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DIACETYL INDUCED SUSPENDED ANIMATION AND CHEMOTAXIS RESPONSE TO VOLATILE CHEMICALS: A COMPARATIVE STUDY IN DIFFERENT STRAINS OF *CAENORHABDITIS ELEGANS*

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ABSTRACT

This study was undertaken to understand the recovery pattern in different strains of *C. elegans* after their resuscitation from suspended animation (SA)/ reanimation in different environments with different chemical stimuli. Emphasis was drawn on understanding the chemosensory plasticity of the worms in terms of their foraging ability after recovery from the 3 and 6hrs of SA with different chemical odorants such as Isoamyl alcohol, Pyrazine and Diacetyl both in the presence and absence of a food source. The merit of the study lies in its ability to holistically enhance our understanding of quiescent states resulting from novel environmental insults and providing a forum of opportunity to understand various responses of *C. elegans* to extremes of environmental situations apart from starvation.

Diacetyl dose was standardized in the N2 strain of *C. elegans* through the mortality, motility and lifespan assays. Furthermore, the chemotaxis assay was done with volatile odorants to look into the olfactory and sensory responses of the worms to different chemicals after their exposure to diacetyl induced SA. N2 behavioral responses were further compared to both the mutant and transgenic strain for clarity.

Since there is reported conservation of molecular and cellular mechanisms between nematodes and mammals, this study could provide valuable insights into understanding the behavioral responses of *C. elegans*, in stressful conditions, with a scope for human translation and application in terms of delayed resuscitation for treatment of ischemic or hypoxic conditions of diseased states.

***Keywords*:** Nematode; *Caenorhabditis elegans*; lifespan, motility, mortality, chemotaxis.

Introduction

The cessation of biological processes can be addressed under different headings such as diapause, quiescence, hibernation, cryptobiosis and reanimation. In particular is suspended animation (SA) that holds a great promise emerging as one of the prioritized fields of research due to its potential application in space travel / cryonics and the cryopreservation of humans after clinical death with the aim to reanimate them in the future (Girard *et al*., 2025; Mauricio Girard1, 2025). This cessation can be introduced via introduction of hypometabolic or ametabolic responses that induce slowing or arrest of gene expression, cell division, metabolism and cell development. SA induced through endogenous natural methods revert to their original state with the cessation of causative environmental stressors (Safar *et al*., 2000). Some of the natural or endogeneous reasons for SA to occur most favourably in metazoan species are the unavailability of food resources, extremes of oxygen deprivation or environmental factors such as

temperature, salinity etc (Mendelsohn *et al*., 2008; Rinkevich *et al*., 2022). SA can further be introduced artificially through exposure to noxious chemicals (Li *et al*., 2023). In case of artificially induced SA, technologically mediated revivals is necessitated. When an organism enters SA, it stops moving, feeding and becomes metabolically inactive in an attempt to conserve energy (Chan *et al*., 2010).

Among the species that successfully undergo SA include Drosophiles (Phillips & Simons, 2024), Zebrafish (Lange *et al*., 2024), and *Caenorhabditis elegans* (*C.elegans*) (Suraci *et al*., 2024). Adverse environmental conditions, including starvation, overcrowding, or anoxia can induce alterations in *C. elegans* existence to either enter the dauer or SA state. Dauer state is distinct from SA in its ability to show a movement response on physical touch or prick (Bodkhe *et al*., 2024). Once *C. elegans* enters a state of SA all perceivable movements in the worm are halted. Upon exposure to standard conditions, this dormant phase shifts back to the active state (Doering *et al*., 2022).

The neurons found in the worms are responsible for sensing various chemicals cues leading to a process known as chemotaxis (Hilliard *et al*., 2002; Qin *et al*., 2025). Young adult worms recognize many volatile compounds either as attractants or repellants. The worm's sensory neurons measure response to different stimulants by measuring their movement towards (attractants) or against (repellents) these chemicals (Lanza *et al*., 2021). The behavior of the worm is known to change depending on their environment, age and health span. Therefore chemotaxis assay can be exploited as a yard stick to measure the sensitivity of olfactory and sensory systems in both health and disease states of the worm (Choi *et al*., 2022).

