Reliable odorant sensing but variable associative learning in *C. elegans*

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Main Text and Figure

Abstract
Animals can move towards or away from an odorant. Such chemotaxis has been used as a paradigm for learning when coupled with pre-exposure to the sensed odorant. Here we develop an assay for the nematode *C. elegans* that avoids the typical use of chemical or physical immobilization when measuring the response of worms to odorants. Using two sets of rectangular arenas that are oriented such that worms in one set must move in the opposite direction to worms in the other set for the same response, we found that unfed worms show reproducible movement towards the odorants butanone and benzaldehyde, and away from the odorant nonanone. In addition to the use of opposing orientations to control for gradients of unknown cues outside the arena, we introduce a measure of dispersal to control for locomotion defects and unknown cues within the arena. Since this assay avoids the use of paralytics or physical constraints, it is useful for the analysis of graded responses to a variety of chemicals and the discovery of underlying molecular mechanisms. Using this setup, we found that pre-exposure of unfed worms to butanone to induce an association of starvation with butanone resulted in different extents of such associative learning during different trials – from no learning to learned avoidance. Given this variation in associative learning despite the artificially controlled lab setting, we speculate that in dynamic natural environments such learning might be rare and highlight the challenge in discovering evolutionarily selected mechanisms that could underlie learning in the wild.

Main Text

The nematode *C. elegans* is expected to be exposed to a rich variety of odorants when growing on rotting vegetation in the wild [1]. Responses to individual odorants in the laboratory have been parsed using controlled conditions [2, 3, 4] and measurement of neuronal responses in physically constrained animals [5] suggest that single odorants can evoke changes in the activities of multiple neurons [6]. A normalized difference measure called chemotaxis index ([number near test odorant – number near vehicle control]/[number total]) is widely used in odorant choice assays (reviewed in [7]), but the odorants and vehicles are often combined with the use of paralytics (e.g., sodium azide [3]) to immobilize worms near either choice before counting. Using these conditions where initial accumulations are captured as choices by paralyzing the worms, both odorant sensing and associative learning paradigms have been developed. Since freely moving nematodes are unlikely to encounter joint gradients of odorants and paralytics in the wild, it is useful to develop an assay that avoids mixing of odorants with immobilizing agents and provides a good measure of the graded response to odorants.

Results & Discussion

To develop an assay that can measure the behavior of populations of freely moving *C. elegans*, we used rectangular arenas where the ~1-mm worms added to a central origin need to move a minimum of ~20 mm towards or away from an odorant by ~1h to contribute to a chemotaxis index. These criteria ensure that minor preference, chance accumulation, or preliminary exploration is not conflated with a clear response. All worms were prepared for the assay by selecting cohorts, growing them to the same stage (staging), and pre-exposing to vehicle or odorant without food (Fig. 1*A*). The impact of this uniform treatment on the mobility of the worms and the state of arenas before the assay were both evaluated by measuring the ability of the worms to disperse in arenas without any added odorants (Fig. 1*A*, *top*). To counter any unknown gradients that may be present in the laboratory, chemotaxis was measured using sets of arenas such that worms in one set must move in the opposite direction to worms in the other set for the same response (Fig. 1*A*, *bottom*). In arenas without any salient chemicals, worms are expected to disperse and occupy all sectors uniformly (q_1 to q_4 quadrants in Fig. 1*B*). Such uniform dispersal results in a calculated entropy [8] of ~2 ($-\sum_{i=1}^{n} q_i \, log_2 \, q_i$, where n = 4 and q_i are quadrants), which can be used as the measure of dispersal. If worms have a movement defect or are attracted to the origin, they will accumulate in q_2 and q_3, which will reduce the dispersal to ~1. If they are attracted or repulsed by an unknown cue in any one quadrant, the dispersal will be reduced to ~0. Thus, by using identically prepared worms, two sets of rectangular arenas oriented in opposite directions, and a measure of dispersal, this assay provides a well-controlled way to ascertain the response to added chemicals without the use of a paralytic while controlling for confounding variables, if any.

