamber (Fig. 1). The glove box custom designed for long term exposure chamber which can hold nitrogen gas up to 99.93% and measured by using various sensors. Sensors were purchased to check the oxygen levels, carbon monoxide levels etc in the chamber and confirmed that the nitrogen gas was filled around 99.93%. Custom fabricated glove box was fitted with the nitrogen, carbon dioxide and oxygen cylinders and the gas was inflow was monitored and recorded digitally. The worm movement was recorded by lumascope -6 20 connected to laptop at 16 fps (Fig. 2).

Worm lab software: Worm Lab is a complete hardware/software for imaging and quantitative analysis of *C. elegans* behavior. It is an easy-to-use worm tracking system with powerful analysis tools. The Worm Lab tracking technology employs a ground breaking algorithm and designed to fully automatically characterize a broad spectrum of behavior of *C. elegans*. like, crawling worms, swimming/thrashing of worms in, whole plate and long-term tracking for measuring speed, direction distance travelled, changes in posture, amplitude of

sinusoidal movement and accurately quantify complex movements such as omega bends, coiling, self-overlap, swimming, and thrashing.

Stimulants Used in the Study

K-Media induced stimulation: K-Medium induced recovery or stimulation as a viscous medium was tested by applying the drop of K-Media to surround the suspended worm and the recovery was recorded. The media was also kept in the glove box chamber till the period of suspended animation was terminated.

Recovery: For the recovery of the worms from suspended animation the Petri plate lid was opened and allowed for the natural aeration and were started recording. Further suspended animation was terminated by the addition of drop of K- Medium on the worms and movement was recorded.

Blue light stimulation: The suspended worms were exposed to blue light by the Lumascope microscope with fluorescence settings and the worm movement was recorded as a function of time.

Observation Recorded:

Track Length of the worm was recorded by measuring the length of forward motion plus length of reverse motion (red) over the total number of frames tracked**.** Speed was recorded by measuring the distance per second covered by the worm along its central axis. The speed is based on the position of the mid-point along the central axis. Peristaltic length was recorded by measuring the length of forward motion minus length of reverse motion is calculated as Peristaltic length. Suspended animation and recovery induction by drop of M9 and blue light exposure.

Statistical Analysis

Hypoxia induced suspended animation of N_2 worms induced suspended animation by exposure to 99.9% of nitrogen gas in the glove box and recorded for three days and the time lapse images were stitched to form the video and were analyzed by WORMLAB software for the various parameters (WormLab® MBF Science).

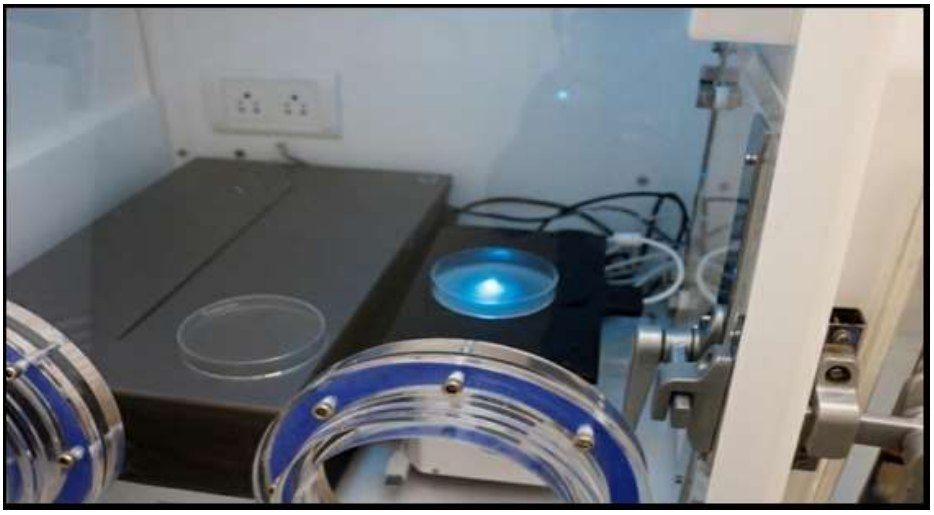


Figure 1: Experimental set up in the glove box and Imaging condition of plate with the worms on the lumascope microscope.

