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Research report

A diacetyl-induced quiescence in young *Caenorhabditis elegans*

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ABSTRACT

Many organisms enter quiescence in response to adverse environmental factors. Here, we show that L1 stage *C. elegans* entered a quiescent state after 3 hours exposure to diacetyl in which movement and growth stopped for hours to days after odorant removal. Entry into quiescence was dependent on neurons affected by the *osm-3* mutation, and by AWA neurons. Conversely, AWB/AWC neurons, the guanylyl cyclase ODR-1, and the TRPV-channel subunit OCR-2 inhibited entry into L1 arrest. This quiescent behavior represents an alternative use of olfactory signaling pathways besides approach or avoidance, and is a novel model in which to characterize genes implicated in quiescence.

1. Introduction

Quiescence is a period in an organism's life cycle when metabolic activity is minimized to conserve energy under adverse environmental conditions. *C. elegans* can enter an alternative larval stage called dauer for up to several months in response to overcrowding or starvation [8], and newly hatched larval 1 (L1) worms enter diapause in response to starvation [10,11]. Under anoxic conditions, *C. elegans* can enter a state of suspended animation characterized by cessation of observable movement [18]. All of these quiescent states have specific triggers–adverse environmental conditions such as starvation, overcrowding or anoxia–and exposure to a better environment induces further development.

C. elegans modifies its behavior in response to diverse environmental cues. For example, many volatile organic compounds elicit approach or avoidance responses upon first exposure, whereas long-term exposure to such compounds leads to behavioral plasticity including adaptation [5,6] and conditioned learning [17]. One such compound, diacetyl (2,3-butanedione), is a byproduct of bacterial fermentation [7,25] and may signal the presence of food. Diacetyl activates the ODR-10 receptor, which is expressed in AWA amphid sensory neurons [23,29], and signals through the ODR-3 Gα protein, which activates downstream targets including the OCR-2/OSM-9 TRPV channel required for diacetyl chemotaxis [5,28].

Here, we report a novel quiescent state in L1 worms exposed to diacetyl in the presence of food. Dauer larvae, worms in starvation-induced L1 arrest, and those in adult embryonic arrest can move in response to touch [1,2,8,10,11], whereas those in diacetyl-induced arrest, like worms in anoxia-induced suspended animation [18], did not move in response to prodding. We examined the time course over which *C. elegans* entered and exited diacetyl-induced L1 arrest, and aimed to identify neurons and signaling molecules that controlled entry into the arrest. Diacetyl-induced quiescence provides a model that could enhance our understanding of quiescent states resulting from novel environmental insults.

2. Materials and methods

Nematode strains were obtained from the *Caenorhabditis elegans* Genetics Centre at the University of Minnesota. The Bristol N2 strain served as the wild-type control in all mutant experiments. Please refer to Supplemental Materials and Methods.

2.1. L1 Synchronization

To obtain a highly synchronized population of worms, we used a bleaching protocol [26]. A description and rationale can be found in Supplemental Materials and Methods.

2.2. Diacetyl-induced Quiescence Assay

Synchronized L1 worms were placed on food plates for 4 hours, to which 0-10 μl diacetyl was added. After three hours of diacetyl exposure, worms were washed off the plates, put on new food plates, and examined daily for recovery. Details can be found in Supplemental Materials and Methods.

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2.3. Chemotaxis Assay

Standard 1-hour chemotaxis assays were performed as previously described [3,17]. See Supplemental Materials and Methods for details.

2.4. Brood Size Count

To check if worms were still fertile after leaving diacetyl-induced arrest, their ability to lay eggs was monitored. See Supplemental Materials and Methods for details.

2.5. Statistical Analysis

A commercial software program (Prism 5.0b) was used for all statistical analyses. $P < 0.05$ (two-tailed) was considered significant. All values are expressed as mean ± SEM. See Supplemental Materials and Methods for details.

