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Bioluminescence: A Fungal Nightlight with an Internal Timer

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http://dx.doi.org/10.1016/j.cub.2015.01.004

A recent study shows that green light emission by *Neonothopanus gardneri* mushrooms, endemic to coconut forests of Northern Brazil, is controlled by a circadian clock. Furthermore, insects are attracted by the light, raising the possibility that bioluminescence functions in spore dispersal and fungal dissemination.

Enchantment and curiosity are immediately evoked when, entering humid woods under a new moon, one sees green, shining mushrooms popping up on the surface of rotten logs. The photo of a colony of *Mycena lucentipis* mushrooms (Agaricomycetes) should help the reader appreciate these rare and splendid creatures [1] (Figure 1). Seventy-one out of thousands of fungus species occurring mainly in tropical and temperate zones of the globe are documented to be bioluminescent, of which twenty-six species belong to the genus *Mycena* [1,2]. As reported in this issue of *Current Biology* by Oliveira *et al.* [3], light emission by *Neonothopanus gardneri* mushrooms found in Brazilian coconut forests is controlled by a circadian clock and serves to attract insects for spore dispersal.

Although observed all over the world and documented by Aristotle and Pliny the Elder, natural philosophers of the Ancient World [4], fundamental questions about bioluminescent fungi — how, when, and why they emit light — have not been answered. Their biochemical and biological features are still murky, partly because it is difficult to spot them in dense forests, even with dark-adapted eyes, and to collect, transport and cultivate their mycelia and mushrooms in the laboratory. The chemical mechanism and function of light production by fungi is still controversial [5,6]. Up until recently, a key question has concerned whether molecular oxygen or hydrogen peroxide oxidizes a luciferin substrate in the presence of the enzyme luciferase. Alternatively, it was possible that light emission is a byproduct of some metabolic process such as lignin degradation? Almost all bioluminescent organisms known use oxygen to produce the electronically excited product (oxyluciferin), which decays to the ground state by photon emission (see reaction scheme below). Thus, the visible and 'cold' light emission results from efficient conversion of energy from chemical bonds to light without heat dissipation.

$$L + NAD(P)H + H^+ \xrightarrow{\text{soluble reductase}} LH_2 + NAD(P)^+$$

$$LH_2 + O_2 \xrightarrow{\text{insoluble luciferase}} LO + H_2O + \text{light}$$

L, luciferin; LH_2, reduced luciferin; LO, oxidized luciferin

In contrast to fungi, the luciferin/luciferase systems of dozens of luminescent organisms — from bacteria to fishes and insects — have long been identified. Furthermore, the bioluminescence produced by such systems has been implicated in courtship and mating, prey attraction or visual localization, predator warning (aposematism), camouflage, and species recognition/grouping [6]. Various chemically and phylogenetically unrelated luciferins have been isolated, identified and synthesized since the 1950s, among them firefly luciferin (a benzothiazole), sea-pansy coelenterazine and jellyfish aequorin (imidazopyrazinones), dinoflagellate luciferin (an open chain tetrapyrrole), bacterial luciferin (flavins), annelid luciferin (an oligoamide) and the limpet luciferin (a formylated aldo-enol) [5,6]. In a number of bioluminescent reactions (e.g., fireflies, crustaceans and coelenterates), chemical electronic excitation of the light emitter involves an intermediate consisting of a highly unstable four-membered ring peroxide named dioxetanone (or α-peroxylactone), whose thermal cleavage yields CO_2 and the excited product [7].

Figure 1. Bioluminescent mushrooms.
Dark exposure of *Mycena lucentipes* mushrooms, abundant in the wetlands of the State Park of Ribeira (PETAR-SP, BR) and in the National Park of Emas (PNE-SP, BR). Photo courtesy of C.V. Stevani.

Only recently has light emission by fungi been confirmed to result from a classical bioluminescent system; i.e., it can be tested by mixing a luciferase-rich 'cold' extract with a luciferin-rich 'hot' extract in normally aerated solutions [8], as described by Dubois in 1886 when studying the bioluminescence of a click-beetle (Elateridae: *Pyrophorus plagiophtalamus*) [9]. Fungal bioluminescence displays a spectral distribution roughly matching flavin fluorescence ($\lambda_{max} \approx 530$ nm), and requires a yet to be identified luciferin, a thermolabile luciferase/NAD(P)H-dependent reductase pair, and molecular oxygen [8].