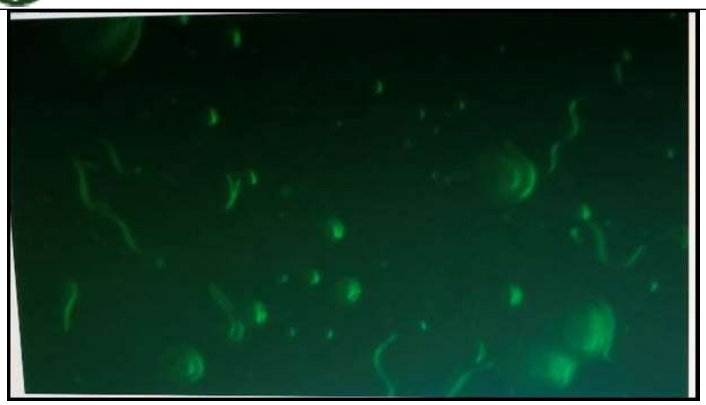


Figure 2: Fluorescence settings of the Lumascope microscope for screening.

Results

Worm Movement

Worm movement during the hypoxic exposure has been quantified by the worm lab software. Worm lab software utilizes the videos captured for the analysis. The videos were captured by utilizing Lumascope 620 which has been placed inside the glove box and the worm images were captured at 30f/s and stitched later on to videos by the program and the videos of one minute duration are fed to the worm lab for the analysis. The analysis before, during and recovery period from the suspended animation are analyzed for the speed, peristaltic movement and track length. The track length of the worm movement measured before hypoxia exposure was 1227.476µm and during suspended animation was recorded to be 74.090µm while 3h of removal or stopping of the flow of nitrogen gas and inflow of oxygen the worms have regained the movement and they have attained a track length of 1083.136µm. These observations (Tabel.1) suggest that the worms were moved with length of 1227.476µm before exposure and the decrease in movement was recorded on exposure/stimulus driven by the pumping in of nitrogen gas. The decrease in track length was 94% on comparison to the worm track length before undergoing suspended animation or normoxia. During recovery from suspended animation after 3hs of removal the track length measured by worm lab was 11.76 per cent reduced on comparison to the normoxia (Fig. 3).

Speed of the worms before hypoxia was 610.707µm/sec and during suspended animation the speed was 74.070µm/sec recorded while 3h of removal or stopping of the flow of nitrogen gas the worms have regained the movement and they have attained speed 1177.621um/sec speed was recorded. Decreasing in the worm speed during suspended animation, this shows that worms were moved with a twofold increased speed 192.82 per cent recorded in comparison with speed of worms (Table 1) (Fig. 4). The observed peristaltic track length of worms in (Table.1) shows that the measured Peristaltic Track Length of worms before Hypoxia exposure was 157.328µm/sec and during suspended animation 50.389µm/sec, while 3hs of removal or stopping of the flow of nitrogen gas the worms have regained speed was 242.416um/Sec (Fig. 5). The embryos of the N2 exposed worms to Nitrogen concentration of 99.9% were not hatched till 72 hrs of exposure and were intact. The larvae were observed on 12hrs of normoxia. Suggesting that the embryo development was also arrested during suspended animation conditions. The representative pictures of hypoxia exposed worms.

Table 1 Worm movement recorded under Lumascope 620

Observations/ Conditions	Normoxia	Suspended Animation	Recovery from Suspended Animation
Track Length	1227.476	74.090	1083.136
Speed	610.707	74.079	1177.621
Peristaltic Track Length	157.328	50.389	242.416

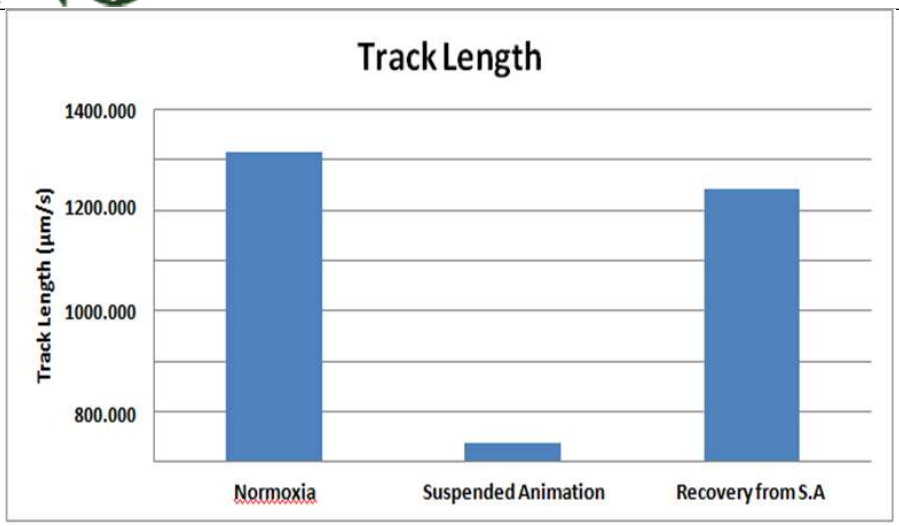


Figure 3: Track length as measured by worm lab on N2 exposure and recovery from suspended animation