Using this assay, we examined odorants that worms have been reported to be attracted to (2-butanone [3] and benzaldehyde [3]) or repulsed by (2-nonanone [3]). The worms and arenas used in every assay showed a dispersal of ~2 in the absence of added odorants (Fig. 1*C*, *left*; e.g., Movie S1), which is the prerequisite for interpreting chemotaxis assays (Fig. 1*B*; e.g., Movie S2 showing response to nonanone). The responses to all three odorants were in agreement with prior assays and were reproducible when assayed on three different days (Fig. 1*C*, *right*). Specifically, worms were attracted to butanone (median chemotaxis index (CI) of 0.83 and median effect size (Cohen's *d*) of 4.0) and benzaldehyde (median CI of 0.58 and median effect size of 6.2) but repulsed by nonanone (median CI of -0.99 and median effect size of 5.7).

Despite their short lives and complex environments, *C. elegans* have been reported to be capable of associative learning (reviewed in [9]). An ~1 hr pre-exposure to 'attractive' odorants in the absence of food eliminates the attraction when tested using a subsequent assay (reviewed in [9]) and such learning, that presumably associates starvation with the odorant, was not observed if the pre-exposure was done in the presence of food [10]. Incorporating the pre-exposure to butanone with starvation into our assay resulted in worms moving away from butanone – an altered response that was reproduced in 6 subsequent trials (Fig. 1D; effect sizes ranging from ~4.1 to ~17). This apparent associative learning was eliminated when the pre-exposure was performed in the presence of food (CI after ethanol pre-exposure = 0.75 ± 0.12 and CI after butanone pre-exposure = 0.8 ± 0.18, resulting in a minimal effect size for associative learning = -0.3; also see Supplemental Dataset 1). However, in 7 subsequent trials the measured associative learning, if any, varied widely (effect sizes from -2.4, indicating increased attraction to butanone, to 3.6, indicating decreased attraction to butanone). The previously detected learned butanone avoidance could not be reproduced despite varying agar (2 trials each using 2 new sources), researcher (3 trials by one and 4 by another), worms (4 trials using a 2nd isolate), and *E. coli* (4 trials using a 2nd isolate). In contrast, the initial response to odorant sensation remained reproducible.

Interpretation of observed responses was aided by requiring worms to move in opposite directions for the same response, thereby controlling for unknown cues in the lab, if any, and measuring dispersal in the arena, thereby controlling for locomotion defects and/or unknown cues within the arena, if any. While we can infer with confidence when effect sizes are large using this simple assay, the ways to control for confounding variables developed here can also be adapted for more elaborate arenas or workflows that aim to increase throughput (e.g., [11]). Our measurements of behavior without immobilization reveal that unlike the reproducible response upon sensing an odorant, the observed response after associative learning is variable and could require artificial environments that are not easily controlled. One possible explanation is that robust learning has been lost through many generations of growth in the laboratory [1]. Alternatively, in the dynamic natural environment of *C. elegans* where evolutionary forces sculpt molecular mechanisms, such learning could be rare.

Materials and Methods

Wild-type *C. elegans* were assayed for dispersal and chemotaxis using rectangular arenas without the use of any paralytic agents (see Supplementary Information for details).

Data, Materials, and Software Availability

All data generated and the code used are available at AntonyJose-Lab/Tasnim_et_al_2024 on GitHub.