Likewise in the present study, using diacetyl (DIA) as a volatile chemical that induces muscle contraction (Hoffmann *et al*., 2010), we initially standardized the induction of SA in the N2 strain of *C. elegans* through the mortality motility and lifespan assays in the worm. Once the dose of DIA was standardized, we conducted chemotaxis assay for chemosensory perception, particularly with volatile odorants, to look into the olfactory and sensory responses after SA. This was done as a comparative response of the worms to different chemicals after their exposure to DIA induced SA. N2 behavioral responses were further compared to that of the mutant strain, CX2205 (mutant with defect in odr-3 gene; resulting in defect to chemical signalling) and the transgenic strain, NL5901 (transgenic strain of Parkinson's disease expressing alpha synuclein in body wall muscles) for further clarity of the neurons that are modulation in such revival mechanisms (Jafri Ali & Sharda Rajini, 2013). This was of particular interest as it would help in understanding the survivability of the worm after recovery from SA. The chemicals chosen for studying chemotaxis were isoamyl alcohol (IAA; they have a fruity smell resembling that of *C. elegans* natural food, OP-50), pyrazine (PZ; as they influence feeding behavior by interacting with olfactory neurons and also on muscle relaxation), DIA; induces hypoxia and SA, and OP-50 (natural food)/M13 (control buffer). The merit of the study lies in the fact that it could contribute in comprehending the impact of DIA induced SA recovery, in terms of the worms' chemical preference and their adaptive behavior thereafter. This study could provide valuable insights into understanding the behavioral responses in stressful conditions, with a scope for human translation due to the conserved molecular and cellular pathways between the two. Furthermore, this study could contribute in its application of understanding delayed resuscitation for treatment of ischemic or hypoxic conditions in diseased states.

Materials and Methods

Chemicals

All chemicals were purchased from Himeda and Sigma Aldrich. Various strains of *C. elegans* namely wild-type (Bristol N2) transgenic (NL5901 transgenic strain of Parkinson's disease expressing alpha synuclein in body wall muscles) and the mutant (CX2205 mutant with defect in odr-3 gene) were procured from the *Caenorhabditis* Genetics Center of the University of Minnesota, USA.

Worm strains culture and maintenance

L4 staged worms were obtained by transferring all strains (N_2, NL5901 and CX2205) to nematode growth medium agar plates containing the following compositions (3 g/L NaCl, 2.5 g/L peptone, 5 mg/L cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM K_2HPO_4 at pH 6.0, and 17 g/L agar) based on the protocol from . Adult L4 stage worms were washed with K medium (53 mM NaCl and 32 mM KCI). Food deprived worm pellets were obtained by washing them with K-media and subsequently centrifuging them at 3000 rpm for 5 minutes (Shaver *et al*., 2021). Approximately 100 worms were collected and placed in appropriate media for various analysis.

Live & dead assay for Diacetyl dose standardization

Approximately 100 mM stock solution of DIA was diluted into various concentration gradients (2-6 µM) in M13 buffer and these concentrations were later

used for exposing the worms for the live/dead assay based on the protocol of (Sillapakong *et al*., 2025) with slight modification. Briefly 50 worms were taken in a 24 well plate and exposed to increasing concentrations of DIA for 24 hrs in a 1 ml assay volume. Thereafter worms observed under a microscope were characterized as dead or alive based on their motility responses. The number of survivors was counted at the end of the treatment period. They were scored dead if they did not respond to a touch stimulus. Percent mortality was calculated from three independent experiments conducted on three different days.

Mobility assay as quantified by the Arena Tracker

The mobility assay was done following protocols from (Bauer *et al*., 2022; Kutzner *et al*., 2024) that employs detecting the changes in the worm's positional movement through repeated scanning. Briefly worms were exposed to various concentrations of DIA (2-6 µM) in M13 buffer in a 24-well plate for 3hrs. Infrared LED micro-beams from the WMicroTracker ARENA System tracked the movement and position of the worms on the culture dishes and interruptions of the LED micro-beam by a worm's movement allowed real-time data to be processed at various concentrations. The accompanying software identified changes in the positions of the interrupted beams during scans and computed an activity score based on the successive scan differences. 70 worms were analyzed in each group of the experiment. This was repeated thrice on three different days.