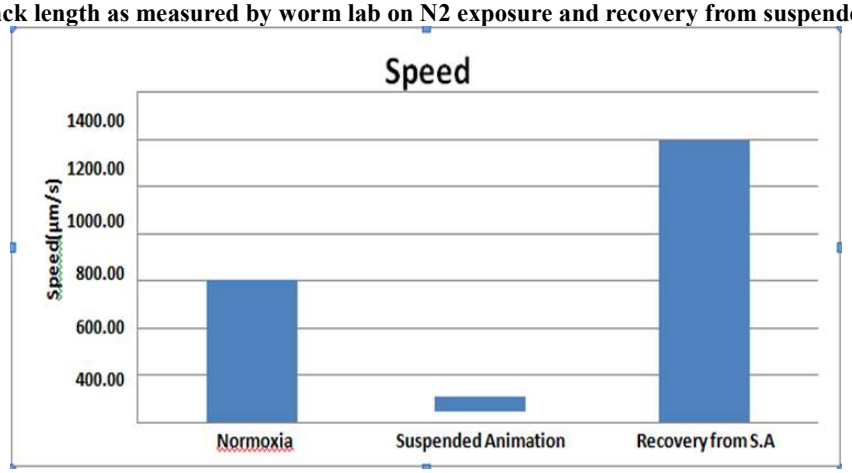


Figure 4: Speed as measured by worm lab on N2 exposure and recovery from suspended animation

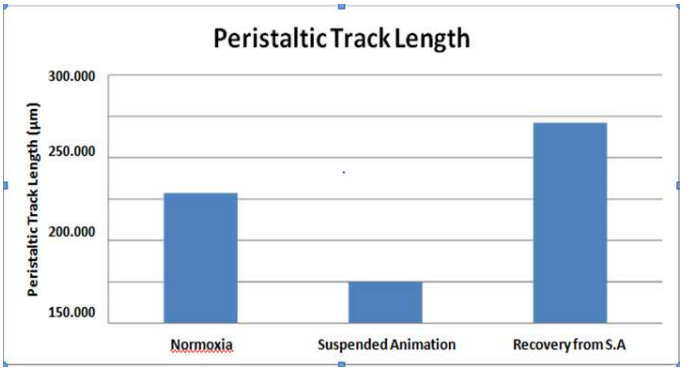
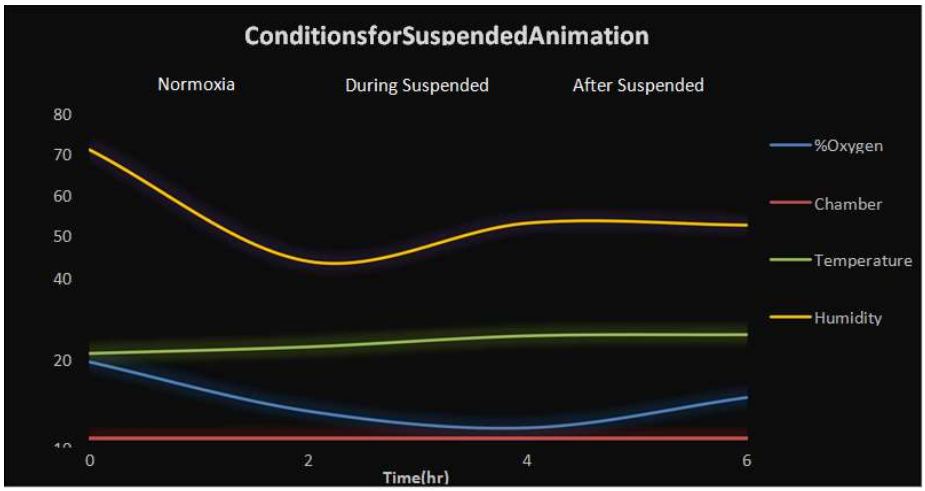


Figure 5: Peristaltic track length as measured by worm lab on N2 exposure and recovery from suspended animation

Suspended animation in *C.elegans* has been induced by Oxygen deprivation. Oxygen deprivation has been induced by placing the worm plate in the environment filled with CO_2, carbon monoxide, exposure to h2s and the chambers filled with N2 an innert gas. Similarly in our study we have utilized the N2 gas as an inducer of suspended animation to create an anoxic environment and utilized to study the effect of suspended animation on worm movement in terms of speed, peristaltic movement, track length, egg hatching and recovery by utilizing the M9 and blue light exposure. Our results are in conformity of non-movement as recorded by various

researchers. The worms have been recovered after removal of the stimulating agent Nitrogen. To understand the recovery process during the experimentation the worms have been stimulated by the K-medium, M9 and blue light stimulation. The worm recovery was faster on stimulus driven by K medium than the other stimulus (Fig. 6).



