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Martina Iapichino, Yen Wen Wang, Savannah Gentry, Anne Pringle, Agnese Seminara. A precise relationship among Buller's drop, ballistospore and gill morphologies enables maximum packing of spores within gilled mushrooms.. 2020. hal-03011381

HAL Id: hal-03011381

<https://hal.science/hal-03011381>

Preprint submitted on 18 Nov 2020

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1 **SHORT TITLE: Maximum spore packing within mushrooms**

2 **A precise relationship among Buller’s drop, ballistospore, and gill morphologies** 3 **enables maximum packing of spores within gilled mushrooms**

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10 **ABSTRACT**

11 Basidiomycete fungi eject basidiospores using a surface tension catapult. A fluid
12 drop forms at the base of each spore and, after reaching a critical size, coalesces
13 with the spore and launches it from the gill surface. It has long been hypothesized
14 that basidiomycete fungi pack the maximum number of spores into a minimal
15 investment of biomass. Building on a nascent understanding of the physics
16 underpinning the surface tension catapult, we modeled a spore's trajectory away
17 from a basidium and demonstrated that, to achieve maximum packing, the size of
18 the fluid drop, the size of the spore, and the distance between gills must be finely
19 coordinated. To compare the model to data, we measured spore and gill
20 morphologies from wild mushrooms and compared measurements to the model.
21 The empirical data suggest that in order to pack the maximum number of spores
22 into the least amount of biomass, the size of Buller’s drop should be smaller but
23 comparable to the spore size. Previously published data of Buller’s drop and spore
24 sizes support our hypothesis and also suggest a linear scaling between spore

radius and Buller's drop radius. Morphological features of the surface tension catapult appear tightly regulated to enable maximum packing of spores. If mushrooms are maximally packed and Buller's drop radii scale linearly with spore radii, we predict that intergill distance should be proportional to spore radius to the power $3/2$.

Keywords: ballistospory; fungi; morphometrics ; biomechanics

INTRODUCTION

Molds, yeasts, and mushrooms are ubiquitous across Earth. Estimates of the number of fungal species range from 1 to more than 5 million (Blackwell 2011), and fungi in ecosystems function as decomposers, mutualists and pathogens. Emerging fungal diseases endanger crops as well as wild plants and animals, threatening food security, but fungal diseases also alter forest dynamics and contribute to the extinction of animals. Losses cost millions of US dollars in damage (Pennisi 2010; Fisher et al 2012; Kupferschmidt 2012).

Most fungal bodies (mycelia) are immobile, typically hidden within substrates. Fungi use spores to reproduce and travel away from a natal habitat. Spores are carried in air currents away from a source and when a spore lands in a favorable environment, it germinates and begins or extends the life cycle. Basidiomycota are among the most common fungi, encompassing pathogens like the honey mushroom as well as charismatic mushrooms like matsutake and the fly agaric. The phylum is defined by the production of sexually derived spores (basidiospores) on a basidium. Basidiospores are launched via a surface tension catapult. Among species of mushroom-forming fungi, e.g. agarics and boletes, spores typically form in groups of four from basidia arranged along the gills or pores of a mushroom, each spore attached to a sterigma. A drop of

liquid, known as Buller's drop, forms extracellularly at the base of each spore when water condenses on the hilar appendix of a spore. Buller's drop then collapses onto another adaxial drop formed along the longitudinal axis of the spore itself (FIG. 1). Upon coalescence, surface energy is converted into kinetic energy. The spore is ejected horizontally away from the basidium and sterigma. The spore decelerates to rest after a few milliseconds and then falls vertically between two gills or within the pore.

Ballistospore discharge was first observed by Schmitz (1843). In the 20th century, Buller (1909) described the phenomenon in more detail, observing the discharge of the spore and describing both the formation of the drop at the hilar appendix and the subsequent launch of the spore together with the drop. The drop is now referred to as "Buller's drop" and the discharge understood as a "surface tension catapult". We also use the phrase "spore-drop complex" to mean the entire spore discharge complex, including the adaxial drop, Buller's drop and the spore. Progress in understanding the anatomy and physics of the surface tension catapult was enabled by the development of cameras. Webster et al. (1984) provided photographic evidence of Buller's drop forming at the hilar appendix just before discharge and proposed a two-phase mechanism for spore ejection: the first phase involved Buller's drop enveloping the spore surface, acquiring momentum; the second entailed the sharing of momentum and movement of the center of mass of the spore-drop complex, a result of rapid wetting. Subsequent works modeled the conversion of surface energy into kinetic energy with different degrees of complexity and imaged ballistospore launch with progressively faster cameras (Pringle et al 2005; Noblin et al 2009; Stolze-Rybczynski et al 2009; Fischer et al 2010b; Liu et al 2017). Pringle et al. (2005) observed coalescence while Noblin et al. (2009) described the process as encompassing four stages and estimated approximately half of the total surface energy was dissipated

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74 during launch. Recently, Liu et al. (2017) moved beyond considerations of energy
75 balance to generate simulations of the fluid dynamics within the Buller's drop and the
76 adaxial drop during coalescence and described experiments with biomimetic drops.
77 These authors found that coalescence occurs in a regime where viscous dissipation in
78 the Buller's drop is negligible. Hence, energy is not dissipated to set Buller's drop in
79 motion, instead, it may be dissipated to break the spore from the sterigma. In addition,
80 Liu et al. (2017) found that the phenomenon known in physics as “pinning” of the
81 contact line (de Gennes 1985) provides directionality for the spore-drop complex as it
82 ejects away from the originating gill.

83 It has long been hypothesized that mushrooms form gills to increase the surface
84 area for spore production and pack the maximum number of spores into a minimal
85 investment of biomass (Buller 1909; McKnight and Roundy 1991; Fischer and Money
86 2010). To achieve an optimal morphology, the size of Buller's drop, the size of the
87 spore, and the distance between gills must be finely coordinated. While spore size and
88 intergill distance may be under genetic control (Kues and Liu 2000), Buller's drop forms
89 extracellularly (Webster et al 1989). Whether and how fungi control the size of Buller's
90 drop remains unknown, although data reporting characteristic sizes of Buller's drop for
91 different species suggest individual species do control size (Pringle et al 2005; Stolze-
92 Rybczynski 2009; Stolze-Rybczynski et al 2009; Fischer et al 2010b).

93 To explore whether the morphologies of gilled mushrooms enable the maximum
94 packing of spores within tissues, we first revisit the theory relating ejection velocity and
95 flight time to the horizontal distance travelled by a spore from the moment of launch to
96 the moment it begins settling underneath the gills (Buller 1909; Pringle et al 2005;
97 Noblin et al 2009; Stolze-Rybczynski et al 2009; Fischer et al 2010b; Liu et al 2017).
98 Using energy balance we obtain the ejection speed and highlight its dependence on the

sizes and densities of the spore and Buller's drop. Combining expressions for ejection speed and flight time, we predict the distance travelled ballistically by the spore-drop complex before downward sedimentation starts. We then use our model to elucidate the criteria enabling maximum packing of spores. In the phase space made up of the three variables (i) drop radius, (ii) spore radius (defined as the radius of a sphere with the same volume as the spore) and (iii) intergill distance, the criterion for maximum packing is that spores must travel ballistically exactly midway between two facing gills. Given two of the three variables (i)-(iii), the model predicts the third, assuming maximum packing. To compare our model to empirical data, we collected mushrooms of eight different species and measured spore size and intergill distance. By placing these morphological data on the phase space generated from our model, we predict that for collected species, the radius of Buller's drop that maximizes spore packing ranges between 23% and 50% of the radius of the spore (depending on the precise value of spore density and efficiency of energy conversion). To validate the prediction, we revisit previously published data for an additional 13 species (Pringle et al 2005; Stolze-Rybczynski 2009; Stolze-Rybczynski et al 2009; Fischer et al. 2010b) and find Buller's drop scales as 32% of spore size, consistent with our prediction. These results suggest that Buller's drop radius scales linearly with spore radius, and combined with our model, generate a second prediction: to enable maximum packing, intergill distance should be proportional to spore radius to the power 3/2. In the aggregate our work synthesizes thinking about the morphologies of Buller's drop, spores, and gills, while providing insights into the principles shaping ballistospory.

