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Short communication

Apparatus for investigating the reactions of soft-bodied invertebrates to controlled humidity gradients

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HIGHLIGHTS

- New experimental set-up for studying behavioral responses to humidity gradient.
- Novel assay design is applicable for soft-bodied invertebrate animals.
- Tunable humidity gradient allows characterization of naturalistic humidity responses.
- Simple, low-cost design is amenable for research labs or K-12 classrooms.

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ABSTRACT

Background: While many studies have assayed behavioral responses of animals to chemical, temperature and light gradients, fewer studies have assayed how animals respond to humidity gradients. Our novel humidity chamber has allowed us to study the neuromolecular basis of humidity sensation in the nematode *Caenorhabditis elegans* (Russell et al., 2014).
New method: We describe an easy-to-construct, low-cost humidity chamber to assay the behavior of small animals, including soft-bodied invertebrates, in controlled humidity gradients.
Results: We show that our humidity-chamber design is amenable to soft-bodied invertebrates and can produce reliable gradients ranging 0.3–8% RH/cm across a 9-cm long × 7.5-cm wide gel-covered arena.
Comparison with existing method(s): Previous humidity chambers relied on circulating dry and moist air to produce a steep humidity gradient in a small arena (e.g. Sayeed and Benzer, 1996). To remove the confound of moving air that may elicit mechanical responses independent of humidity responses, our chamber controlled the humidity gradient using reservoirs of hygroscopic materials. Additionally, to better observe the behavioral mechanisms for humidity responses, our chamber provided a larger arena. Although similar chambers have been described previously, these approaches were not suitable for soft-bodied invertebrates or for easy imaging of behavior because they required that animals move across wire or fabric mesh.
Conclusion: The general applicability of our humidity chamber overcomes limitations of previous designs and opens the door to observe the behavioral responses of soft-bodied invertebrates, including genetically powerful *C. elegans* and *Drosophila* larvae.

1. Introduction

Moisture is essential for life. Therefore many animals display behavioral mechanisms to migrate toward their preferred moisture level (hygrotaxis) (Gunn, 1937; Thomson, 1938; Bursell and Ewer, 1950; Warburg, 1964; Reshetnikov, 1996; Yu et al., 2010; Russell et al., 2014). These behaviors are critical to keep an animal within its niche and regulate essential processes like growth and reproduction. Humidity is one of the most fundamental environmental factors determining the distribution of species (Andrewartha and Birch, 1954). Although the neuro-molecular underpinnings of many sensory modalities have progressed a great deal in the past 30 years, how animals sense and orient to humidity remains enigmatic (Montell, 2008). One proven approach to reveal potentially conserved molecular bases for sensory perception

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is by studying the invertebrate model organisms *Caenorhabditis elegans* and *Drosophila*. Although some progress has been made using adult flies (Sayeed and Benzer, 1996; Liu et al., 2007), it has been difficult to assay how Drosophila larvae respond to moisture gradients without confounding sensory stimuli from food and gravity (Johnson and Carder, 2012). Moreover, humidity responses had not been conducted with *C. elegans*, perhaps because they quickly desiccate upon removal from a moist substrate.

The need for investigating the humidity responses of soft-bodied invertebrates arose with our observations that *C. elegans* avoids puddles. Although this behavior suggested their ability to sense humidity levels, it was not reproducible enough gain formal understanding of the genes and neurons essential for the response. Controlled humidity gradients using hygroscopic materials have been described in previous literature, however, none were amenable for use with *C. elegans* because they required the animals to move across a wire or fabric mesh (Supplemental Fig. 1A and 1B) (Gunn and Kennedy, 1936; Barlow and Nicholls, 1961). More recent studies determining the genetic basis for humidity sensation of *Drosophila* were conducted utilizing a binary choice-chamber apparatus (Supplemental Fig. 1C) (Sayeed and Benzer, 1996; Liu et al., 2007). Their experimental design relied on circulating dry and moist air to produce very steep humidity gradients. Because *C. elegans* reacts to air currents, we were wary of this approach due to its potentially confounding stimuli. Additionally, we were interested in observing animals under more naturalistic humidity gradients in order to characterize physiologically meaningful behavior. Here we describe the construction and implementation of our simple, low-cost experimental set-up for characterizing humidity responses in the soil nematode *C. elegans* (Supplemental Fig. 1D). We also illustrate its potential for broader use with other soft-bodied invertebrates as both a research and teaching tool.