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Figures

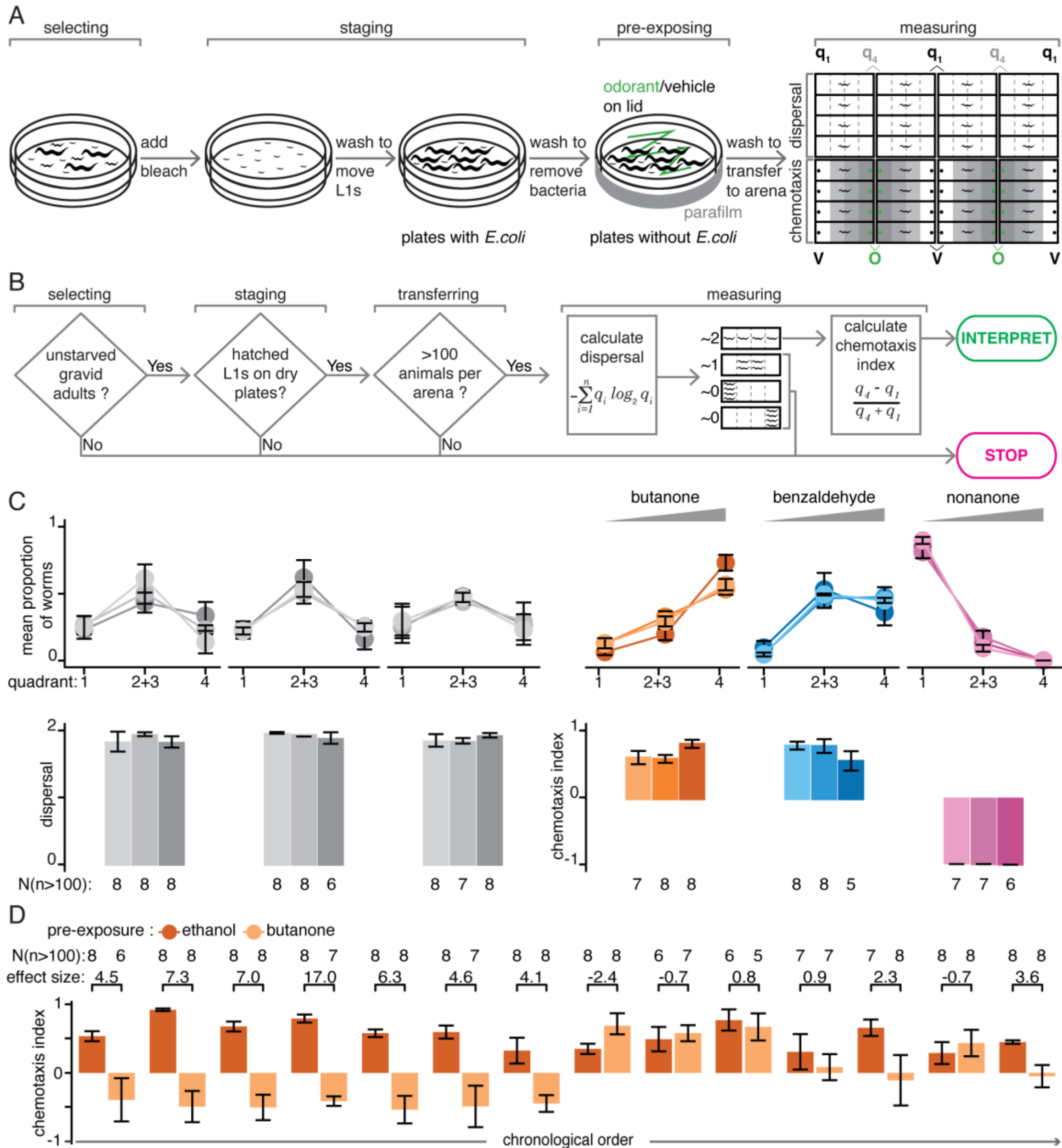

Fig. 1. Assay for measuring the response of freely moving *C. elegans* reveals reproducible odorant sensing but variable learning. *(A)* Procedure for preparing worms and measuring their response to a volatile odorant. Select plates with unstarved gravid adults for the addition of bleach to dissolve worms while preserving embryos. Move the hatched L1 worms by washing onto plates with *E. coli* OP50 and grow to young adulthood (~96 hrs after bleaching). Wash young adults to remove bacteria and move to plates without *E. coli* to pre-expose them with either the vehicle (e.g., ethanol; V) or the odorant (e.g., butanone; O). Transfer pre-exposed worms to the center of each rectangular arena to measure dispersal with no odorants (top) or chemotaxis towards an odorant (q_4) or the vehicle (q_1) (bottom). Count the number of worms in each quadrant (q_1 to q_4) of the arena using a video taken after 1h. *(B)* A decision chart for interpreting behavior. Results can be interpreted only if sufficient numbers of worms (>100 per plate) of comparable age (young adults) were assayed