MATERIALS AND METHODS

Spore ejection speed.—The reduction in surface energy following coalescence is $\sim \pi \gamma R_B^2$ where γ is surface tension of Buller's drop and R_B is Buller's drop radius. By

balancing surface energy to kinetic energy of the spore-drop complex $1/2(m_s + m_B)v_0^2$, we obtained:

$$v_0 = \sqrt{\frac{2\pi\gamma\alpha R_B^2}{m_s + m_B}} \quad (1)$$

where v_0 is the ejection velocity and m_s and m_B are the mass of the spore and of Buller's drop. Note that we have neglected viscous dissipation because ballistospory operates in a regime of low Ohnesorge number (Liu et al 2017). By simple algebra we can express v_0 in the following form $v_0 = U \sqrt{\frac{y^2}{y^3 + \beta}}$ where $U = \sqrt{3\alpha\gamma/(2\rho_B R_s)}$ is a velocity scale independent of Buller's drop radius; R_s is the radius of a sphere with the same volume as the spore, this is the "equivalent radius" of the spore, and we will call it "spore radius" for short; $y = R_B/R_s$ is the normalized Buller's drop radius, i.e. the ratio of Buller's drop to spore radii; ρ_B and ρ_s are densities of Buller's drop and spore respectively and $\beta = \rho_s/\rho_B$ is the ratio of Buller's drop to spore densities. The parameter α accounts for the fraction of available energy dissipated when the spore breaks apart from the hilum, the point of attachment between the spore and the sterigma (FIG. 1).

Relaxation time of the spore-drop complex.—The complex's relaxation time is determined by the air drag that causes rapid deceleration and is well approximated by the Stokes time (Stokes 1851; Fischer et al 2010a):

$$\tau = \frac{2R^2}{9\bar{\rho}v} \quad (2)$$

where ρ_a , ρ_s and ρ_B are the densities of the air, of the spore and of Buller's drop respectively; $\bar{\rho} = \rho_a(R_s^3 + R_B^3)/(\rho_s R_s^3 + \rho_B R_B^3)$ is the density of air divided by the density of the spore-drop complex; v is the kinematic viscosity of air and R is the radius of the spore-drop complex, which we consider as a sphere with volume equal to the sum

of the spore and drop volumes $R = (R_B^3 + R_s^3)^{\frac{1}{3}}$. When Buller's drop is considerably smaller than the spore which is often the case, $\bar{\beta} \sim \rho_a / \rho_s$. Following simple algebra we obtain the Stokes time of the spore-drop complex in the following non-dimensional form $\tau = T(y^3 + 1)^{\frac{2}{3}}$ where $T = \frac{2R_s^2}{9\nu\bar{\beta}}$, ν is the air kinematic.

Data collection and published data.—Between 15–17 Sep 2017 we collected mushrooms from the Upper Peninsula of Michigan. On 15 Oct 2017 we collected mushrooms from the University of Wisconsin-Madison Lakeshore Natural Preserve. We collected opportunistically, taking any mushroom that appeared in good shape, but focusing on gilled (not pored) fungi. We collected specimens of eight morphologically distinct species (TABLE 2).

We also retrieved published data from the literature; these data were generated from a different group of species (TABLE 3) and provide measurements of Buller's drops and spores, but do not provide information about intergill distances (Pringle et al 2005; Stolze-Rybczynski 2009; Stolze-Rybczynski et al 2009; Fischer et al 2010b). In this series of papers, the authors captured ballistospory from high speed video microscopy and obtained Buller's drop and spore size (but not intergill distance) from image analysis. We discarded the species for which measures of Buller's drop size was inferred indirectly. When spore volume was available, spore radius was calculated as $R_s = (\frac{3V_s}{4\pi})^{1/3}$. When spore volume was not available directly, we calculated it from spore length and width, assuming spores are prolate spheroids, $V_s = \frac{4}{3}\pi L_s W_s^2$. Values of spore densities range from 0.8 to 3.8 g/cm³ (Hussein et al 2013).

Data from the literature were used to estimate the efficiency parameter α as follows. For two species of basidiomycota (*Auricularia* and *Sporobolomyces*), for

170 which geometry of the spore and drop was observed directly, velocity of ejection was
171 73% and 68% of the theoretical maximum (Noblin et al. 2009), yielding a fraction of
172 usable energy $0.73^2 = 0.53$ and $0.68^2 = 0.46$, hence $\alpha = (50 \pm 5)\%$, consistent with
173 estimates presented in (Stolze-Rybczynski 2009), based on a different hypothesis about
174 the geometry of the adaxial drop.

175 ***Preparing specimens for morphometrics.***—On the same day mushrooms were
176 collected, a scalpel was used to separate caps from stems (FIG. 2A). Caps were left face
177 down for 8 to 12 hr on a piece of paper covered with aluminum foil in order to create
178 spore prints (FIG. 2B). Spore prints are generated when spores fall from gills and settle
179 directly underneath the cap. Spore prints reflect the morphology of each collected
180 specimen, and the location of stems and patterns of gill spacing are easily seen from a
181 spore print. To image spores, three small pieces of foil, each measuring approximately 1
182 mm $\times$ 1 mm, were cut (i) from close to each stem, (ii) equidistant between the stem and
183 the cap edge, and (iii) from near the edge of each cap. Spores were washed off each foil
184 piece and suspended in a 0.01%vol solution of Tween 80. 15 μ L of each spore
185 suspension were immediately spread onto a glass slide and spores imaged. Microscope
186 slides were sealed with nail polish to minimize evaporation of the Tween solution and
187 prevent the movement of spores during imaging. To measure distances between gills, a
188 photograph of each cap's underside, with a ruler included in the photograph, was taken
189 immediately after spore printing using a Canon EOS400D.

190 ***Identification of species using DNA barcoding.***— Tentative field identifications were
191 confirmed by sequencing the nuc rDNA ITS1-5.8S-ITS region (ITS barcode; Schoch et
192 al. 2012) of each mushroom. To generate DNA barcodes for each collected mushroom,
193 we extracted DNA with an NaOH extraction method modified from Wang et al (1993).
194 First, the tissues of each sporocarp were ground finely with a pestle in 40 μ L of 0.5 M

NaOH and the solution centrifuged at 13 000 rpm for 10 min. 5 μ L of supernatant were transferred to 495 μ L of 100 mM Tris-HCl (pH 8) and centrifuged at 13 000 rpm for another min. Next, the resulting supernatant was used as template for PCR. We amplified the ITS using primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) following PCR protocols outlined in White et al. (1990) modified for Lucigen's EconoTaq Plus Green 2x Master Mix (Lucigen Corp., Middleton, Wisconsin). Amplified products were cleaned and then Sanger sequenced by Functional Biosciences (Madison, Wisconsin). Sequences were deposited in GenBank under accession numbers MK829236-MK829244. Two specimens, one *Mycena* (MK829242) and one *Russula* (MK829243), could not be confidently identified to species despite their barcode data.

Microscopy and image analysis to measure spore geometry.—

Images of spores were taken using microscopes housed at the Newcomb Image Center at the University of Wisconsin-Madison. Spores were imaged either individually or in groups (FIG. 2C), depending on whether a particular microscope's field of view housed one or more than one spore, using Zeiss Elyra LSM 780 and Zeiss LSM 710 confocal microscopes. Spores were not stained as all collected species proved to be autofluorescent. The laser wavelength used to excite autofluorescence was 405 nm. The average area S and average radius of spores $R_s = \sqrt{S/\pi}$ of each species were then calculated from images of between 155–1180 spores using an image analysis tool implemented in ImageJ v.1.51. A single pixel's dimension in μm was calculated from the microscope and the images converted to greyscale (8-bit or 16-bit). ImageJ was then used to threshold each image and convert the greyscale to a binary image, highlighting all the spores to be counted and using the measurement of a single pixel to calculate the area of each spore as shown in FIG. 2C-D. Spores touching other spores were not

measured, nor were particles smaller than $2\ \mu\text{m}^2$. Particles bigger than $2\ \mu\text{m}^2$ were identified either as spores or not by eye.