3. Results

3.1. Diacetyl induced L1 quiescence

Exposure to 4-10 μl of diacetyl induced quiescence in L1 worms (Fig. 1A). Further experiments used a diacetyl exposure time of three hours, since at this time point the proportion of worms in arrest stabilized (Figure 1A, Two-way repeated-measures ANOVA, volume X time $F_{40,192} = 19.68$, $P < 0.05$; Bonferroni-corrected t-tests between 120, 150, 180, 210 and 240 minute time points, $P > 0.05$ at all diacetyl volumes). Once movement stopped, worms were unresponsive to prodding and pharyngeal pumping stopped (our unpublished observations). Our second experiment examined recovery from diacetyl-induced L1 quiescence. More worms entered quiescence and fewer worms recovered with increasing diacetyl volumes (Fig. 1B). The majority of arrested L1 worms recover during Day 1 after diacetyl exposure; at the 4 μl volume, a significant fraction of worms recovered during Day 2 after exposure (Fig. 1B, $n = 62$ plates; Day 0: 61 ± 2%, Day 1: 9 ± 2%, Day 2: 3 ± 1; One-way repeated measures ANOVA on 4 μl $F_{2,124} = 660$, $P < 0.05$; Bonferroni-corrected t-tests, Day 0 vs. 1, $t_{61} = 29.4$, $P < 0.05$; Day 1 vs. 2, $t_{61} = 3.8$, $P < 0.05$).

Worms exposed to 4, 6 or 8 μl of diacetyl recover at similar rates as evidenced by parallel recovery curves (Fig. 1B). What differs between the 4 vs. 6 and 8 μl–treated worms is that at higher volumes, many worms enter a state from which they do not recover. We observed worms for two weeks following diacetyl exposure, and negligible numbers of worms recovered after Day 3 following diacetyl exposure; worms that did not recover were likely dead since they were immobile for two weeks. Thus, the diacetyl-induced state consists of two distinct processes. The first is a diacetyl-specific quiescence from which worms recover; this is the dominant process after treatment with 4 μl diacetyl. The second is a non-specific effect that appears to kill the worms, and is observed in many worms after treatment with 6–10 μl diacetyl. Since the specific effect was of more interest, we investigated the effect of a 3-hour exposure to 4 μl diacetyl for subsequent mutant analyses.

To test whether fertility or behavior was altered after diacetyl treatment, we assayed chemotaxis to diacetyl and brood size as adults. Although egg laying was delayed for 17-24 hours in those exposed to diacetyl compared to those not exposed to diacetyl (our unpublished observations), this time period corresponds to the approximate time in arrest. Neither brood size nor diacetyl approach differed between adult worms that were exposed for 3 hours to 8 μl diacetyl in the L1 stage vs. untreated adults (Fig. 1C, $n = 5$ plates. Student's t-test, $t_8 = 0.13$, $P > 0.05$; Fig. 1D, $n = 13$ plates (treated), $n = 14$ plates (untreated), Student's t-test, $t_{25} = 1.1$, $P > 0.05$). This suggests that diacetyl treatment did not change the worms' behavior or fertility once development was restarted. Unarrested L1 worms on Day 0 become adults on Day 2. It was therefore expected that if arrested L1 worms started recovering on Day 1, they would become adults on Day 3. Many single, isolated, L1 diacetyl arrested worms became adults on Day 3, suggesting that development was halted in the L1 stage after 3-hour diacetyl treatment.