Lifespan Assay

Lifespan assay was conducted following protocol from (Jafri Ali & Sharda Rajini, 2013). As mentioned above, worms were exposed to various concentrations of DIA (2-6 µM) in M13 buffer in a 24-well plate. After the exposure period, they were washed thrice with K-medium and subsequently re-exposed in a 12-well plate containing K–medium with OP50. 5-fluoro-2′-deoxyuridine (FudR, 50µM) was added to arrest worm reproduction. Worms were maintained at 20°C. The survivability was scored every day. Worms that failed to respond to touch were considered dead. This survivability was checked until all the worms were classified as being dead

Chemotaxis Assay

Chemotaxis assay was conducted following protocol from (Koh *et al*., 2025). 100 young adult synchronized worms were placed at the center of the 25mm NGM plate that was divided into four quadrants with two opposite ends being labelled as control containing either M13 buffer / OP50 media and the other two quadrants being spiked with the test chemical (PZ, IAA, DIA). The lid of the petri-plates was closed for 180 and 360 min (3-6 hrs) so that the worms could go into the state of SA at a temperature of 20 °C. We allowed all the plates to stand for 180 minutes initially, then identified the movement of worms towards different chemicals (40ul directly from the respective stock bottles) and then analyzed their chemotaxis behavior by counting the number of worms that were attracted/repelled to different test solutions using a microscope and imaged them using an EPSON V800 scanner at the 180- and 360-min time point. We performed the chemotactic response during the daytime to mitigate the effects of circadian rhythm (Olmedo *et al*., 2012). Our experiments were conducted thrice on three different days using young adult worms.

Scoring and Analysis

Chemotaxis index (CI) for all test chemicals was calculated using the following equation (Crombie *et al*., 2022):

Chemotaxis index (CI) = (A+C)-(B+D) / (A+B+C+D)

Where

(A+C) =No. of worms exposed to same test chemicals in opposite quadrants

(B+D) = No of worms in control quadrants (B+D)

(A+B+C+D) = Total number of worms on the plate

The chemotaxis index had a positive value when the nematodes exhibited an attractive response and a negative value when they exhibited an avoidance response. Values were presented as Mean ± SEM. Data analysis was done through 1- way ANOVA followed by post-hoc analysis (Tukey Multiple Comparison Test) ($p<0.0001$) using GrapPad Prism software version.

Results

Mortality Assay

100 worms were exposed to varying concentrations of DIA ranging from (2-6 µM) in a 24 well plate. The results of our study showed that 20µl corresponding to 2mM of DIA reported 2% , 30 µl (3mM) reported 4%, 40 µl(4mM) reported -4%, 50 µl (5mM) reported -10%, 60 µl (6mM) reported -12%) mortality in the assay as seen in Fig. 1 A & B. Mortality was recorded as a “no respond phenomenon” to a touch stimulus.

Motility Assay

The mobility assay was based on detecting the changes in the worm's positional movement as IR units of activity through repeated scanning for 6hrs using

WMicroTracker ARENA software. The results of our study showed that maximum IR activity was observed in control group that had no DIA and was 35.0 IR units. From 10 µl to 60 µl DIA spiking arms, IR activity saw a concentration independent fluctuation with 10 µl (1mM) reporting motility activity corresponding to 5.07 IR units, 20 µl (2mM) reporting motility activity corresponding to 4.79 IR units**,** 40 µl (4mMl) reporting motility activity corresponding to 6.71 IR units, 50 µl (5mM) 5.86 IR units and 60 µl (6mM) reporting 4.86 IR units. As compared to 10 µl arm, highest fold IR activity was observed in 40 µl arm with 1.33 fold increased IR activity. Lowest fold change was observed in 60 µl arm with 0.95 fold increase in IR activity as compared to the 10 µl arm as seen in Fig. 2. Values were represented as Mean ± S.E (n=50). Data was analyzed by 1 way ANOVA followed by post-hoc analysis (Tukey Multiple Comparison Test) which showed a significant difference between control and the varying concentration gradients of DIA groups at a significance of **** for ($p<0.0001$).

Lifespan Assay

After 3 hrs of exposure to diacetyl, worms were washed thrice with K-medium and then re-exposed in a 12 well plate containing K–medium with OP-50. 5-fluoro-2′-deoxyuridine (FudR, 50µM) was added to arrest reproduction and maintained at 20°C. The survivability was scored every day. Worms that failed to respond to touch were considered as dead. The result of the lifespan assay showed highest lifespan expectancy in control at 21 days with IR activity corresponding to 33 units. Similarly, the lowest lifespan expectancy was recorded at 9 days with an IR activity index at 5.1 units as seen in Fig. 3 A & B. Data was analyzed by 1 way ANOVA followed by post-hoc analysis (Tukey Multiple Comparison Test) that showed significant difference between the control and the varying arms of DIA group (10-60 µl) **** ($p<0.0001$). Similarly, a significant difference was further observed between the different concentrations of DIA arm against the 1mM (10ul) DIA arm.