Image analysis to measure intergill distances.—To measure distances between gills, we first identified the center of each cap by eye (FIG. 2E). We then drew between 6 and 10 circles (depending on the size of each specimen's cap) concentrically around the center of the cap (FIG. 2E). We then used ImageJ v1.51 to open each picture, set pixel length in mm using the image of the ruler included in each photograph, and convert images to greyscale (8-bit or 16-bit). The Oval Profile plugin was used to obtain greyscale profiles traced along each of the concentric circles drawn onto an image. Profiles were sampled at 3600 equally spaced points along each circle. Next, the area of each circle was measured to calculate its average distance from the cap center, and these measurements were later used to convert the distance between gills from radians to mm. The greyscale profile obtained from ImageJ along each circle was imported into Matlab R2017b (one example in FIG. 2F) and analyzed with the function Findpeaks. Peaks in the greyscale image identify the centers of gills, which appear white in greyscale images. Peaks that were closer than 0.3° were discarded as noise. We visually inspected data to confirm that minor peaks did correspond to gills. Finally, we quantified gill thickness as the width of the peak, defined as the distance where grey value drops half way below peak prominence, which is a measure of peak height. The distance between two gills, d , was defined as the distance between their centers minus the half-width of each of the two gills (see close up of two peaks in FIG. 2G).

RESULTS

Ejection speed.—We first focused on the velocity achieved by the surface tension

catapult, which depends on various physical parameters as well as on the dimensions of the spore and Buller's drop. Let us first recapitulate the physical processes that lead to spore launch. Buller's drop coalesces with the adaxial drop to power the surface tension catapult; both drops are made from condensed water vapor and appear following the secretion of hygroscopic substances by the fungus. When Buller's drop coalesces with the adaxial drop, the resulting reduction of surface area provides the surface energy to accelerate the spore. Because the adaxial drop is pinned to the surface of the spore, Buller's drop accelerates along the axis of the spore towards its distal tip. Once the moving drop reaches the tip of the spore, capillarity and contact line pinning decelerate water, and its momentum is transferred to the spore. Momentum transfer causes the force that breaks the contact between spore and sterigma, resulting in spore ejection away from the basidium.

In this physical process, surface energy is converted in kinetic energy and by using the physical principle of energy conservation we obtained equation (1) (MATERIALS and METHODS): this equation predicts there will be a radius of Buller's drop that maximizes v_0 (FIG. 3A). By zeroing the derivative in (1) we obtained the size of Buller's drop that maximizes ejection speed: $y_{max} = (2\beta)^{1/3}$ and when considering spores with densities once to twice the density of water (Hussein et al 2013), $\beta = 1$ to 2, this equation implied that at y_{max} Buller's drop radius is comparable to the equivalent radius of the spore $R_B \sim 1.26R_s$ to $1.59R_s$ (the gray shade in FIG. 3A marks all values of y_{max} , for β ranging from 1 to 2). Note that at y_{max} control of the ejection speed is robust, i.e. ejection speed becomes insensitive to small deviations from the exact value of Buller's drop size. Buller's drop is generally assumed to scale with spore length (Fischer et al. 2010b), and this scaling appears to hold for at least 13 species of basidiomycetes, as shown in Pringle et al. (2005), Stolze-Rybczynski (2009), Stolze-

Rybczynski et al. (2009), and Fischer et al. (2010b). FIG. 3B uses these published data to calculate spore radius R_s pointing to $y_{literature} = R_B/R_s \sim 0.32 \pm 0.08$ where we report the best fit through the data and $\pm$ the mean residual. $y_{literature}$ are represented in cyan on the horizontal axis in FIG. 3A. Note that only three out of their 13 species were gilled mushrooms (TABLE 3), but these three species lined up with the rest of the data and showed no clear departure from the rest of the collected data (FIG. 3B).

Ballistic range.—In order to understand how far a spore-drop complex travels after launch, we analyzed the timescale τ over which the spore-drop complex decelerates to rest, or relaxation time (see MATERIALS AND METHODS). We found that after discharge, spores travel horizontally a distance

$$x = v_0 \tau$$

with v_0 and τ from equations (1) and (2). Next, a spore stops abruptly and starts to sediment vertically, out from beneath a pair of facing gills, following a trajectory commonly known as a "sporabola" (FIG. 4A).

Maximum spore packing.—In order to successfully escape the mushroom, a spore must travel away from its basidium a distance x into the clear space between gills before settling downwards; it must travel far enough to avoid entrapment within the basidia and spores underneath it. If x is in fact dictated by this criterion that ensures spores escape the gills without sticking to tissue underneath, and assuming all gills are reproductive, then the distance between two facing gills, d , should be at least twice x , hence $d > 2x$. To pack as many spores as possible within a mushroom and avoid inefficient empty spaces, the distance between gills must be close to this minimum value:

$$d = 2x$$

where $x = v_0\tau$. Maximum packing was first suggested by Buller (1909), and this relationship appeared explicitly in more recent literature (Stolze-Rybczynski et al. 2009). Plugging in the values of v_0 and τ given by equations (1) and (2) we obtain:

$$\left(\frac{y_{pack}^2}{y_{pack}^3 + \beta}\right)^{1/2} (y_{pack}^3 + 1)^{2/3} = \frac{d}{2UT}$$

(3)

where U and T are the velocity and time scales derived in the previous two paragraphs and summarized in TABLE 1 and y_{pack} is the normalized Buller's drop radius, we add the suffix $_{pack}$ to stress that this prediction is valid at maximum packing. Equation (3) predicts the relationship between three variables: nondimensional Buller's drop radius y_{pack} , spore radius R_s (R_s is contained in the expressions for U and T summarized in TABLE 1) and intergill distance d - at maximum packing. Hence *given two of these three variables, equation (3) predicts the third*, assuming maximum packing and given the values of the parameters (spore density, energy conversion efficiency). For example, for any combination of spore radius and intergill distance, equation (3) predicts the optimal radius of Buller's drop that achieves maximum packing. We solve Equation (3) numerically and show the result for the normalized radius of Buller's drop, y_{pack} , in FIG. 4 color-coded from 0 (cyan) to 5 (black) for different combinations of intergill distances and spore radii using parameters listed in TABLE 1. At each point in the phase space defined by spore radius and intergill distance, the color represents the value of normalized Buller's drop radius that achieves maximum packing. Symbols and error bars represent our own data, described, analysed, and discussed below.

Data collection.— To compare our model to data, we measured spore and gill morphologies (see MATERIALS and METHODS) for eight wild mushroom species (TABLE 2). While spore size varied from species to species, spores within a single mushroom cap were considerably more similar and there were no consistent pattern of variation, e.g. spores were not larger nearer the center or edge of a cap) (FIG. 5). Moreover, the average intergill distance also remains constant with distance from the center of the cap. The phenomenon is driven by the appearance of secondary gills (lamellulae) towards the edge of a mushroom cap; as two gills with origins at the stipe diverge, often a secondary gill will appear. The appearance of secondary and tertiary gills keeps intergill distances constant. The only species in our dataset with variable intergill distances was an unidentified species of *Russula*, which did not produce secondary gills (Phillips 1991; Fischer and Money 2010). Among measured species, intergill distances varied from about 0.25 mm to 1.5 mm (FIG. 6), but there was no obvious correlation between intergill distances and the size of the mushroom cap.

To ensure maximum packing in real species, we predicted Buller's drop radius should be 23% to 50% of spore radius.—Here we used equation (3) and data about spore radius and intergill distance to predict Buller's drop radius at maximum packing. We computed average and standard deviation of spore radius and intergill distance across single individuals and placed these data on the phase space generated by our hypothesis for maximum spore packing (symbols and error bars in FIG. 4B). While most parameters of our model are known (TABLE 1), α and ρ_s are not. In order to understand how the model depended on parameters, we derived an approximate formula from equation (3), showing that in our model the optimal radius of Buller's drop depended only weakly on these free parameters. Indeed, when $y_{pack} < 1$, we could

expand equation (3) to leading order, i.e. neglecting the smallest terms in the equation.

In equation (3) we neglected $y_{pack}^3 \ll 1$ and $y_{pack}^3 \ll \beta$, to obtain:

$$R_B \sim d \sqrt{\frac{27\rho_a v^2 \rho_a}{8\alpha\rho_s R_s \gamma}} \quad (4)$$

This compact formula explicitly connected the radius of Buller's drop at maximum packing with the parameters of the problem. Particularly, it showed that Buller's drop radius at maximum packing depended on $(\alpha\rho_s)^{-1/2}$. For the range of variation reported in the literature (see TABLE 1), $\alpha\rho_s = 0.4 \text{ g/cm}^3$ to 1.9 g/cm^3 , and plugging these values as well as data collected in this study in our model, we predicted that at maximum packing $\langle y_{this \text{ study}} \rangle = 50\%$ to 23% . Importantly, the observed value of y from the literature is within this range of variation: $\langle y_{literature} \rangle \sim 32\%$, which supports the hypothesis of maximum packing. The value of the fitting parameters for which the predicted Buller's drop radius for maximum packing averaged over our collected data fits the observed value, $\langle y_{this \text{ study}} \rangle = \langle y_{literature} \rangle = 32\%$ is $\alpha\rho_s = 0.9 \text{ g/cm}^3$. For the eight species analyzed in this study, we found a standard deviation of 12%. A dataset of Buller's drop radius, spore radius, and intergill distance measured on the same specimen is needed to further confirm the hypothesis of maximum packing.