3.2. Diacetyl-induced quiescence is odor-specific

Since different odors are sensed by different receptors and neurons [3,5,23,27], we examined the odor specificity of the arrest by exposing N2 L1 worms to 8 μl of benzaldehyde, butanone, isoamyl alcohol, pyrazine, or 1-nonanol. We chose an 8 μl exposure volume to increase the probability that an arrest phenotype induced by other odorants would be observable. Pyrazine is an attractant sensed by AWA neurons [4], whereas benzaldehyde, butanone, and isoamyl alcohol are attractants detected by AWC neurons [4]. 1-nonanol is a repellent sensed by ASH neurons [4]. None of these odorants drove L1 worms into quiescence (Fig. 1E, $n = 3$ plates, One-way ANOVA, $F_{4,10} = 235.4$, $P < 0.05$; post-hoc Dunnett's test, $P < 0.05$). Since benzaldehyde immobilized a small proportion of worms, we exposed worms to 20, 40 or 80 μl benzaldehyde, which immobilized 44%, 62% and 92% of worms, respectively. Recovery after benzaldehyde exposure was modest at best; 20% of immobilized worms exposed to 20 μl of benzaldehyde recovered by three days after treatment; negligible proportions of worms recovered at higher exposure volumes. Since virtually all worms recovered after 4 μl diacetyl exposure, which arrested 44% of worms (Fig. 1B), benzaldehyde-induced immobility likely represents toxicity and not behavioral quiescence.

Butanone is structurally similar to diacetyl; if it induced quiescence, this would suggest that quiescence occurs in response to chemicals that are structurally related. However, no N2 L1 worms entered a quiescent state when exposed to 80 μl butanone (our unpublished observations), suggesting that L1 arrest occurred specifically in response to diacetyl.

3.3. Diacetyl induces a reversible arrest phenotype selectively in L1 worms

To determine if the arrest was developmentally specific, *C. elegans* in L2, L3, L4, and adult stages were exposed to 8 μl diacetyl. 24%, 24%, 2%, and 0.5% of L2, L3, L4, and adult worms, respectively, became immobile compared to 96% of L1 worms (Fig. 1F, $n = 5$ plates, One-way ANOVA, $F_{4,25} = 44.7$, $P < 0.05$; Dunnett's test, $P < 0.05$.). To test the possibility that animals in developmental stages other than L1 could arrest after exposure to higher volumes of undiluted diacetyl, we exposed L3 worms to 20 μl, 40 μl, and 80 μl diacetyl. They stopped moving in a dose dependent manner (60%, 88%, and 95%, respectively) but, unlike L1 worms, L3 worms did not recover from this state by three days after diacetyl treatment (data not shown), suggesting that diacetyl only induces quiescence in L1 stage worms.

3.4. Diacetyl-induced L1 quiescence is mediated through sensory neurons

C. elegans detects environmental changes using immotile cilia located on sensory neurons. We tested the effect of two ciliary mutants, *osm-6* and *osm-3*, on entry into arrest. These two mutants had opposite effects, suggesting that different subsets of sensory neurons may inhibit or promote entry into diacetyl-induced arrest (see Supplemental Results and Supplemental Figure 1 for details).