Chemotaxis Assay

Chemotaxis in a worm is based on its response for a chemical odorant either as a movement towards it or one that is away from it. Those movement responses that are towards the odorant are classified as being "attractants." Similarly, those movements that are away from the chemical odorant are classified as "repellent." In the present study, we analyzed the chemotactic responses of the worms based on two treatment scenarios. Responses of the worms were analyzed in reference to their movement either in the presence of OP-50 as food source on the plate after 3 and 6 hrs of SA or their responses in the presence of M-13 buffer again analyzed after 3 and 6 hrs of SA. The results of the study were as follows:

For the plates containing OP-50 food source, we observed that after 3 hrs of SA, N2 and CX2205 worms showed no response for IAA, whereas strain NL5901 was repelled with a CI index of -0.14. Similarly for all the three strains, DIA acted as a repellent with a CI index of -0.41 (N2), -0.09 (CX2205) and -0.55 for NL5901. Similarly, PZ also acted as a repellent for all the three strains with CI values of -0.09 (N2), -0.36 (CX2205) and --0.27 for NL5901. Analysis of the data by 1 way ANOVA followed by post-hoc analysis (Tukey Multiple Comparison Test) showed a significant difference in chemotaxis behavior between the strains for DIA and PZ odors and between N2 and NL5901 for IAA at **** ($p<0.0001$). However, no significance was observed between N2 and CX2205 for IAA as seen in Fig. 4A. Similarly in plates with OP-50 we observed that after 6 hrs of SA, for IAA; N2 strains showed neutral response with CI value (0), CX2205 showed a positive attractive response (0.15) while NL5901 showed a negative repellent response (-0.10). Similarly for the other two chemical odorants viz., DIA and PZ, all three strains showed a negative CI value with a repellent response as seen in Fig. 4B.

Likewise for the plates containing M-13 buffer, we observed that after 3 hrs of SA, for IAA; N2 worms showed neutral response, whereas strains CX2205 and NL5901 showed a positive CI index where IAA acted as an attractant **** ($p<0.0001$). Similarly for the other two odorants, namely DIA and PZ, all three strains of the worms showed a repellent effect with negative CI values. Plates containing M-13 buffer, after 6 hrs of SA, for IAA showed more positive response for all the three strains. For PZ, all three strains showed a repellent negative CI index. Similarly, their resposes for DIA were also that of repellent negative CI values. Analysis of the data by 1 way ANOVA followed by post-hoc analysis (Tukey Multiple Comparison Test) showed that interactions between IAA and PZ were significant difference for all the strains as seen in Fig. 4C with significance at **** ($p<0.0001$). Likewise in plates with M-13 buffer we observed that after 6 hrs of SA, for IAA; all three strains showed attractive response with positive CI values at **** ($p<0.0001$). However, for the other two chemical odorants viz., DIA and PZ, all three strains showed a negative repellent response with negative CI values as seen in Fig. 4D.

Discussion

This study was undertaken to understand the recovery pattern in different strains of *C. elegans* after their resuscitation from SA or reanimation in different environments with different chemical stimuli. Emphasis was drawn on understanding the chemosensory plasticity of the worms in terms of their foraging ability after recovery from the 3 and 6hrs of SA with different chemical odorants such as IAA, DIA and PZ both in the presence and absence of a food source.

The merit of the study lies in its ability to holistically enhance our understanding of quiescent states resulting from novel environmental insults and providing a forum of opportunity to understanding various responses of *C. elegans* to extremes of environmental situations apart from starvation and to define the underlying chemosensory mechanisms.