Scaling of Buller's drop radius with intergill distance.—Buller's drop is often assumed to scale *proportionally* with spore size (Fischer et al. 2010b), and published data shown in FIG. 3B corroborated this assumption (although note that only three out of 13 species in these published data correspond to gilled mushrooms). However, the assumption appears at odds with the prediction for maximum packing because equation (4) implies that Buller's drop radius scales linearly with intergill distance d and with the *inverse* square root of spore radius R_s . To resolve the paradox, intergill distance must increase with increasing spore radius in the following way:

$$d \sim 0.32 \sqrt{\frac{8\gamma\alpha\rho_s}{27\rho_a^2 v^2}} R_s^3$$

where we have simply plugged $R_B \sim 0.32R_s$ into equation (4). In order to convincingly prove or disprove this relationship, further data monitoring spore, drop, and gill morphologies, as well as spore density and ejection velocity, are needed.

DISCUSSION

The intricate morphologies of gilled mushrooms are hypothesized to maximize surface to volume ratios, an adaptation enabling the maximum packing of spores within a minimal investment of biomass. The hypothesis requires intergill distances to be exactly twice the horizontal range of an ejected spore: an ejected spore must both clear its natal gill and avoid lodging into the gill across from it, and assuming gills are crowded together as closely as possible (to efficiently use the space within a mushroom cap), the spore will be finely tuned to reach midway between facing gills. Intergill distances may be shaped by the reach of a spore, but because spore range is dictated by the dimension of Buller's drop and its density relative to the dimension and density of the spore, the three parameters – intergill distance, spore size, and Buller's drop size – emerge as highly interdependent in the context of maximum packing. We find that intergill distances and spore sizes from empirical observations populate a region of phase space where our model predicts the radius of Buller's drop enabling maximum spore packing ranges from 23% to 50% of spore radius. Previously published data (Pringle et al 2005; Stolze-Rybczynski 2009; Stolze-Rybczynski et al. 2009; Fischer et al. 2010b) suggest Buller's drop radius scales with spore dimensions as $R_B \sim 32\% R_s$ and support the hypothesis of maximum packing. A further prediction of our model is

that the linear scaling of Buller's drop and spore radii implies intergill distance must scale as $R_s^{3/2}$ within an optimally packed mushroom.

The fungi used in our analyses (FIG. 3A) do not operate at maximum ejection speed; the velocity of ejection for these spores remains well below the maximum. Whether other species operate at maximum ejection speed remains to be elucidated. This result may not be surprising because robust control of ejection speed may not contribute an obvious selective advantage. By contrast, the maximum packing of spores is expected to contribute significantly to the fitness of an individual.

More data are needed: our conclusions are based on a total of 21 species. However, the data collected to date are consistent with the hypothesis of maximum packing, confirming Buller's drop radius is likely to be finely tuned to control range and speed. How tuning evolves and the biomechanics underpinning ballistospory, a purely extracellular process operating in the context of fluctuating environments, remains a fascinating question for future research.

ACKNOWLEDGEMENTS

This work was supported by the Agence Nationale de la Recherche Investissements d'Avenir UCA^{JEDI} #ANR-15-IDEX-01, by the Centre National de la Recherche Scientifique Project international de cooperation scientifique (PICS) "2FORECAST", by the Thomas Jefferson Fund a program of FACE and by the Global Health Institute at the University of Wisconsin-Madison. Research at the Huron Mountains was supported by the Huron Mountain Wildlife Foundation. We would like also to thank Sarah Swanson for all her help and discussions about confocal microscopy.

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FIGURE LEGENDS and FOOTNOTES

Figure 1. Our current understanding of the surface tension catapult. Left to right: Spore and structure holding spore; Buller's drop forms and grows by condensation at the base of the spore (blue arrows). At the same time, the adaxial drop (visible to some extent in the central frame, and indicated by blue arrows) grows on the surface of the spore also by condensation (blue arrows). At a critical size, Buller's drop collapses onto the adaxial drop and reduces the total liquid surface, thus releasing energy. The released energy is converted into kinetic energy, catapulting the spore away from its parent. The size of Buller's drop, together with material parameters, determines the speed of spore discharge. Image adapted from (Webster et al. 1984).

Figure 2. Collection and analysis of gill spacing in wild mushrooms. A. An image of the underside of a mushroom cap collected fall 2017 in Michigan. B. Spore print obtained by placing the spore cap on half aluminum foil/ half paper overnight. C. Confocal microscope image of a sample of spores from the spore print. D. Segmentation of image, used to recover spore contours. E. Concentric circles around the center of the cap mark where intergill distances were measured and define the azimuthal angle θ , used to compute intergill distance. F. Gray scale values from image in E, as a function of azimuthal angle θ . Peaks correspond to gills (white in the image of E), while troughs correspond to the spaces between gills (dark or black in the image of E). To obtain intergill distance, we marked all peaks (note arrows) and kept track of their azimuthal angles. G. Close-up image showing locations of two peaks, marked by arrows 1 and 2. Intergill distance $\Delta\theta$ is defined in radians, as the peak-to-peak distance (difference in azimuthal angle of two adjacent peaks) minus the width of the gills themselves (width 1

and width 2 - see Materials and Methods). We calculated intergill distances in *mm* by multiplying $\Delta\theta$ for the radius of the circle (r_c in panel E).

Figure 3. A. Energy balance from eq (1) predicts discharge speed. We represent the normalized speed v_0/U as a function of y defined as the ratio of Buller's drop radius R_B divided by spore radius R_s (red line). The thickness of the red line represents predicted speed for β ranging from 1 to 2, where β is the ratio of spore to drop density. B. Linear scaling between spore radius and Buller's drop radius from data published in the literature (see TABLE 3). Spore radius is calculated as described in the main text.

Figure 4. Mushroom cap morphology and the maximum packing of spores. A. From left to right: Representative mushroom; detail of gill arrangement indicating sporobolae (trajectories of individual spores); magnified view of adjacent gills with basidia and basidiospores. Sporobolae are represented in faint gray in the center panel and black arrows in the right panel; adapted from Buller's drawing (Buller 1909). Maximum packing implies that spores initially travel a distance $x = v_0\tau$ to reach the midpoint between two facing gills separated by $d = 2v_0\tau$ with v_0 and τ given by equations (1) and (2). B. Prediction for normalized Buller's drop radius at maximum packing, y_{pack} , obtained by numerically solving Equation (3) with the parameters listed in Table 1. y_{pack} is color coded from 0 (cyan) to 4.4 (black); white corresponds to the value $\langle y_{literature} \rangle = 32\%$. Symbols correspond to data of intergill distances and spore radii measured from 8 species collected by us in Michigan and Wisconsin (see Figure 4). Each symbol is linked with a species as in Figure 5, 6 and Table 2. The optimal Buller's drop radius for the 8 collected species is $y_{pack} \sim 0.32 \pm 0.12$, where we report average $\pm$ standard deviation. The free parameters α and ρ_s are chosen within their range of natural variation. The prediction varies little across the range of variation of α and ρ_s .

522

523 **Figure 5.** Spore size does not vary across a single mushroom cap. Gray thin lines mark
524 measurements of spore radius taken at three different distances from cap stipes with
525 errorbar representing standard deviation; means and standard deviations for spore radius
526 at all distances are shown in dark red vertical lines. Horizontal red line guides the eye.

527

528 **Figure 6.** Average gill spacing varies little with distance from cap stipes, a result driven
529 by the appearance of secondary gills at greater distances from the cap stipe. Gray lines
530 show all measures at various distances from the center, vertical red lines show the
531 average and standard deviation. Horizontal red line guides the eye. The only exception
532 is *Russula* sp. which has no secondary gills.