and they dispersed uniformly in the absence of odorant (dispersal ~2). In the absence of added odorants, assays where the worms remain in the middle of the arena (dispersal ~1, resulting from attraction to center and/or defective movement) or accumulate at one quadrant (dispersal ~0, indicative of response to an unknown sensory gradient in the arena) cannot be interpreted. *(C)* Odorant sensing by wild-type worms is reproducible. Mean proportions of worms in each quadrant (*top*) and calculated dispersal (*left bottom*) or chemotaxis index (*right bottom*) for three repeats of the assay are shown. *(D)* Learned response to butanone by wild-type worms is variable. In the absence of food, worms pre-exposed to the vehicle ethanol consistently showed attraction to butanone, however worms pre-exposed to butanone showed variable responses (including aversion, reduced attraction, and increased attraction) when different cohorts of worms were tested over a period of ~2.5 years. Populations tested (N), numbers of worms in each population required for interpretation (n), effect sizes (Cohen's *d*) and 95% confidence intervals (error bars) are shown.

Supporting Information for

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This PDF file includes:

- Supporting text
- Legends for Movies S1 and S2
- SI References

Other supporting materials for this manuscript include the following:

- Movie S1
- Movie S2
- Supplemental Dataset 1 (available on GitHub)
- R code (available on GitHub)

Supporting Text

Materials and Methods

Worm growth and selection

The *C. elegans* wild type Bristol N2 was obtained from the Caenorhabditis Genetics Centre (University of Minnesota, Minneapolis, MN, USA). Worms were grown at 20ºC, and chemotaxis assays were performed at room temperature (~25ºC).

Growth, pre-exposure, and assay plates were prepared using appropriate volumes of Nematode Growth Medium (NGM): 4 ml per 35 mm plate [Falcon™ Bacteriological Petri Dishes with Lid, FisherScientific, cat#08-757-100A], 20 ml per 100 mm plate [Falcon® Petri Dishes, VWR, cat#25373-100] and 8 ml per assay plate [Nunc® Rectangular Dishes, VWR cat#73521-424]. NGM was prepared by combining 17 g bacteriological agar [VWR cat#97064-334 unknown lot for Fig. 1*C* butanone and benzaldehyde assays and for Fig. 1*D* assays 1 to 7, VWR cat#97064-334 lot#23K0156549 for Fig. 1*D* assays 8 to 10, Carolina® cat#842130 for Fig. 1*D* assays 11 and 12, VWR cat#97064-334 lot#24G2356953 for Fig. 1*C* nonanone assays and Fig.1*D* assays 13 and 14], 2.5 g bacto-peptone [Gibco™, Thermo Fisher Scientific cat#211677 unknown lot for Fig. 1*C* butanone and benzaldehyde assays and for Fig. 1*D* assays 1 to 11, Gibco™, Thermo Fisher Scientific cat#211677 lot#4024890 for Fig. 1*C* nonanone assays and Fig. 1*D* assays 12 to 14], and 3 g NaCl [Supelco®, VWR cat# EMD-SX0420-1] in 1 L water, autoclaving, cooling to 60ºC, then adding 1 ml each of cholesterol [Sigma-Aldrich cat# C8667-5G] (5mg/ml in ethanol), 1M $CaCl_2$ [MilliporeSigma™, FisherScientific cat#M1023820500] and 1M $MgSO_4$ [MilliporeSigma™, FisherScientific cat#MMX00751], and 25 ml of potassium phosphate buffer (pH 6.0) [made using K_2HPO_4 VWR Chemicals BDH® cat#BDH9266-500G and KH_2PO_4 VWR Chemicals BDH® cat#BDH9268-2.5KG]. The use of various sources of agar was inspired by anecdotal reports that behavior can vary between batches of agar (e.g., [1]).