The primarily dose of DIA was standardized through mortality and motility studies in the worm. Different concentrations of DIA in the range of 1 to 6 mM were taken to exposure the worms for 24 hrs after which the mortality pattern was ascertained. Furthermore, using an automated infrared LED scanning protocol from ARENA tracker, motility profile of the worms was derived. Based on our results, DIA dose was standardized at 4 mM (40ul) that was taken directly from the bottle which bore similarity to the studies conducted by other authors who also studied quiescence behavior in *C. elegans* using DIA as a chemical inducer of hypoxia. Similar to our work studies by other authors have also investigated the dose for DIA in chemotaxis assay (Hoffmann *et al*., 2010; Koh *et al*., 2025). Since genes involved in induction of SA in *C. elegans* are also reported to be involved in longevity (Chan *et al*., 2010; Padilla & Ladage, 2012), we conducted lifespan studies on the worms that were treated with variable concentrations of DIA. The maximum lifespan of the worms was estimated as 21 days and the least as 9 days. S. Park *et al*., 2020 in their study showed that DIA decreases the activity of DAF-16/FOXO, a longevity-promoting transcription factor acting downstream of insulin/IGF-1 signaling. The odor of DIA shortened longevity of the worms. Food odor has been shown to trigger metabolic and physiological changes in various other species like *Drosophila*(Lushchak *et al*., 2015), mice (Wang *et al*., 2024), and humans (Morquecho-Campos *et al*., 2020)

It is known that *C. elegans* modulates its behavior in response to diverse environmental cues in the form of approach or avoidance responses upon first exposure, whereas long-term exposure to such compounds could lead to behavioral plasticity including adaptation and conditioned learning (Zhao *et al*., 2025). The sensory neurons in *C. elegans,* such as AWA, AWB, AWC, ADL, and ASH, are responsible for sensing volatile chemicals, particularly the AWA and AWC neurons, which play a crucial role in chemosensory behavior (Xue *et al*., 2025). Likewise in the present study, we used two treatment scenarios for conducting chemotaxis assay, one in which the petriplates had OP-50 food source and the other in which the food source of OP-50 was replaced by M-13 buffer. In both case scenarios, worms were introduced to a combination of volatile odorants along with DIA that led the worm to a state of SA. Resuscitation and chemical foraging were assessed after the respective time points of 3 and 6 hrs to understand the chemosensory behavior of the worms in short- and long-term exposure of the DIA.

In the plate with OP-50 food source, all the three strains of C. elegans, showed different responses to the three chemical odorants namely, IAA, PZ and DIA.

For Chemotaxis responses to IAA, CI of N2 worms was zero (0), reflecting a neutral response of the worm to the odorant. For CX2205 worms, with a mutation in odr-3 gene, olfactory driven chemotaxis behavior is altered since the odr-3 gene mutation affects the G-protein coupled sensing behavior of volatile odorants. In our study we found that CX2205 strain showed a positive CI index for IAA which acts like an attractant. IAA being a ketone is known to cause a positive CI index for CX2205 strain for concentrations upto 500mg/l as also shown by other researchers in their study (Qin *et al*., 2025; Worthy *et al*., 2018). Similarly, after 6hrs of SA, the CI index of CX2205, is further reflective of a positive attractant behavior of the worm towards IAA. For NL5901 strain, IAA chemo sensitivity response both at 3hrs and 6 hrs study was that of repulsion, with a negative CI index. Not much has been reported about the interaction of NL5901, with IAA, although, IAA has been reported to affect the longevity of the NL5901 model of Parkinson's disease in the worm. This CI index of IAA response in NL5901 was same at 3 and 6 hrs of study design.

For Chemotaxis responses to PZ, CI of N2 worms was negative, reflecting a repellant response of the worm to the odorant. For CX2205 and NL5901 worms, both strains showed a negative CI index for PZ at 3hrs and 6hrs of study. PZ is known to cause a positive CI index in N2 strain (Dror Cohen *et al*., 2019), although in our study we did not observe the same. Similarly for DIA, our study showed that all the three strains of the worm showed a repulsion to DIA with a negative CI

index, which could have been because of the presence of OP-50 in the plates. Normally DIA is reported as a chemo attractant in chemotaxis studies with the N2 strain of *C. elegans*.

In the plate with M-13 buffer, all the three strains of C. elegans, showed different responses to the three chemical odorants namely, IAA, PZ and DIA.

We observed that in the absence of OP-50 food, in the M-13 buffer, N2 strain does not show much alterations in its chemical preferences for IAA, PZ and DIA both at 3 and 6 hrs of the study. In CX2205 strain CI index for IAA is more positive and IAA is a strong attractant for both 3 and 6 hrs of the study. However, we observe that in the NL5901 strain, IAA acts as an attractant at both time points of the study. IAA is known to have a fruity smell like OP-50, which could cause this shift to occur specifically in the NL5901 strain.

Taken together, the results of our study reiterate that IAA has a neutral effect on N2 strain, attractant effect on CX2205 and the NL5901 strain in M-13 buffer plates. PZ and DIA both act majorly as repellents in our study for all the three strains with and without the OP-50 food source.