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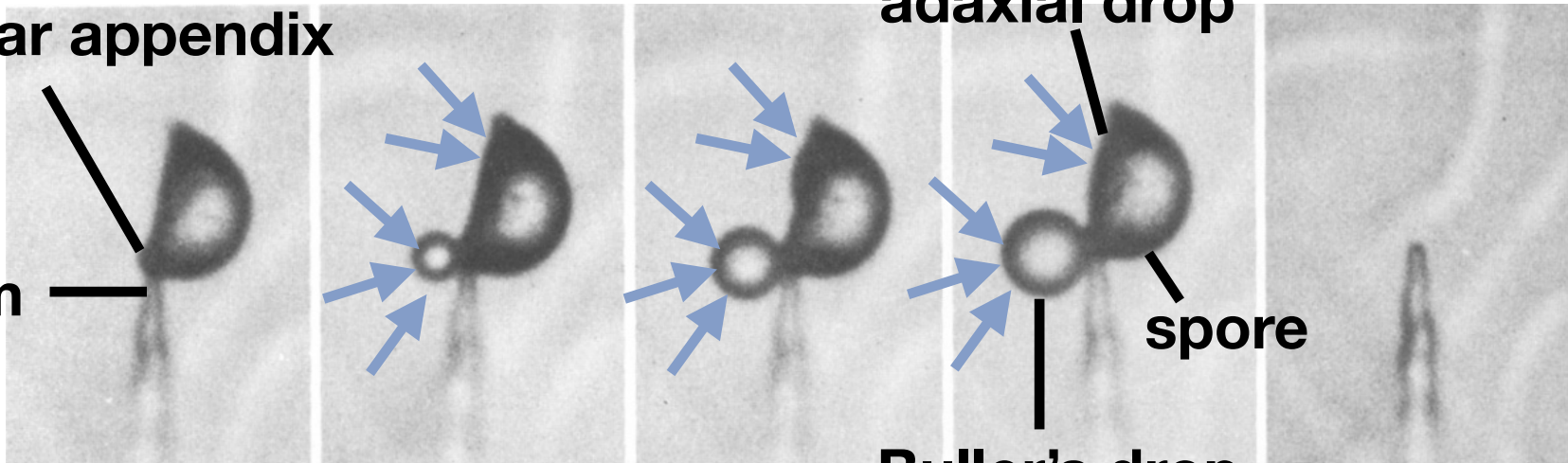
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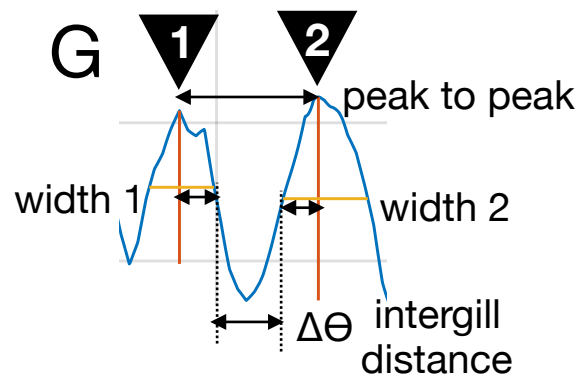
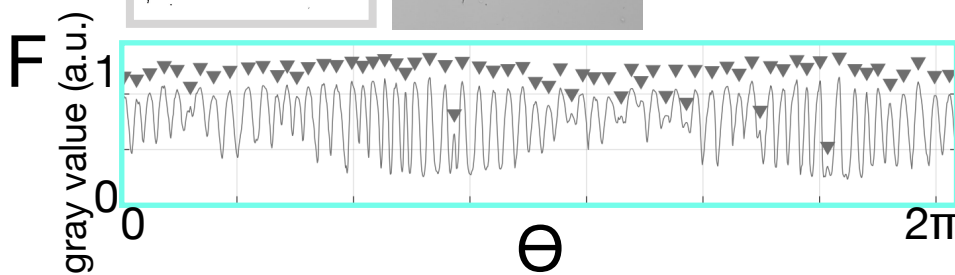
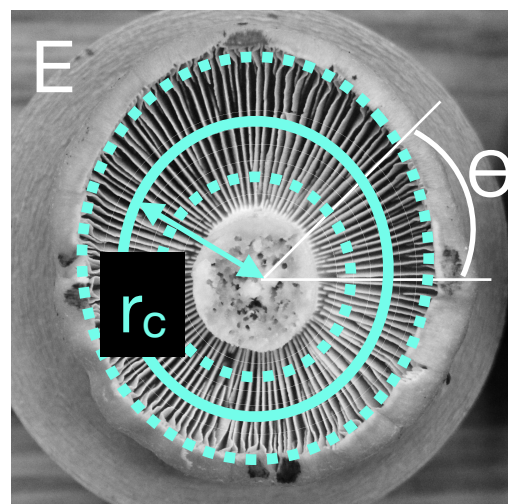
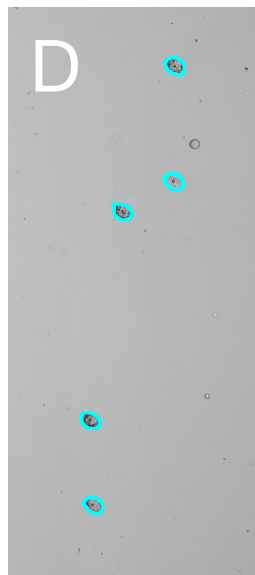