2. Materials and methods

2.1. Culturing of C. elegans for hygrotaxis assays

Wild-type and mutant *C. elegans* are raised on agar plates seeded with an OP50 strain bacterial lawn for food as described (Brenner, 1974). *C. elegans* displayed the strongest humidity preference when they were starved (Russell et al., 2014). Therefore we learned to qualify the degree of starvation by observing the condition of the worms and the bacterial lawn. Worms may be rinsed off of seeded plates onto unseeded plates to control the starvation conditions – 12–18 h works well. We found, however, that we could achieve similar results by more conveniently allowing the worms to eat all of the bacteria on their original culture plate. We found that a plate was optimal for testing this way when the lawn was completely devoured. This was evident by the absence of trails left by the worms in the bacterial lawn when viewed by oblique illumination. In either case, starved plates were optimum if they still contained plenty of unhatched eggs. If left to starve to the point where all of the eggs hatched, the hatched larvae would display L1-stage larval arrest and the adult worms would appear unhealthy and perform poorly in our assay.

2.2. Humidity chamber

In brief, the assay consists of a polymerized methyl methacrylate (Lucite) chamber with troughs on each end which hold substances to control humidity (Supplemental Fig. 2). Steep humidity gradients (10%–90% RH over 9 cm) can be generated using a desiccant such as calcium sulfate (Drierite) and water poured in opposite toughs. Shallow humidity gradients can be produced with the same chamber using different concentration aqueous NaOH solutions (Supplemental Fig. 3). The behavioral field that the worms are placed on consists of a semi-desiccated agarose gel placed on a glass plate (Fig. 1). This allows the worms to be illuminated from below facilitating observation via steromicroscope. Once the worms are introduced on the agarose field, the chamber is sealed with plastic wrap to establish the humidity gradient. The response of the worms to the humidity gradient is observed through the plastic wrap. We quantified humidity preference by counting the number of worms that reach the dry and humid sides of the chamber at 60 min. The humidity gradient forms within 10 min and is maintained for over 90 min (see Section 2.3 below), however, so other time-points could be recorded to accommodate the performance of slower worms. Once the behavioral assay is complete, the chambers should be cleaned with distilled water or a dilute (70%) ethanol solution and reused.

2.3. Quantifying humidity gradient

To quantify the humidity gradient within the apparatus we utilized the colorimetric moisture indicator cobalt (II) chloride. Each molecule of the indicator absorbs five water molecules. Each water bond shifts the indicator's color from bright blue to magenta. We uniformly infused cobalt (II) chloride powder into blotter paper to generate re-useable humidity measurement strips. The water was driven out of the strip by microwaving until it was bright blue. Dried strips were kept in an air-tight container with desiccant until use.

Because we were interested in the humidity gradient that animals experience across the behavioral field, we placed the humidity strip across the entire length of the field on the glass substrate without agarose. Then we placed desiccant and water respectively in the two troughs and enclosed the apparatus in plastic wrap allowing the humidity gradient to form. We then photographed the indicator strip under full-spectrum lighting with a digital SLR camera at different time intervals beginning at 10 min. To quantify the moisture absorption along the length of the strip, we extracted the red and blue values along the length of the photo of the strip via Image J. The ratio of the red and blue values at the respective points along the strip were then plotted. This revealed a mostly linear gradient of moisture absorption across the indicator strip. Photographic analysis of the humidity strip at multiple time points suggested that a linear humidity gradient persisted from 10 min through 90 min.

3. Results and discussion

3.1. Constructing chamber and preparation of assay components

3.1.1. Assemble chamber and glass substrate (Supplemental Video 1)

The humidity chamber was constructed out of inexpensive and widely obtainable polymerized methyl methacrylate (Lucite). A sheet of Lucite was cut into rectangular pieces according to specifications outlined in diagram (Supplemental Fig. 2). The individual pieces were then secured together with a clear, thin solvent cement (Weld-On #4, Compton, CA). Once the chambers are assembled we tested them for water-tightness by filling with water. Small leaks were filled through treating with a low viscosity polymer (Rain-X, Houston, TX). The chambers were allowed to air out for 72 h prior to assay worms.

Tempered glass plates, to be used as a substrate, were cut 0.5 mm more narrow than the inside chamber dimensions (~74.5 mm). This allowed the glass to remain tightly positioned in the chamber through placing strips of Parafilm (West Chester, PA) on the inside of one chamber wall and securing the glass in place through friction just prior to the assay. Tempered glass should be used since the glass plates will be repeatedly exposed to hot molten agar.