Growth plates were seeded with overnight cultures of *E. coli* OP50 grown at 37ºC in Luria-Bertani (LB) broth (100 µl per 35 mm NGM plate and 500 µl per 100 mm NGM plate) and left at room temperature for ~48 hrs, by when a lawn forms. These seeded plates were stored for up to 4 weeks at 4ºC until use. Worm washes and transfers were done using M9 buffer prepared by adding 5 g NaCl [Supelco®, VWR cat#EMD-SX0420-1], 11.32 g dibasic Na_2HPO_4*$7H_2O$ [unknown for Fig. 1*C* and Fig. 1*D* assays 1 to 8, Spectrum Chemical, The Lab Depot cat#S1400-500GM-EA for Fig. 1*D* assays 9 to 14] and 3 g KH_2PO_4 [VWR Chemicals BDH® cat#BDH9268-2.5KG] to 1 L water, autoclaving, and cooling to ~60ºC before addition of 1 ml 1M $MgSO_4$ [MilliporeSigma™, FisherScientific cat#MMX00751]). A worm bleaching solution (a stock mix of 45ml 5M NaOH [Supelco®, MilliporeSigma cat#SX0590-3], 15ml Clorox® bleach (8.25% NaOCl) and 90ml water aliquots diluted to 50% before use) was used to dissolve worms while leaving the embryos protected by the eggshells. Subsequent hatching resulted in populations of L1-staged animals that were then moved to seeded plates and grown to adulthood before their behavior was assayed.

Staging

Plates were set up on day 1 by washing unstarved but a crowded 35 mm plate of worms with 1 ml M9 and transferring 10 µl of the suspension onto 12 OP50-seeded 35 mm plates. These plates were grown for 36-48 hrs at 20ºC until large numbers of gravid adults were observed, then worms were bleached by dropwise addition of 250-300 µl of worm bleaching solution, ensuring that all worms were covered using the least volume of bleaching solution. Bleached plates were kept at 20ºC for 24 hrs until the surviving eggs hatched, then L1 worms were pooled from 2 plates by washing with 500 µl M9 and transferred onto a seeded 100 mm plate. These worms were then allowed to grow for an additional 72 hrs (total 96 hrs post-bleach) to get a synchronized cohort of young adult worms which were then pre-exposed and tested for their response to volatile odorant(s). Six 100mm plates of young adult worms were sufficient to pre-expose with one odorant (or vehicle) and to measure both dispersal and chemotaxis in 8 arenas each.

Pre-exposure

M9 buffer (5-6 ml) was used to pool worms from six 100 mm plates into 1.5 ml microcentrifuge tubes and washed a total of 3 times by adding 1 ml of M9 and centrifuging at 11,000 rpm for 2 min. This use of centrifugation is different from the gravity-aided settling that was used in some assays for associative learning [2] (we estimate gravity-aided settling until the solution above the worm 'pellet' is clear to take >7 min.). However, centrifugation was consistently used in all trials, including the 9 repeats that showed learning with an effect size >2 (Fig. 1*D*). At the end of the third wash, the supernatant was discarded, retaining 200 µl of liquid with worms and one such tube of worms was used per 100 mm plate during pre-exposure. A P1000 pipette with was used to transfer worms onto 100 mm plates with (for one experiment) or without *E. coli* OP50 food, taking care not to transfer any bacterial pellets and pipetting from the top of the worm "pellet" so as not to transfer any worm carcasses from the bottom of the tube. Ethanol [Sigma Aldrich, cat#E7023-500ML] was used as a vehicle and fresh odorant dilutions (10% 2-butanone [Sigma Aldrich, cat#360473-500ML], 20% benzaldehyde [Sigma Aldrich, cat#418099-100ML] or 10% 2-nonanone [Sigma Aldrich, cat#W278505-SAMPLE-K]) were made on the day of the behavioral assay in 1.5ml microcentrifuge tubes. 5 µl of either the vehicle or the diluted odorant was streaked onto the lid of the plate and the covered plate was then sealed with parafilm. Plates were kept undisturbed at room temperature for 1h, then pre-exposed worms were collected and washed three times using M9 buffer. At the end of the third wash the supernatant was discarded, retaining 200µl of liquid with worms.