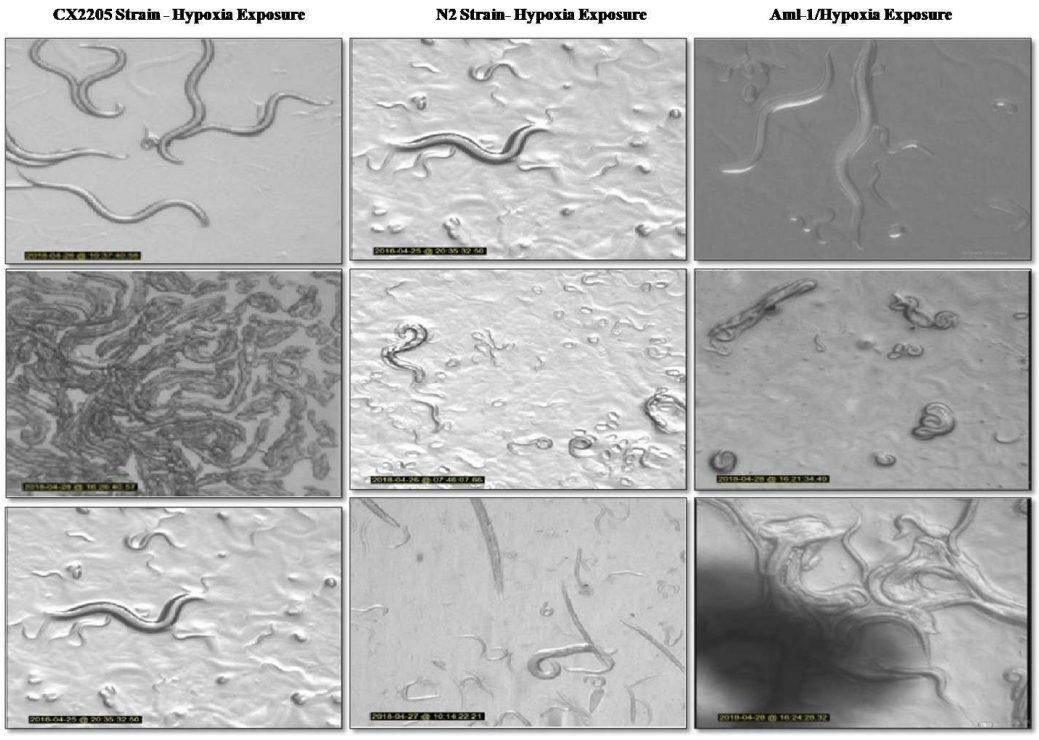


Figure 6: Conditions for suspended animation under normoxia, during suspended animation and after suspended animation

Discussion

It has been of enormous interest to the researchers to know how the suspended animation occurs and the ways the organisms can be induced to enter into suspended animation and exit in live conditions from suspended animation is of greater interest. Various studies have shown /instated the utility of the hypoxic conditions to

induce the suspended animation in various organisms at various stages of life cycle and development undergoing hibernation, quiescence and cues required for to enter into suspended animation is of various interest. The studies have shown that various organisms can enter and exit from suspended animation.

In the current study worm movements (speed, track length and peristaltic track length) suggests that worms have undergone the arrest in movement during the exposure period and recovery was confirmed by the reverting the worm movement in terms of the speed traversed by worms on removal or withdrawal of the flow of nitrogen gas. During this period as measured by the various sensors the nitrogen gas present in the chamber was around 97.8%. This confirms that the high hypoxia conditions are leading to the worm arrest in their movement. worms peristaltic track length was decreased by 67.98 per cent whereas, increased peristaltic length track of worms was 54 per cent after suspended animation during recovery was noticed (Table 1). The studies by Nystul and Roth 2004, in response to anoxia in *C. elegans* enters a state of suspended animation in which no movement is visible, including cell division and developmental progress. When returned to normoxia after being suspended for 24 hours or more, *C. elegans* will recover with a high viability. Similarly worm speed has been enhanced during the recovery and all the parameters measured were increased during recovery from the suspended animation. Suggesting the increase in activity has been recorded for the recovered worms. When turtle hepatocytes are starved of oxygen, the cell makes a concerted attempt to inhibit processes including protein synthesis, ion channel activity, and anabolic pathways. This reduces the need for ATP by 94 %. This shows that worms are suspended and the movement of head part was observed even eggs are also did not develop.