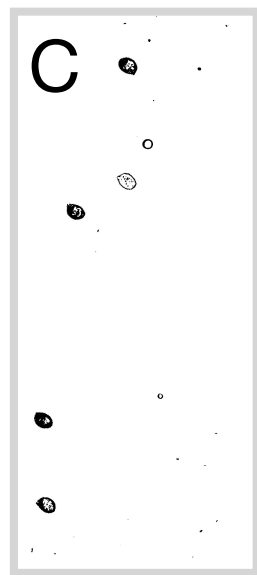
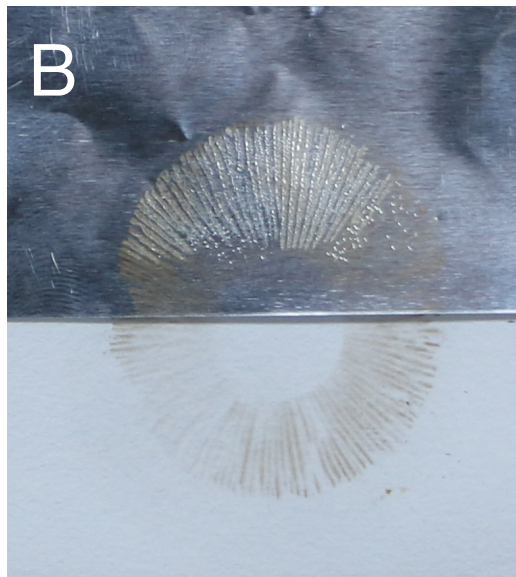
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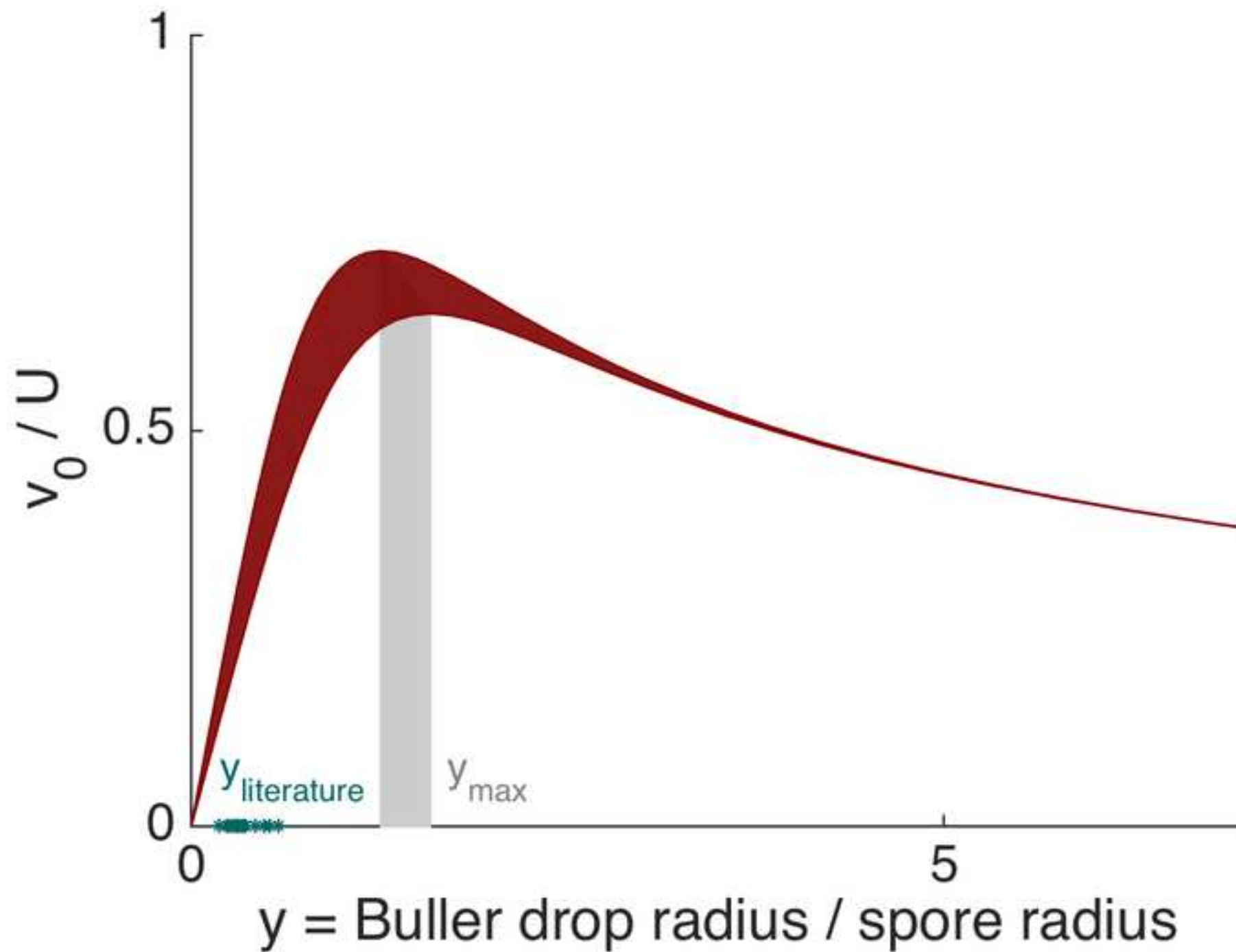
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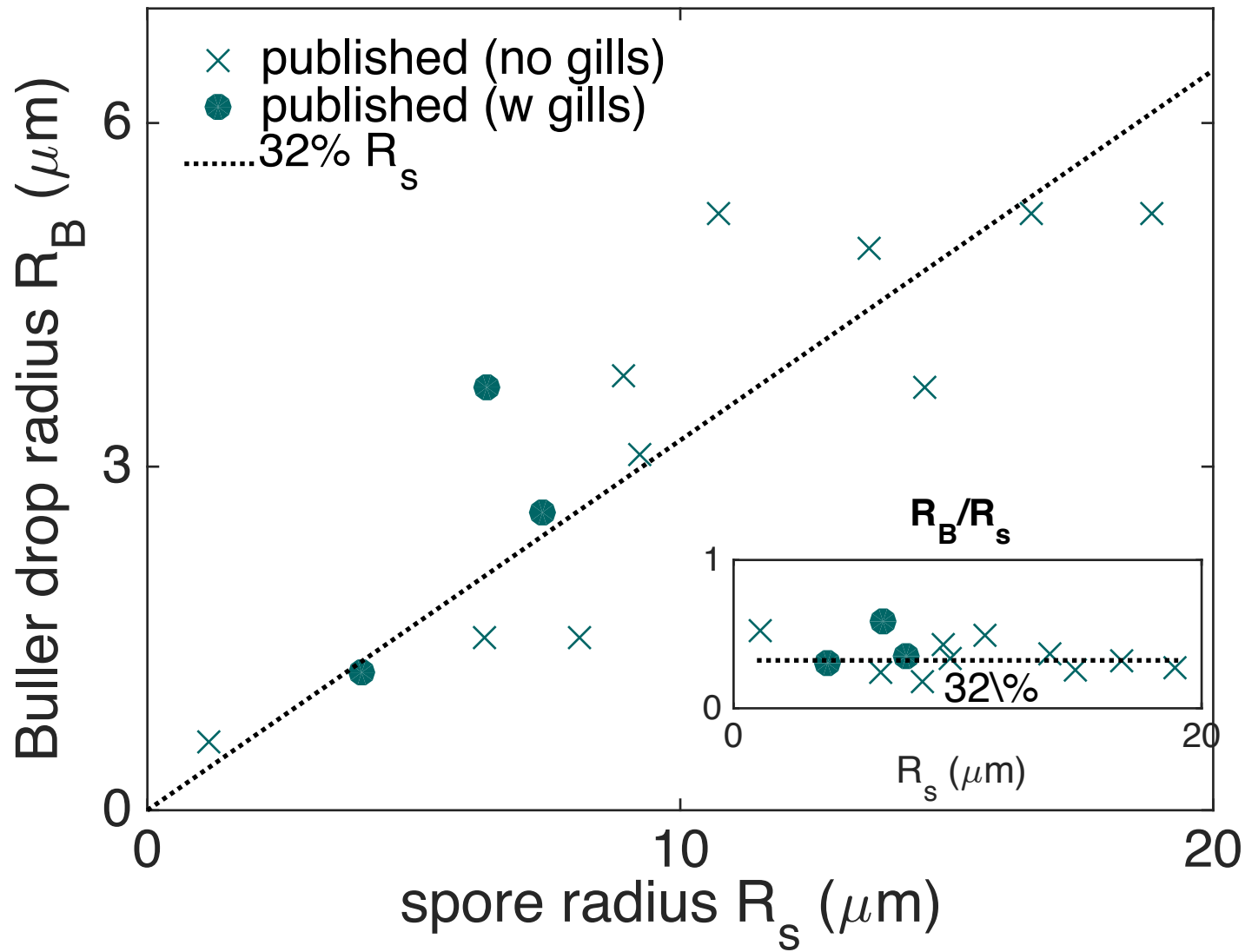
spore