Conclusions

Our findings suggest that under the influence of the DIA, *C. elegans* show varying behaviors towards different odorants. After SA of 3 and 6 hrs, both mutant strain CX2205 and NL5901, naturally gravitates towards IAA odor akin to the fruity smell of OP50 as their first preference, in the M-13 plate which could be due to the worm's adaptation to their altered environment and behavior in response to IAA.

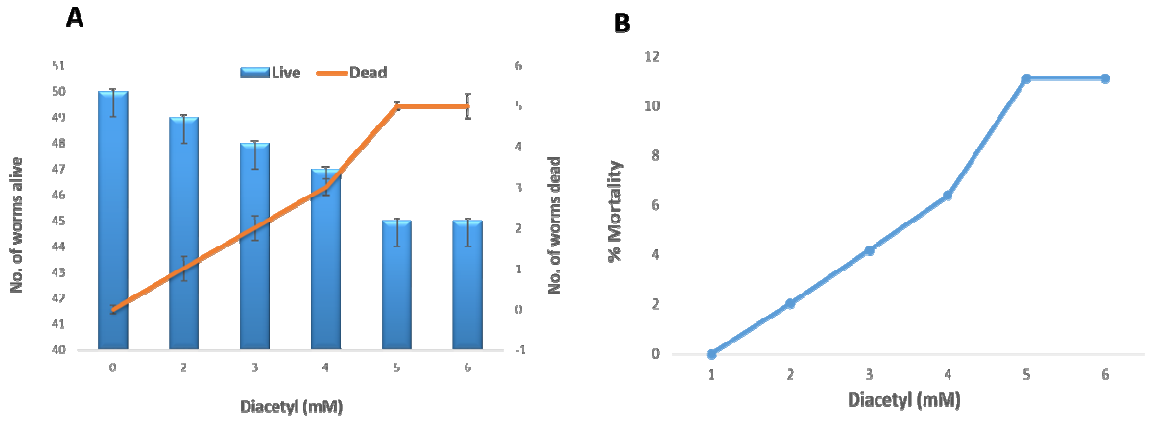

Fig. 1 : Live & dead assay for standardization of diacetyl dosing in *C. elegans*
A: No. of live and dead worms in different treatment groups **B:** Percentage mortality in different treatment groups
Values represented are Mean ± S.E (n=50).

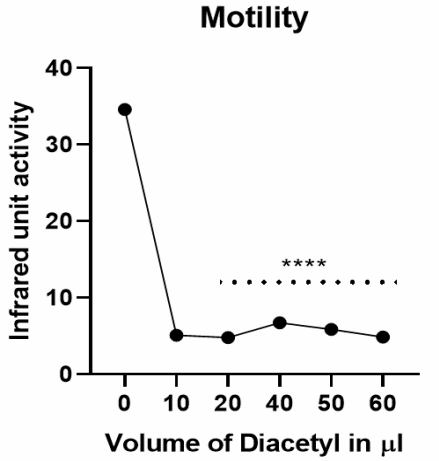

Fig. 2 : Evaluation of the mobility responses in *C. elegans* as quantified by the Arena Tracker
Mobility assay was conducted by detecting the changes in the worm's positional movement through interruptions of the LED micro-beam.
Values represented are Mean ± S.E (n=50). Data analysed by 1 way ANOVA followed by post-hoc analysis (Tukey Multiple Comparison Test). **:** *** significant different as compared to control ($p<0.0001$).