Hypoxia is a typical natural stress, and there are a number of well-conserved mechanisms that help cells adapt to hypoxic situations. The cell must either enhance anaerobic energy generation or decrease energy demand to make up for the loss in the capacity for aerobic energy production under hypoxia. In metazoans, examples of both of these reactions are frequent, and the particular reaction employed is typically based on the amount of oxygen accessible to the cell (J A Powell-coffman 2010).

Nystul and Roth 2003 Assessed the survivability of wild-type C. elegans after exposing embryos to various oxygen pressures between moderate hypoxia and anoxia for 24 hours in order to know the ranges in which each of these responses are active. Anoxic-exposed embryos entered suspended animation and had a high viability after the exposure. Embryos in 0.5 kPa O2 survived with a high viability and shown suspended animation throughout the exposure. They concluded that the embryos did not survived, when exposed to an intermediate range of oxygen tensions (0.1 to 0.01 kPa O2) between moderate hypoxia and anoxia.

The production of carbon monoxide by heme oxygenase in response to hypoxia is one recently identified reaction (Dulak and Jozkowicz 2003). Exogenous carbon monoxide perfusion in transplant animals can produce similar cytoprotective benefits to endogenous carbon monoxide in terms of activating signaling cascades that reduce hypoxia damage through antiapoptotic and antiinflammatory activities. Although the potential cytoprotective role of this activity in hypoxia has not been explored, carbon monoxide competes with oxygen for binding to iron-containing proteins, such as mitochondrial cytochromes, at greater quantities (Otterbein *et al*., 2000, Otterbein *et al*., 2003, Amersi *et al*., 2002 and Gormann *et al*., 2003)

The suspended animation induced by high oxygen that is 0.25 kPa O_2 was protected by excess carbon monoxide. The levels of carbon monoxide may induce suspended animation in hypoxia by directly interacting with oxygen sensing through competitive inhibition. It was interesting to know that the accumulation of carbon dioxide naturally in hypoxia tissue to induce local regions of suspended animation (Nystul and Roth 2004).

These measurements suggests that worms have undergone the arrest in movement during the exposure period and recovery was confirmed by the reverting the worm movement in terms of the track length traversed by worms on removal or withdrawal of the flow of nitrogen gas. During this period as measured by the various sensors the nitrogen gas present in the chamber was around 97.8%. This confirms that the high hypoxia conditions are leading to the worm arresting their movement. Shows that worms are suspended and the movement of head part was observed even eggs stage were not developed.

Similarly previous studies in response to anoxia, *C. elegans* enters a state of suspended animation in which no movement is visible, including cell division and developmental progress. When returned to normoxia after being suspended for 24 hours or more, *C. elegans* will recover with a high viability (Padilla *et al.,* 2002 and Voorhies and Ward 2000). When turtle hepatocytes are starved of oxygen, the cell makes a concerted attempt to inhibit processes including protein synthesis, ion channel activity, and anabolic pathways. This reduces the need for ATP by 94% (Hochachka *et al.,*1996).

Nystul and Roth 2003 assessed the survivability of wild-type *C. elegans* after exposing embryos to various oxygen pressures between moderate hypoxia and anoxia for 24 hours in order to know the ranges in which each of these responses are active. Anoxic-exposed embryos entered suspended animation and had a high viability after the exposure. Embryos in 0.5 kPa O2 survived with a high viability and shown suspended animation throughout the exposure. They concluded that the embryos did not survived, when exposed to an intermediate range of oxygen tensions (0.1 to 0.01 kPa O2) between moderate hypoxia and anoxia. Embryos did not hatch when subjected to this moderate level of hypoxia, demonstrating that the HIF-1-mediated signaling was not carried out. The exposed *C. elegans* embryos to intermediate range of hypoxia did not shown suspended animation activity due to Hif-1 non signaling mode. Then they conduced that embryos exposed to lethal oxygen tensions did not arrest embryogenesis and also the increased amount of oxygen correlated with an increase in the extent of development progression in the embryo. This statement shows that the intermediate range of hypoxia is a unique stress in which O_2 levels are neither sufficiently high to facilitate continued animation nor sufficiently low to induce suspended animation. *C.elegans* can survive for about a day in normal culture conditions with complete lack of oxygen (anoxia) by entering into a state of reversible arrest that is suspended animation. Recovers from anoxia induced arrest does not require hif -1 but all cells checkpoints are crucial for embryonic survival in anoxia long term anoxia or survey hypoxia at the temperature is ultimately lethal. Several studies have demonstrated that the induction of suspended animation in mammals is possible for a few hours at a time through a procedure in which the exsanguinations is followed by an aortic flush with cold saline (Bellamy *et al*., 1996 and Alam *et al*., 2002).