Buller's drop

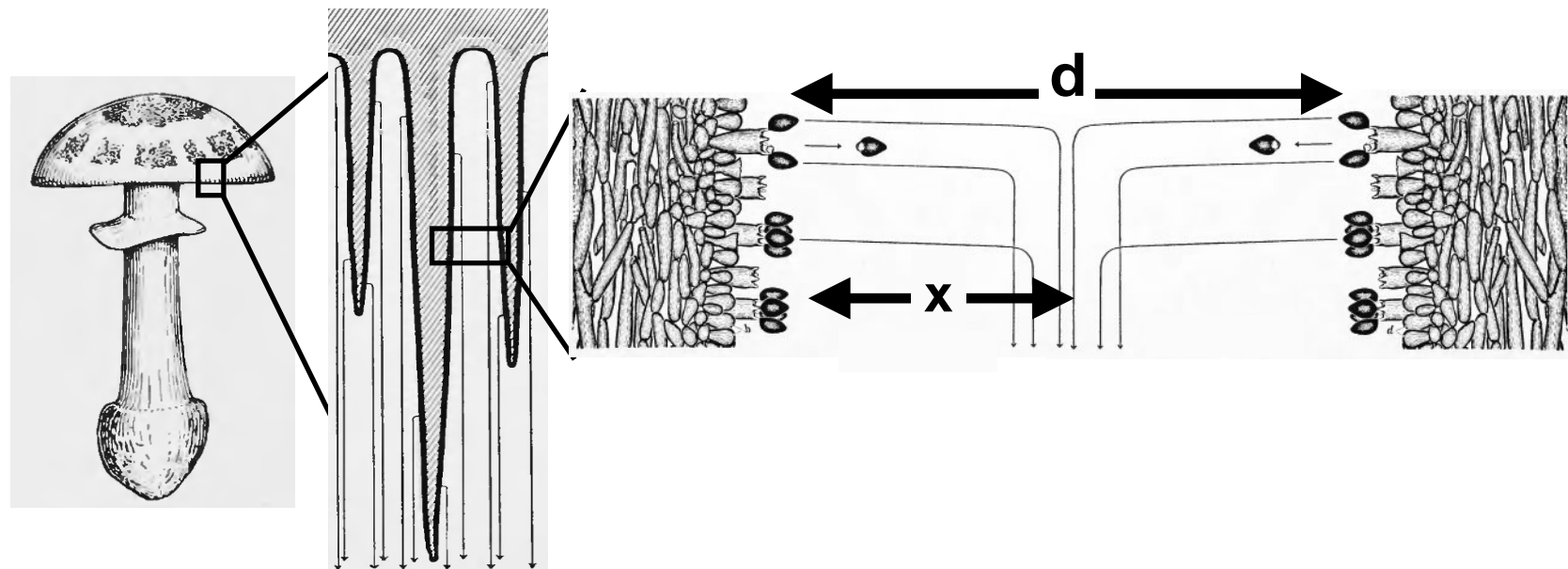






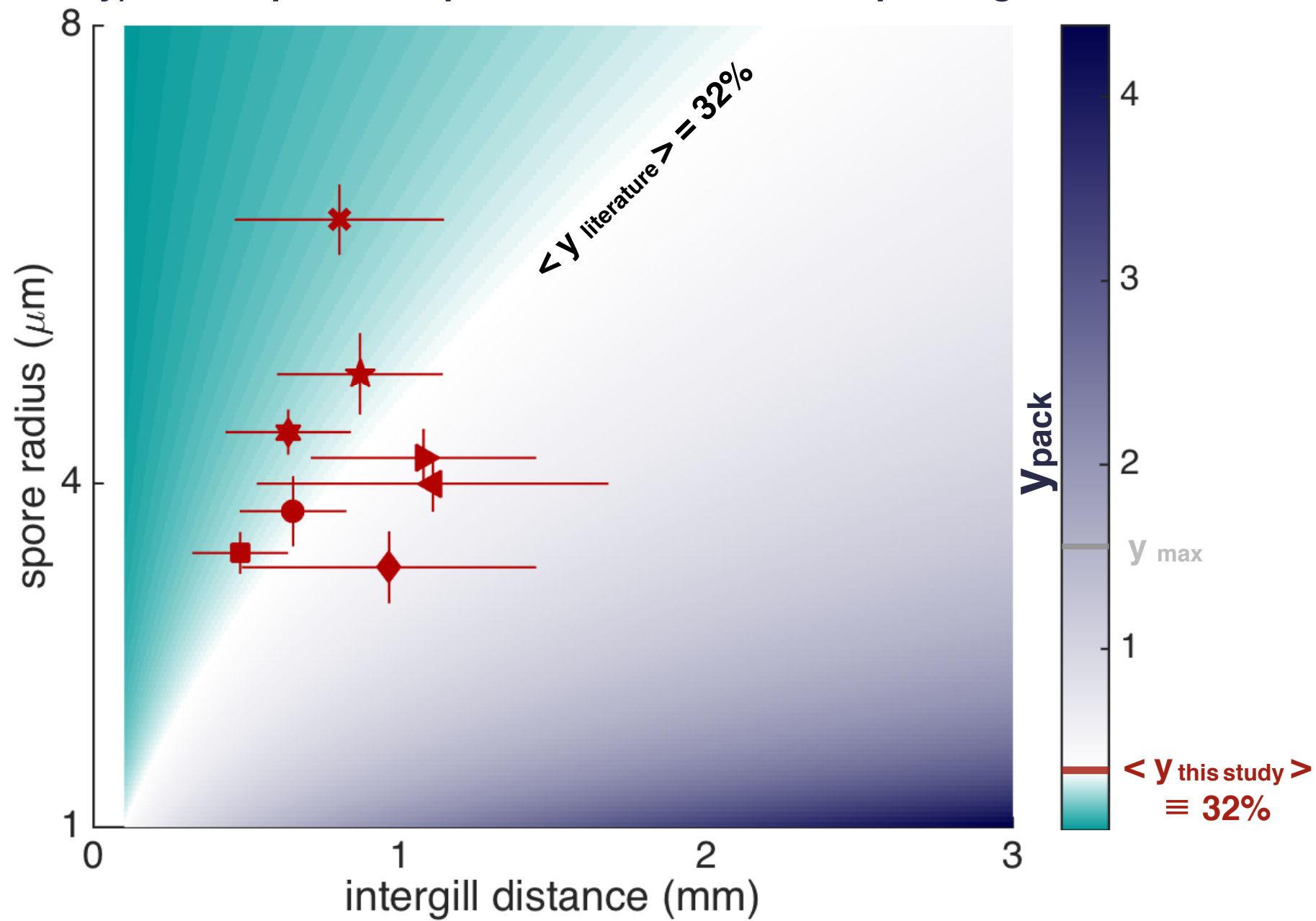


A



B

$y_{\text{pack}} = \text{drop radius/spore radius at maximum packing}$



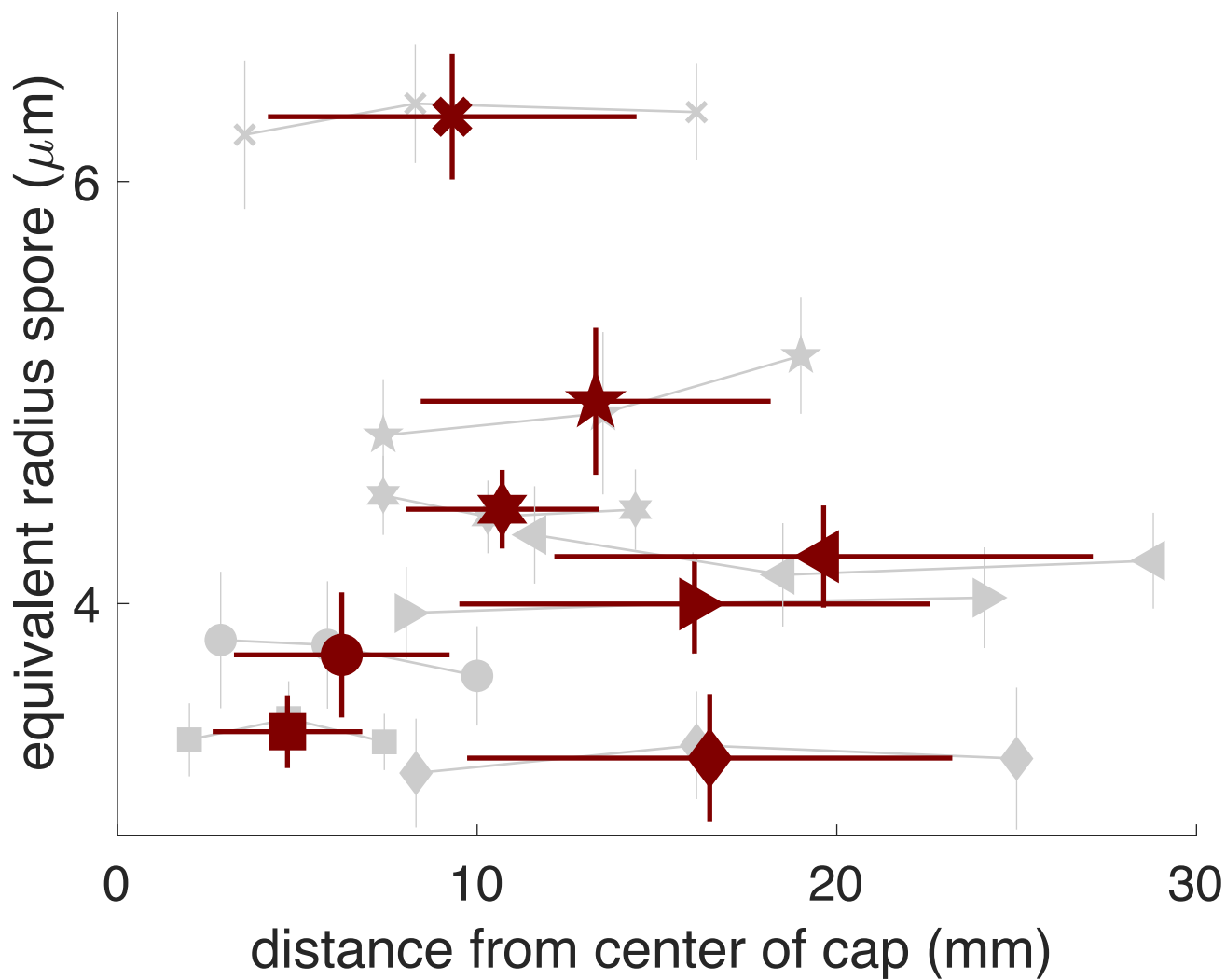


Figure 6

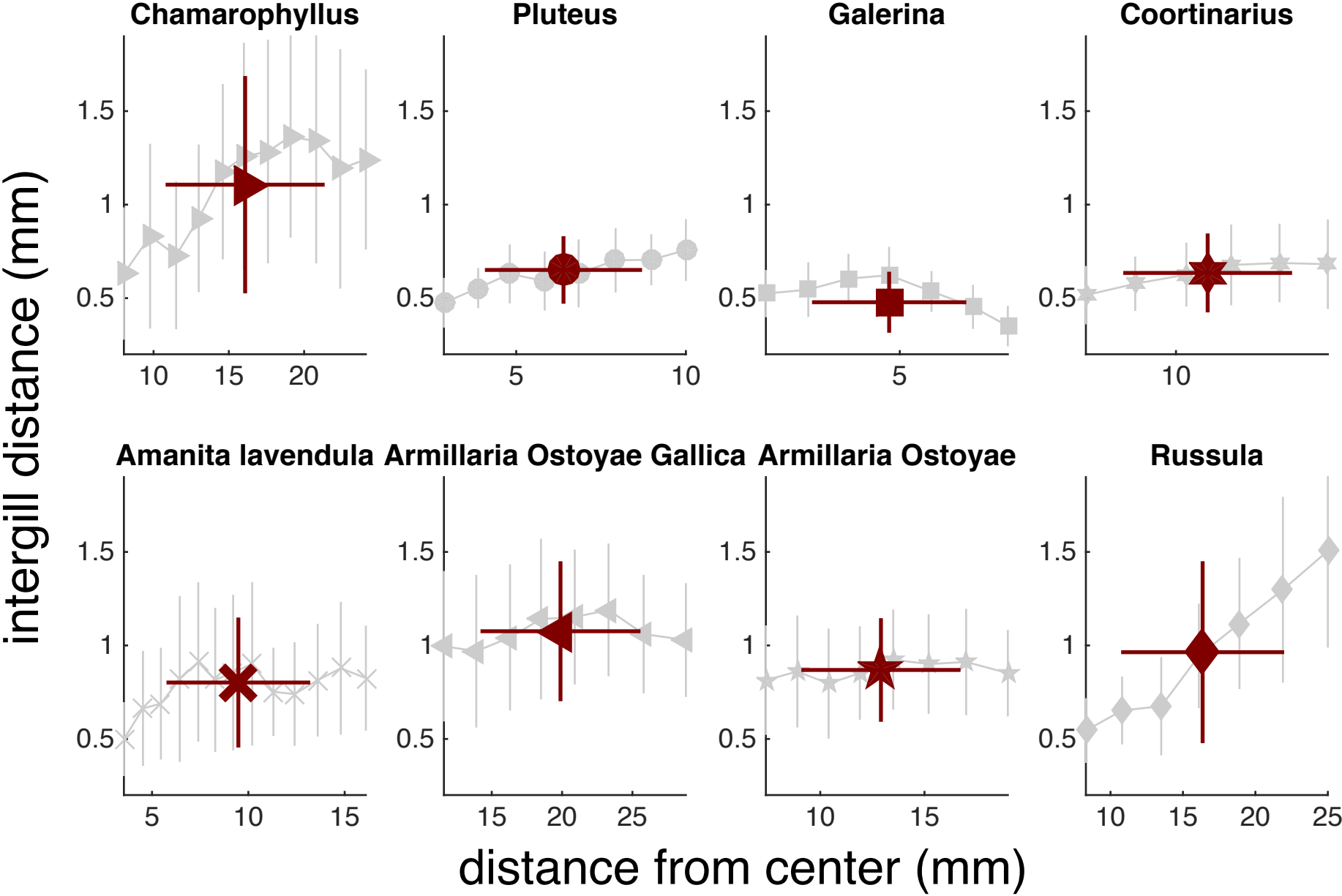


Table 1. List of parameters and their estimated or measured values from the literature.

Parameter	Symbol	Value	Reference	used in Fig 3
Air density	ρ_a	1 kg/m^3		1 kg/m^3
Spore density	ρ_s	0.8 to 3.8 g/m^3	(Hussein et al. 2013)	1.8 g/m^3
Buller's drop density	ρ_B	1 g/m^3	same as water ¹	1 g/cm^3
Buller's drop surface tension	γ	0.07 N/m	same as water ¹	0.07 N/m
Efficiency of energy conversion	α	$(50 \pm 5)\%$	(Noblin et al. 2009)	0.5
Spore to Buller's drop density ratio	$\beta = \rho_s/\rho_B$			
Air to Buller's drop density ratio	$\bar{\beta} = \rho_a/\rho_B$			
Reynolds number	$Re_B = R_s\gamma/(v^2\rho_B)$			
Reynolds number	$Re_s = R_s\gamma/(v^2\rho_s)$			
Intergill distance	d			
Spore radius	R_s			
Buller's drop radius	R_D			
Normalized Buller's drop radius	$y = R_B/R_s$			
y at maximum packing	y_{pack}			
Time scale	$T = 2R_s^2/(9v\bar{\beta})$			
Velocity scale				

¹Webster and coauthors estimated that Buller's drop contain about 1% in mass of mannitol and sucrose (Webster et al. 1995), hence γ and ρ_B are well approximated by the surface tension and density of water.

Table 2. List of collected species, collection location, number of spores imaged and analyzed, corresponding symbol used in Figures.

Collected species	Location	No. spores analyzed	Symbol
<i>Camarophyllus borealis</i>	Marquette County, Michigan	231	▶
<i>Cortinarius caperatus</i>	Marquette County, Michigan	1180	★
<i>Amanita lavendula</i>	Marquette County, Michigan	155	×
<i>Armillaria mellea</i> complex (A)	Marquette County, Michigan	301	★
<i>Armillaria mellea</i> complex (B)	Marquette County, Michigan	257	◀
<i>Mycena</i> sp.	UW-Madison Lakeshore Natural Preserve, Wisconsin	530	●