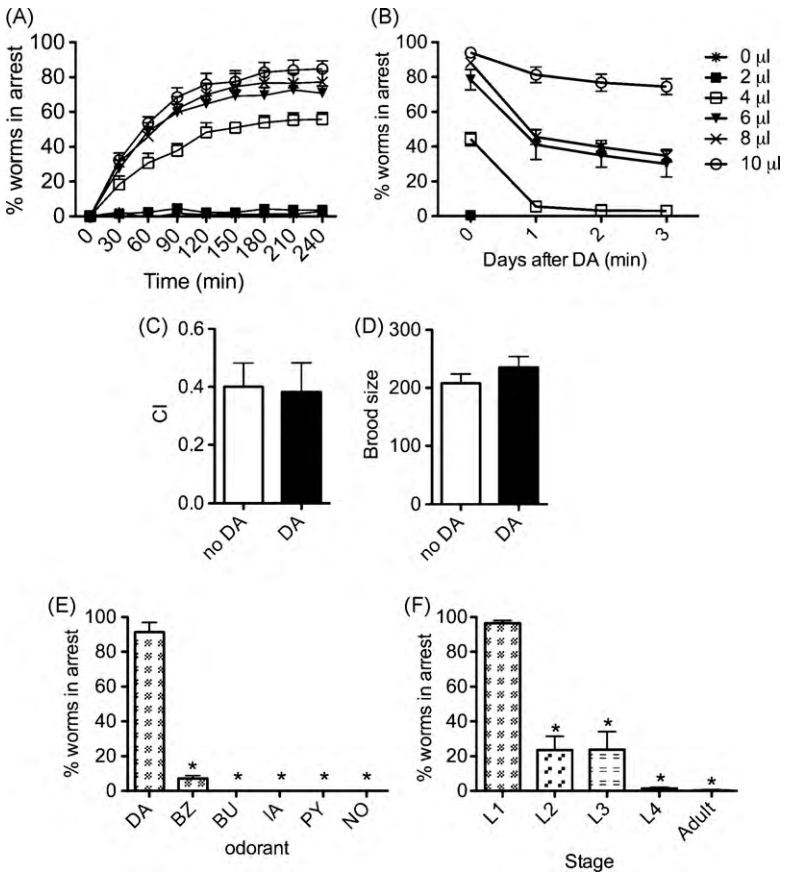

Fig. 1. A novel diacetyl-induced quiescence in *C. elegans*. A: L1 N2 worms were exposed to 0–10 µl of undiluted diacetyl for 240 minutes. The proportion of worms in arrest leveled off between 120 and 240 minutes ($n = 5$ plates/volume). B: L1 N2 worms were exposed to 2–10 µl of undiluted diacetyl for 3 hours ($n = 5$ plates), and the number of worms exiting diacetyl-induced arrest was quantified every 24 hours for three days. With increasing volume, the fraction of worms entering the arrest increased and the fraction of worms recovering from the arrest decreased. C: Worms arrested in the diacetyl-induced arrest that recovered (DA) were compared to untreated worms (no DA) for 0.01% diacetyl chemotaxis. There was no significant difference between the two groups ($n = 5$ plates/group). D: The brood size of worms that recovered from diacetyl-induced arrest ($n = 13$) vs. untreated worms ($n = 14$) did not differ significantly. E: Entry into diacetyl-induced arrest is odorant-specific, since only worms exposed to diacetyl (DA) and not to benzaldehyde (BZ), butanone (BU), isoamyl alcohol (IA), 1-nonanol (NO), or pyrazine (PY, 50 mg/ml) entered arrest ($n = 3$ plates/odorant). F: Entry into diacetyl-induced arrest is larval stage-specific, since a significantly larger proportion of L1 worms went into arrest than any other developmental stage ($n = 5$ plates). * indicates $P < 0.05$.

3.5. AWA neurons promote entry into quiescence via the ODR-10 receptor and the ODR-3 Gα protein

The *osm-3* mutant revealed that a specific subset of neurons can promote entry into diacetyl-induced L1 arrest (Supplemental Figure 1B). Since a significant proportion of *osm-3* worms still entered arrest, another mechanism must also promote entry. Since diacetyl is sensed by AWA neurons, which are unaffected by the *osm-3* mutation [3,22], we tested the *odr-7(ky4)* mutant, which lacks a transcription factor required to specify AWA neurons [13,22]. Fewer *osm-7* than N2 worms entered quiescence (Fig. 2A, $n = 8$ plates/genotype, two-way repeated measures ANOVA genotype X time $F_{2,14} = 6.3$, $P < 0.05$; Bonferroni-corrected t-tests, Day 0, $t_{14} = 3.9$, $P < 0.05$; Day 1, $t_{14} = 0.11$, $P > 0.05$; Day 2, $t_{14} = 0.40$, $P > 0.05$), suggesting that AWA neurons promote entry into diacetyl-induced arrest.