Several studies have described the new roles for *C. elegans* HIF-1 in stress responses and aging [Budde and Roth 2010, Mehta, R. *et al.,* 2009, Chen D *et al*., 2009, Bellier, A. *et al.,*2009 and Zhang, Y. *et al*., 2009]. Compared to wild-type animals, egl-9-deficient mutants are more resistant to heat, hydrogen cyanide, hydrogen sulfide, and certain pathogens [Budde and Roth 2010, Bellier, A. *et al.,*2009, Gallagher and Manoil 2001, Darby *et al*., 1999, Anyanful, A. *et al.,*2005 and Treinin, M. *et al*.,2003]. In all cases so far studied, deletion of hif-1 suppresses these egl-9 loss-of function phenotypes [Budde and Roth 2010, Bellier, A. *et al.,* 2009 and Treinin, M. *et al*., 2003]. Moderate over expression of HIF-1 causes dose-dependent increases in adult lifespan. Nematodes exhibit diseases in a variety of tissues, including muscle, the nervous system, and the pharynx, after being incubated for more than 20 hours at high temperatures in conditions of severe hypoxia or anoxia. Homozygous animals receive protection from these hypoxic injuries and maintain their mobility for several hours because of the daf-2 (e1370) mutant (Scott, B.A. *et al.,* 2002 and Mendenhall, A.R. *et al*. 2006). Loss-of-function mutations in daf-2 result in nuclear localization of the DAF-16 fork head transcription factor and confer resistance to a range of other stresses [Panowski and Dillin 2009]. There are interesting differences in the daf-2 mutations that confer hypoxic resistance phenotypes and those that cause dauer formation or longevity phenotypes. These allelic differences have been used as tools to identify gene expression differences that correlate with resistance to severe hypoxia [Mabon. M.E. *et al.*2009]. By enhancing the expression of genes involved in anaerobic energy production, such as glycolytic enzymes and glucose transporters, hypoxia-inducible transcription factor 1 (HIF-1) is intended to compensate for the decreased aerobic energy output (Semenza, G.L. 2001 and Guillemin & Krasnow 1997). As a defense against the harm caused by free radicals, this response also encourages the up-regulation of antioxidants like catalase and superoxide dismutase. As a result, even in mild hypoxia, the cell can continue to function at nearly normoxic levels. When turtle hepatocytes are starved of oxygen, the cell makes a concerted attempt to inhibit processes including protein synthesis, ion channel activity, and anabolic pathways. This reduces the need for ATP by 94%.

Conclusion:

Hypoxia was induced by the 99.00% of nitrogen in glove box which induces hypoxic conditions. The nematodes were exposed to these conditions and as soon as the 99.00 of oxygen is reached worms enter into reversible state of suspended animation. Till the conditions were returned to normoxia. Recovery was recorded by normoxia conditions. Worms were recovered from suspended animation by re-oxygenation and by the way of normoxia and recovery was tested by addition of K-media drops on the worms and tested for their movement. The worms started moving as soon as K- medium drops were circulated through its body and the worms started moving and normal crawling was recorded. Blue light induced activation was not recorded for the tested worms during suspended animation but the blue light activated movement was recorded during after recovery period. Recovery was recorded by normoxia conditions and movement was observed.

References

1. Nystul TG, Goldmark JP, Padilla PA, Roth MB. Suspended animation in *C. elegans* requires the spindle checkpoint. Science 2003; 302:1038–41.