Chemotaxis and dispersal assays

Arenas were set up as shown in Figure 1*A* (right) and for each test (dispersal or chemotaxis), 2 sets of 4 rectangular arenas were used per pre-exposure treatment (N=8). A template was used to trace quadrants onto extra lids of plates and these lids were placed under each set of 4 arenas to enable transfer of worms to the origins to initiate each assay. A P20 pipette with the tip cut off to increase the bore was used to transfer 10 µl of worms from the top of the worm "pellet" onto the center (origin) of each rectangular plate, minimizing transfer of worm carcasses or remnant bacteria from the bottom of the tube. Typically, one tube of pre-exposed worms yielded enough for testing >100 worms per plate on 4 sets of 4 rectangular arenas (N=16). After transferring worms, 2.5 µl of the odorant in vehicle or the vehicle alone was pipetted at either end of the chemotaxis plates in opposite ends such that the worms in one set of arenas must move in the opposite direction to worms in the other set for the same response. With this orientation, if worms were responding to a gradient of an unknown cue outside the arena, then they will move in the same direction in both arenas, thereby reducing effect size in response to the odorant within the arena. The dispersal plates did not have any odorant added to them (see Figure 1*A*). The transfer of worms and odorants/vehicle typically took a total of 5 min per 4 set of 4 rectangular arenas (N=16). The plates were left with the lids on at room temperature for 1h. At the end of 1h, videos were taken of the plates keeping the extra lid underneath to allow visualization of the quadrants, and the videos were used to count the numbers of young adult worms in each quadrant [see Movie S1 & S2]. The video was taken using an iPhone positioned over the objective using an adapter and typically took 5 min per 4 set of 4 rectangular arenas (N=16).

Measures and Inference

The proportions of worms in each sector or quadrant (q_i with n total) was used to calculate a measure of dispersal that is based on Shannon's entropy [3]:

$$\text{dispersal} = -\sum_{i=1}^{n} q_i \, log_2 \, q_i$$

Chemotaxis towards or away from a volatile odorant was calculated (similar to [4]) by considering proportions of worms in the quadrant with the odorant in vehicle (q_4) and the quadrant with the vehicle only (q_1) using the formula

$$\text{chemotaxis index} = \frac{q_4 - q_1}{q_4 + q_1}$$

Chemotaxis indices could range from +1 (interpreted as attraction towards the odorant) to -1 interpreted as aversion).

Dispersal and chemotaxis index are both necessary for inference. For example, two experiments using 100 worms could result in the same calculated chemotaxis index (= 0.8, say) either when a few worms are in the extreme quadrants ($q_4 = 9, q_3 = 45, q_2 = 45, q_1 = 1$) or many worms are in the extreme quadrants ($q_4 = 45, q_3 = 35, q_2 = 15, q_1 = 5$). The first case ($q_4 = 9, q_1 = 1$) could arise because of a locomotion defect or an attraction to the origin, which would both be revealed by a dispersal <2 and can be used to raise caution in the interpretation of some mutant strains (e.g., *tax-4 osm-9* mutants in Fig. 2*A* of [5]).

Effect sizes were measured using Cohen's *d* [6], which not only provides a measure of differences in the means (e.g., as indicated in [5]) but also accounts for the variance. This relative measure is calculated using the formula

$$\text{effect size} = \frac{mean_{test} - mean_{control}}{\sqrt{\frac{sd_{test}^2 + sd_{control}^2}{2}}}$$

Two experiments with the same difference in mean, but different standard deviations will be appropriately reported as having different effect sizes by this measure. For example, for $mean_{test} = 0.8$ and $mean_{control} = 0$, the effect size = 8 when $sd_{test} = sd_{control} = 0.1$ but the effect size = 1.6 when $sd_{test} = sd_{control} = 0.5$. In theory, effect sizes can be influenced by unknown odorants or other cues that are present in the arena before the test odorant is added. For example, the extent of the aversion to nonanone could be modulated by the presence of other attractive cues in the arena even if such unknown cues are uniformly distributed.

Supplemental Movie Legends

Movie S1. Representative video panning through 2 sets of 4 arenas with a marked lid underneath the arena at the end of a dispersal assay.

Movie S2. Representative video panning through 2 sets of 4 arenas with a marked lid underneath the arena at the end of an assay for the chemotaxis response to nonanone.

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