Diacetyl is a ligand of the ODR-10 seven-transmembrane G protein-coupled receptor [23,29]. The *odr-10(ky38)* mutant lacks functional ODR-10 receptors, and fewer *odr-10* worms entered quiescence (Fig. 3B, $n = 7$ plates/genotype, two-way repeated measures ANOVA genotype X time, $F_{2,12} = 121.6$, $P < 0.05$; Bonferroni-corrected t-tests, Day 0, $t_{12} = 5.3$, $P < 0.05$; Day 1, $t_{12} = 0.017$, $P > 0.05$; Day 2, $t_{12} = 0.26$, $P > 0.05$). The ODR-3 Gα protein acts downstream of the ODR-10 receptor [3,14], and the *odr-3(n2150)* mutant is defective in diacetyl chemotaxis [20,23]; as with *odr-7* and *odr-10* mutants, fewer *odr-3* worms entered arrest vs. N2 (Fig. 2C, $n = 8$ plates/group, two-way repeated measures ANOVA genotype X time, $F_{2,14} = 26.6$, $P < 0.05$; Bonferroni-corrected t-tests, Day 0, $t_{14} = 7.6$, $P < 0.05$; Day 1, $t_{14} = 0.18$, $P > 0.05$; Day 2, $t_{14} = 1.2$, $P > 0.05$), suggesting that ODR-10 signaling through the ODR-3 Gα protein promotes entry into diacetyl-induced arrest.

Fig. 4.

3.6. OCR-2 inhibits entry into diacetyl-induced L1 quiescence

Downstream of the ODR-3 protein is a TRPV-related channel composed of two subunits: OSM-9 and OCR-2 [5,24,28]. Compared to N2 worms, more *ocr-2* mutants entered quiescence, and fewer recovered, whereas *osm-9* mutants showed no significant difference. See Supplemental Results and Supplemental Figure 2 for details.

3.7. The ODR-1 guanylyl cyclase inhibits entry into quiescence

We examined the effect of entry into quiescence of the *odr-1* mutant, which lacks the guanylyl cyclase ODR-1. This protein is expressed in AWB and AWC chemosensory neurons. Significantly more L1 *odr-1* mutants than wild type worms entered quiescence (Fig. 3A, $n = 7$ plates/group, two-way repeated measures ANOVA genotype X time, $F_{2,12} = 2.6$, $P < 0.05$; two-way repeated measures ANOVA genotype, $F_{1,12} = 8.7$, $P < 0.05$). To determine if AWB and AWC olfactory neurons inhibit entry into quiescence, we exam-

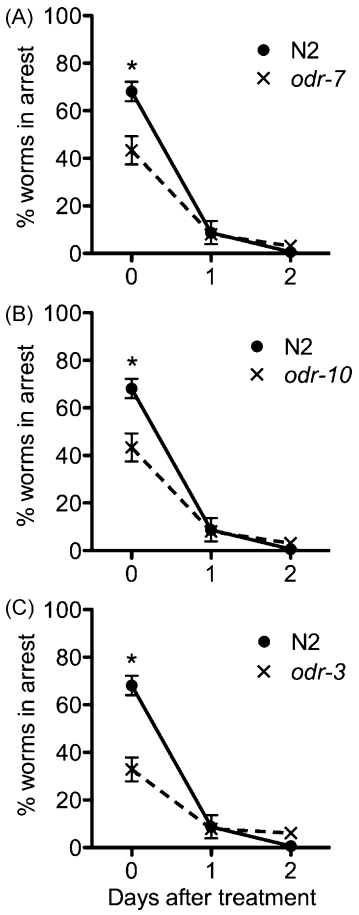

Fig. 2. AWA neurons promote entry into diacetyl-induced quiescence through ODR-10 receptor and ODR-3 Gα protein signaling. A: Fewer *odr-7* (A), *odr-10* (B) and *odr-3* (C) mutant worms entered the arrest than N2 worms, suggesting that entry into arrest is promoted through ODR-10 signaling in AWA neurons.