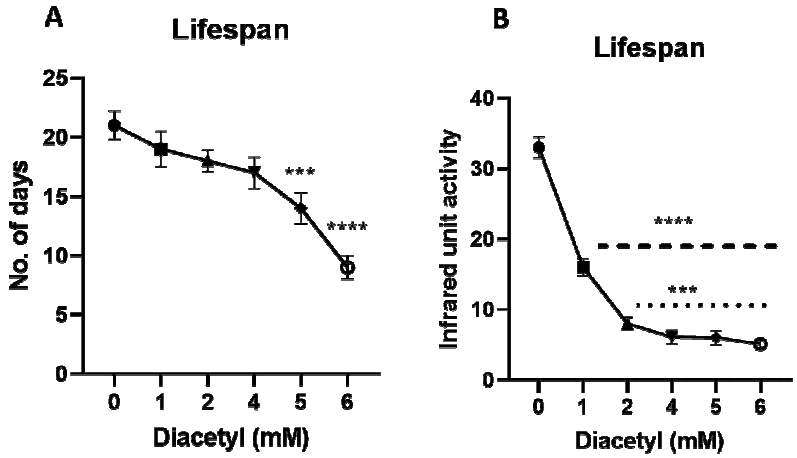

Fig. 3 : Evaluation of lifespan in *C. elegans* on exposure to varying concentrations of diacetyl
A: Lifespan estimation on the basis of number of day's survival **B:** Lifespan estimation on the basis of infrared unit activity
Values represented are Mean ± S.E (n=50). Data analysed by 1 way ANOVA followed by post-hoc analysis (Tukey Multiple Comparison Test). **:** **** significant different as compared to control *** significant different as compared to 1mM concentration of diacetyl ($p<0.0001$).

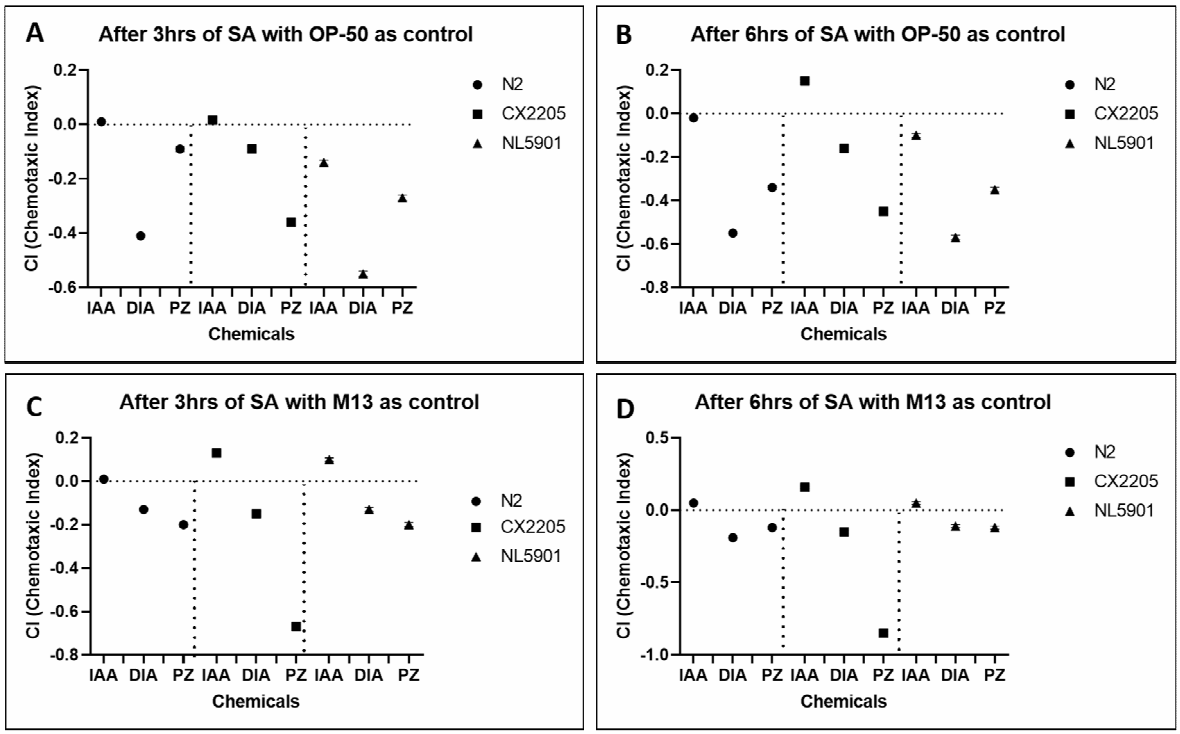

Fig. 4 : Evaluation of the Chemotaxis response to different volatile chemicals after recovery from diacetyl-induced suspended animation in *C. elegans*
A: Chemotaxis response after 3hrs of suspended animation with OP-50 as control
B: Chemotaxis response after 6hrs of suspended animation with OP-50 as control
C: Chemotaxis response after 3hrs of suspended animation with M-13 buffer as control
D: Chemotaxis response after 6hrs of suspended animation with M-13 buffer as control
Values represented are Mean ± S.E (n=100). Data analysed by 1 way ANOVA followed by post-hoc analysis (Tukey Multiple Comparison Test). **:** *** significant observed between groups ($p<0.0001$).

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