2. [2] Shen, Chuan, Powell-coffman JA. Genetic analysis of hypoxia signaling and response in C. elegans. Annals of the New York Aca Sci 2003; 995.1: 191-199.
3. [3] Chen D. HIF-1 modulates dietary restriction-mediatedlifespan extension via IRE-1 in C. elegans. PLoS Genet 2010; 5:e1000486.
4. [4]Angstman NB, Frank HG, Schimz C. Advanced behavioral analyses show that the presence of food causes subtle changes in C.elegans movement. Front Behaviour Neurosci 2016;10:60.
5. [5] Hajeri VA, Trejo J, Padilla PA. Characterization of sub-nuclear changes in Caenorhabditis elegans embryos exposed to brief, intermediate and long-term anoxia to analyze anoxia-induced cell cycle arrest. BMC Cell Biol 2005;6:47. https://doi.org/10.1186/1471-2121-6-47
6. [6] Budde MW, Roth MB. Hydrogen sulfide increaseshypoxia-inducible factor-1 activity independently of von Hippel–Lindau tumor suppressor-1 in C. elegans. Mol. Biol Cell 2010;21:212-217.
7. [7] Miller Dana L, Mark B, Roth. *C. elegans* are protected from lethal hypoxia by an embryonic diapause. Curr Biol 2009;19.14:1233-1237.
8. [8] Douglas RM. Hypoxia induces major effects on cell cycle kinetics and protein expression in Drosophila melanogaster embryos. American J of Physiol Regul Integr Compar Physiol 2005;288.2:R511-R521.
9. [9] Fischer, Matthias G. The mitotic arrest in response to hypoxia and of polar bodies during early embryogenesis requires Drosophila Mps1. Curr Biol 2004; 14.22: 2019-2024.
10. [10] Wang D, Xing X. Assessment of locomotion behavioral defects induced by acute toxicity from heavy metal exposure in nematode *Caenorhabditis elegans*. J Environ Sci 2008;0.9: 1132-1137.
11. [11] Yu, Zhenyang, Jiang l, Yin D. Behavior toxicity to *Caenorhabditis elegans* transferred to the progeny after exposure to sulfamethoxazole at environmentally relevant concentrations. J Environ Sci 2011; 23.2: 294-300.
12. [12] Massie MR, Lapoczka EM, Boggs KD, Stine KE, White GE. Exposure to the metabolic inhibitor sodium azide induces stress protein expression andthermotolerance in the nematode Caenorhabditiselegans. Cell Stress Chaperones 2003;8:1-7PMID:12820649;http://dx.doi.org/10.1379/14661268.
13. [13] McCarter J, Bartlett B, Dang T, Schedl T. Soma-germ cell interactions in *Caenorhabditis elegans*: multiple events of hermaphrodite germline development requirethe somatic sheath and spermathecal lineages. Dev Biol 1997;181:121-143. doi: 10.1006/dbio.1996.8429.
14. [14]SurafelM. Tegegne , Markandeya Jois , MatthewR. Flavel , DamienL. Callahan , Devin Benheim R apid induction of vitamin B12 deficiency in *Caenorhabditis elegans* cultured in axenic medium ·Journal of Nutrition & Intermediary Metabolism Volume 13, September 2018, Pages 20-25.
15. [15] Nicholas B. Angstman, Hans-Georg Frank, and Christoph Schmitz. Advanced Behavioral Analyses Show that the Presence of Food Causes Subtle Changes in *C. elegans* Movement. Front Behav Neurosci. 2016; 10: 60.
16. [16] Boivin GP, Hickman DL, Creamer-Hente MA, Pritchett-Corning KR, Bratcher NA. Review of CO(2) as aEuthanasia Agent for Laboratory Rats and Mice. J Am Assoc Lab Anim Sci. 2017; 56(5):491–9. PMID:28903819.
17. [17] Sharp J, Azar T, Lawson D. Comparison of carbon dioxide, argon, and nitrogen for inducing unconsciousnessor euthanasia of rats. J Am Assoc Lab Anim Sci. 2006; 45(2):21-5. PMID: 16542038.
18. [18] Queenie Hu, Dayana R. D'Amora,Lesley T. MacNeil, Albertha J. M. Walhout, and Terrance J. Kubiseski. The *Caenorhabditis elegans* Oxidative Stress Response Requires the NHR-49 Transcription Factor. G3 (Bethesda). 2018 Dec; 8(12): 3857–3863.
19. [19] Powell-Coffman, Jo Anne. Hypoxia signaling and resistance in *C. elegans*. Trends Endocrinol Metabol 2010;21.7: 435-440.
20. [20] Agnieszka Loboda, Milena Damulewicz, Elzbieta Pyza, Alicja Jozkowicz & Jozef Dulak. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cellular and Molecular Life Sciences .Volume 73, pages 3221–3247, (2016)
21. [21] Brian S. Zuckerbraun, Beek Yoke Chin, Martin Bilban, Joana de Costa d Avila, Jayashree Rao, Timothy R. Billiar, Leo E. Otterbein. Carbon monoxide signals *via* inhibition of cytochrome *c* oxidase and generation of mitochondrial reactive oxygen species. The FASEB JournalVolume 21, Issue 4Apr 2007Pages963-1284.
22. [22] Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA, Choi AM. Cardioprotective Actions by a Water-Soluble Carbon Monoxide–Releasing Molecule. Nat Med 2003;6:422–428.