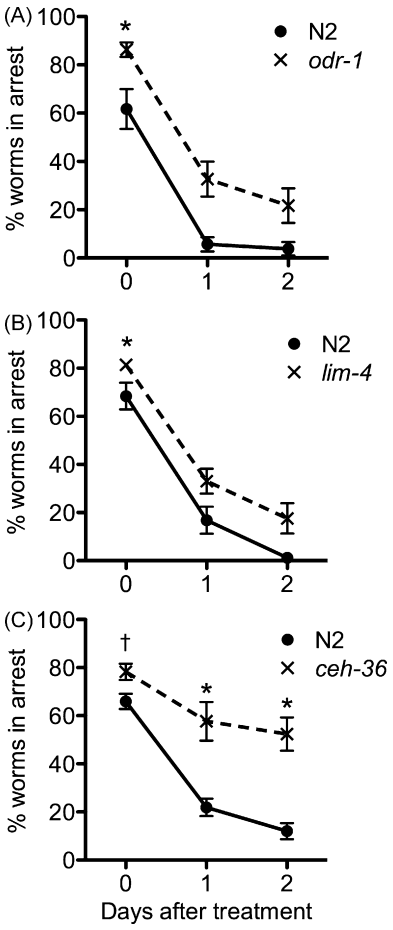

Fig. 3. ODR-1 protein signaling, and ODR-1 expressing neurons AWB and AWC inhibit entry into diacetyl-induced quiescence. A: Significantly more *odr-1* vs. N2 worms entered arrest. B: Likewise, more *lim-4* mutant worms, which lack functional AWB neurons, entered arrest. C: More *ceh-36* worms, which lack AWC neurons, tended to enter arrest than N2 worms ($P < 0.10$). * indicates $P < 0.05$, † indicates $0.05 < P < 0.10$; $n = 7$–8 plates/group.

ined the *lim-4* mutant, which lacks AWB neurons [21,27] and the *ceh-36* mutant, which lacks a transcription factor required for specification of the AWC neurons [12]. Significantly more L1 *lim-4(ky403)* mutants entered arrest (Fig. 3B, $n = 7$–8 plates/group, two-way repeated measures ANOVA genotype X time interaction, $F_{2,12} = 0.088$, $P > 0.05$; two-way repeated measures ANOVA genotype factor, $F_{2,12} = 10.9$, $P < 0.05$) whereas *ceh-36* mutants tended to do the same (Fig. 3C, $n = 8$ plates/group, two-way repeated measures ANOVA time X genotype, $F_{2,14} = 16.86$, $P < 0.05$; Bonferroni-corrected t-tests at each time point, $P = 0.057$ (time = 0), $P < 0.05$ (time = 1 or 2)).

4. Discussion

Organisms that can enter a quiescent state do so in response to cues that indicate adverse environmental conditions. The present report shows that when L1 stage worms are exposed to high volumes of undiluted diacetyl, they enter a quiescent state that is larval stage- and odorant-specific. This quiescent state differs phenotypically from dauer formation and starvation-induced L1 arrest, since Diacetyl-arrested L1 worms do not move even when provoked by touch whereas dauer larvae, and worms in L1 starvation-induced arrest can move while in the arrested state [2,8,10,11]. Superficially, diacetyl-induced arrest resembles paralysis. However, paralysis is usually accompanied by neuronal damage that prevents impulse conduction to muscle. Paralysis is a plausible explanation for the non-specific component of the diacetyl-induced arrest from which worms do not recover after exposure to ≥6 μl diacetyl. However,

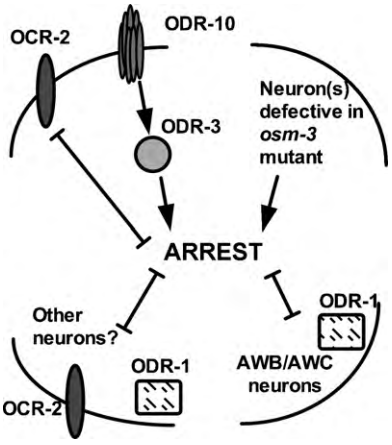

Fig. 4. Pathways involved in diacetyl-induced L1 quiescence entry. An ODR-10/ODR-3-dependent signaling pathway acting in AWA neurons promotes entry into arrest in parallel with neurons affected by the *osm-3* mutation. The AWB and AWC neurons inhibit entry into arrest. ODR-1 and OCR-2 signaling also inhibit entry into the arrest, possibly through AWB/C neurons and the AWA neuron respectively, although signaling through these proteins may also be important in other neurons. Pointed arrowheads indicate facilitation whereas flat arrowheads indicate inhibition of entry into arrest.

it is unlikely that worms that exit diacetyl-induced arrest have suffered neuronal damage, for three reasons. First, recovery from diacetyl-induced paralysis would indicate a reversal in this damage over a short time course. Second, diacetyl-induced arrest was ODR-10 signaling-dependent, suggesting that arrest is a specific effect of diacetyl rather than a toxic effect. Third, *C. elegans* can enter a state of suspended animation in which movement ceases [18], suggesting that cessation of movement can accompany quiescent states in *C. elegans*.