<i>Russula</i> sp.	UW-Madison Lakeshore Natural Preserve, Wisconsin	1053	◆
<i>Galerina marginata</i>	UW-Madison Lakeshore Natural Preserve, Wisconsin	1159	■

Table 3. Data of Buller's drop radius and spore radius from the literature. From left to right: Name of species, structure bearing spores, Buller's drop radius R_B , spore length L_S , spore width W_S , spore volume V_S , spore equivalent radius R_S , reference; species that produce gilled mushrooms are in boldface.

Species (order or class)	Spore-bearing structure	R_B (μm)	L_S (μm)	W_S (μm)	V_S (μm)	R_S (μm)	Reference
<i>Trametes versicolor</i> (Polyporales)	Pores	0.6	-	-	6.4	1.15	Fischer et al. 2010
<i>Aleurodiscus oakesii</i> (Russulales)	Smooth, discoid	5.2	23.2	17.0	-	18.86	Pringle et al. 2010
<i>Itersonilia perplexans</i> (Tremellales)	Yeast	4.9	15.1	12.8	-	13.52	Pringle et al. 2010
<i>Tilletiopsis albescens</i> (Exobasidiomycetes, incertae sedis)	Yeast	3.7	13.4	4.4	-	6.38	Pringle et al. 2010
<i>Laccaria amethystina</i> (Agaricales)	Gills	1.5	8.1	8.1	-	8.1	Stolze-Rybczynski 2009
<i>Stereum hirsutum</i> (Russulales)	Pores	1.2	7.2	3.0	-	4.02	Stolze-Rybczynski 2009
<i>Xerula radicata</i> (Agaricales)	Gills	3.7	16.8	13.6	-	14.59	Stolze-Rybczynski 2009
<i>Gymnosporangium juniperi-virginianae</i> (Pucciniales)	Gelatinous telial horns	5.2	20.0	15.1	-	16.58	Stolze-Rybczynski 2009
<i>Tilletia caries</i> (Tilletiales)	Host tissue	5.2	21.4	7.6	-	10.73	Stolze-Rybczynski 2009
<i>Sporobolomyces salmonicolor</i> (Sporidiobolales)	Yeast	3.8	11.5	7.9	-	8.95	Stolze-Rybczynski 2009
<i>Auricularia auricula</i> (Auriculariales)	Smooth, jelly-like	3.1	12.9	7.8	-	9.22	Stolze-Rybczynski 2009
<i>Polyporus squamosus</i> (Polyporales)	Pores	2.6	14.0	5.4	-	7.41	Stolze-Rybczynski 2009
<i>Armillaria tabescens</i> (Agaricales)	Gills	1.5	6.8	6.1	-	6.32	Stolze-Rybczynski 2009
<i>Clavicornia pyxidata</i> (Russulales)	Smooth, coral-like	1.2	4.7	3.5	-	3.86	Stolze-Rybczynski 2009