23. [23] Amersi F, Shen XD, Anselmo D, Melinek J, Iyer S, Southard DJ, Katori M, Volk HD, Busuttil RW, Buelow R,Kupiec-Weglinski JW. Ex vivo exposure to carbon monoxide prevents hepatic ischemia/reperfusion injury through p38 MAP kinase pathway. Hepatology 2002;35:815-823.
24. [24]Gorman D, Drewry A, Huang YL, Sames, C. The clinical toxicology of carbon monoxide.
25. [25] Padilla, Pamela A, Mark B. Roth. Oxygen deprivation causes suspended animation in the zebrafish embryo. Proceedings of the Nat Aca Sci 2001;98.13: 7331-7335.
26. [26] Voorhies, Wayne A, Van, ward S. Broad oxygen tolerance in the nematode I. J Exp Biol 2000;203.16: 2467-2478.
27. [27] Hochachka PW, Buck LT, Doll CJ, Land SC. Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. Proc Natl Acad Sci USA 1996; 93:9493−9498.
28. [28] Bellamy R, Safar P, Tisherman SA, Basford R, Bruttig SP, Capone A, Dubick MA, Ernster L, Hattler Jr HJ, Hochachka P, Klain M, Kochanek PM, Kofke WA, Lancaster JR, McGowan Jr FX, Oeltgen PR, Severinghaus JW, Taylor MJ, Zar H. Suspended animation for delayed resuscitation. Crit Care Med 1996;24: S24-S47.
29. [29] Alam HB, Bowyer MW, Koustova E, Gushchin V, Anderson D,Stanton K, Kreishman P, Cryer CM, Hancock T, Rhee P. Learning and memory is preserved after induced asanguineous hyperkalemic hypothermic arrest in a swine model of traumatic exsanguination. Surgery 2002;132:278–288.
30. [30] Mehta R. Proteasomal regulation of the hypoxic responsemodulates aging in *C. elegans*. Sci 2009;324:1196–1198.
31. [31]Bellier A. Hypoxia and the hypoxic response pathwayprotect against pore-forming toxins in C. elegans. PLoS Pathol 2009;5: e1000689.
32. [32]Zhang Y. The HIF-1 hypoxia-inducible factor modulateslifespan in *C. elegans*. PLoS ONE 2009; 4:e6348
33. [33]Gallagher LA, Manoil C. Pseudomonas aeruginosa PAO1kills Caenorhabditis elegans by cyanide poisoning. J Bacteriol 2001: 183:6207–6214
34. [34] Darby C. Lethal paralysis of Caenorhabditis elegans byPseudomonas aeruginosa. Proc Natl Acad Sci USA 1999;96:15202-15207
35. [35] Anyanful A. Paralysis and killing of Caenorhabditiselegans by enteropathogenic Escherichia coli requires the bacterialtryptophanase gene. Mol Microbiol 2005;57:988–1007.
36. [36] Mark W. Budde and Mark B. Roth. Hydrogen Sulfide Increases Hypoxia-inducible Factor-1 Activity Independently of von Hippel–Lindau Tumor Suppressor-1 in C. elegans. Molecular Biology of the Cel l Vol. 21, 212–217, January 1, 2010.
37. 37] Audrey Bellier, Chang-Shi Chen, Cheng-Yuan Kao, Hediye N.Cinar, Raffi V.Aroian. Hypoxia and the Hypoxic Response Pathway Protect. Against Pore-Forming Toxinsin *C.elegans.* Plos pathogens December 11, 2009 https://doi.org/10.1371/journal.ppat.1000689.
38. [38] Treinin M. HIF-1 is required for heat acclimation in the nematode *Caenorhabditis elegans*. Physiol Genomics 2003;14:17–24.
39. [39] BARBARA A. SCOTT, MICHAEL S. AVIDAN, AND C. MICHAEL CROWDERAuthors Info & Affiliations. Regulation of Hypoxic Death in C. elegans by the Insulin/IGF Receptor Homolog DAF-2. ***SCIENCE***13 Jun 2002 Vol 296, Issue 5577 DOI: 10.1126/science.1072302.
40. [40] Alexander Mendenhall, Matthew M. Crane, Patricia M. Tedesco,2Thomas E. Johnson, and Roger Brent. Caenorhabditis elegans Genes Affecting Inter individual Variation in Life-span Biomarker Gene Expression. Journals of Gerontology: Biological Sciences cite as: J Gerontol A Biol Sci Med Sci, 2017, Vol. 72, No. 10, 1305–1310 doi:10.1093/gerona/glw349.
41. [41] William Mair ,Siler H. Panowski, Reuben J. Shaw, Andrew Dillin. Optimizing Dietary Restriction for Genetic Epistasis Analysis and Gene Discovery in C. elegans. Pols one February 20, 2009 https://doi.org/10.1371/journal.pone.0004535
42. [42] Meghann E. Mabon, Xianrong Mao, York Jiao, Barbara A. Scott and C. Michael Crowder. Genetics Society of America DOI: 10.1534/genetics.108.097188.
43. [42] Semenza GL. How animal cells signal hypoxia to the nucleus. Cell 2001; 107:1-3.
44. [43] Guillemin K, Krasnow MA. The hypoxia response: hufing and HIFing. Cell 1997;89: 9-12.