Two sets of hypotheses could explain entry into diacetyl-induced L1 arrest. First, diacetyl may permeate the cuticle, causing non-specific neuronal excitation or fatigue. This could explain why many worms do not exit diacetyl-induced quiescence after exposure to high volumes. However, it is unlikely that non-specific neuronal activation explains the phenomenon we observed using 4 μl of diacetyl, since two antagonistic mechanisms (*osm-3* vs. *osm-6*; Supplemental Figure 1) act in response to diacetyl to promote or inhibit the arrest. A second hypothesis is that diacetyl-induced arrest arises from specific processes. Indeed, two parallel processes, one controlled by a subset of the neurons defective in *osm-3* mutants and the other acting in the AWA neuron through activation of the ODR-10 receptor, promote entry into the diacetyl-induced L1 arrest. Likewise, OCR-2 and ODR-1 were shown to be important for inhibiting the arrest likely through different pathways. This evidence supports the second hypothesis - quiescence results from signaling events occurring in specific chemosensory neurons.

Fewer worms carrying mutations in *odr-1*, *ceh-36*, *lim-4* or *ocr-2* entered diacetyl-induced L1 quiescence vs. N2 worms. LIM-4 and CEH-36 are transcription factors required to specify AWB and AWC neuronal fates respectively [12,21,27], and ODR-1 is a guanylyl cyclase required for chemotaxis to AWC- and AWB-sensed odorants [12,16,21]. It seems unlikely that OCR-2 is working in the same pathway as these genes since OCR-2 is not expressed in neurons expressing the *odr-1* gene. Moreover, *ocr-2* has a much stronger phenotype than the other two mutations. While the *odr-1* and *lim-4* mutations affect entry into arrest, mutant recovery curves are parallel to the N2 curve suggesting that these mutants do not participate in recovery from arrest; the *ceh-36* mutation did affect exit from arrest, but to a lesser magnitude than the *ocr-2* mutation.

Once worms recovered from the diacetyl-induced quiescence, they appear to develop normally, since they produced normal brood sizes and showed normal chemotaxis towards diacetyl. By gross observation, adults exposed to diacetyl as L1 larvae were not discernible from those unexposed. These lines of evidence, combined with the delay of both egg laying and onset of adulthood in diacetyl-treated worms by approximately one day after non-arrested worms, suggest that diacetyl-induced quiescence may represent a developmental arrest.

Two functions of diacetyl provide clues to why it may induce quiescence. First, diacetyl is an antimicrobial agent at high concentrations [9,19]. Since *C. elegans* prefers live bacteria as a food source [15], high concentrations of diacetyl may signal a dead or dying bacterial food source. Second, diacetyl is a byproduct of bacterial fermentation [7,25]. It seems counterintuitive that *C. elegans* would enter quiescence in response to a signal associated with food. *C. elegans* uses oxidative phosphorylation to derive much of its energy, and ODR-10 can respond to pyruvate in addition to diacetyl [29], suggesting that the diacetyl receptor would be activated during hypoxia. Under anoxic conditions, *C. elegans* enters a state of suspended animation that resembles diacetyl-induced quiescence, since worms in both conditions are immotile and appear to cease development [18]. Further experiments investigating a possible relationship between anoxia-induced suspended animation and diacetyl-induced quiescence could speak further to this issue.

Diacetyl-induced developmental arrest gives some insight into roles of olfactory signaling in *C. elegans* outside of approach to or avoidance of odors. This arrest provides an excellent opportunity to study the *C. elegans* response to extreme environmental situations apart from starvation and to define the underlying chemosensory mechanisms.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2010.05.021.

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