Light emission by fungi was earlier proposed by O. Shimomura [10,11] to result from a non-enzymatic, superoxide radical-dependent chemiluminescent reaction whose substrate is an aldehyde called panal. To support this hypothesis, he used the Fenton reagent (Fe^{2+}-H_2O_2) in model studies to spark the chemiluminescent oxidation of a panal-methylamine Schiff adduct, and performed inhibition studies with fungal extracts in the presence of superoxide dismutase. More recently, Shimomura co-authored a study with a Siberian group, where they reported a luciferin/luciferase-dependent mechanism of light emission [12], thus corroborating Airth and McElroy's [13] and Oliveira and Stevani's [8] findings demonstrating an enzymatic, luciferase-dependent source of fungal bioluminescence.

The biological function(s) of fungal light emission and whether light emission is continuous or regulated by a biological clock is now answered by the new study by Oliveira *et al.* [3]. The authors provide precise and compelling data to answer these questions using experiments performed with a Brazilian luminescent mushroom, *Neonothopanus gardneri*. The mushroom abounds in the coconut forests flourishing in the transitional biome from the humid Amazonas Forest (North) to the dry Caatinga Forest (Northeast). *N. gardneri* resembles the *Omphalotus* spp. (basidiomycetes), displaying the traditional cap and a long and thick, fleshy stem, and emits green light from either mycelia or basidiomes. Tens of kilograms of the mushrooms were easily collected and brought to the laboratory by the authors for biological and biochemical studies. After two days entrainment to a 12/12 hour light/dark cycle at 21°, 25° or 29°C, a circadian rhythm in bioluminescence was maintained for 6 days. The processed photographical data revealed light intensity oscillations repeatedly peaking at around 22 hours at 25°C. This rhythm was only slightly affected by the temperature and accompanied by about a 4-fold increase in the content of luciferin in hot extracts and in the activity of reductase and luciferase measured in cold extracts.

Aiming to disclose the biological function of light emission by the mushrooms, the authors photographed, collected, and identified various specimens of flies, beetles, ants, wasps and other bugs attracted by the green light emitted by the mushrooms. The authors argue that especially in dense forests, where humidity and spore germination are high and there is little wind, nocturnal transport of spores by insects offers some advantage for fungus dispersion. As all these insects display maximum optical sensitivity in the green spectral region, they designed a clever field experiment to lure them with artificial green lights. The mushroom's insect visitors were also attracted to same-sized acrylic 'toy mushrooms' installed in the forest habitat and illuminated by green-emitting LEDs with similar light intensity and wavelength, but not when the LED had been turned off. This finding strongly advocates a role for the bioluminescence in fungus spore dispersal.

In conclusion, the parallel oscillation of luciferin/luciferase levels and of the mycelium light emission in culture attests to the circadian control of fungal bioluminescence. The adaptive significance of this regulatory system is evident as a system that maximizes light intensities at night would prevent the squandering of metabolic energy by day. Finally, the fungal bioluminescence undoubtedly functions in insect attraction and spore dispersal, thereby enhancing propagation. The disclosure of circadian-controlled bioluminescence of mushrooms will certainly be useful in determining the right time for the isolation of highly rich and active luciferin and luciferase extracts in order to facilitate biochemical and structural studies of this extraordinary and scientifically challenging phenomenon. Furthermore, the authors suggest the use of *N. gardneri* in molecular studies of circadian rhythms as its circadian period, being shorter than 24 hours and temperature-compensated,

resembles that of *Neurospora crassa,* a non-luminescent ascomycete model routinely employed for such investigations [14].

Further experimental effort is required to better characterize the molecular bases and biological functions of fungal bioluminescence. This work will include the isolation and structural identification of the luciferin and the luciferase/reductase pair to determine the chemical mechanism of light emission. It will also be interesting to establish metabolic connections between bioluminescence and the organism's redox balance, to investigate other possible roles of bioluminescence, such as aposematism (several fungal species may be distasteful to predators), and to develop analytical applications for the fungal luciferase. Despite the large time window spanning from Aristotle's three centuries B.C. writings, the publication of Harvey's herculean "Bioluminescence" in 1956 [4], to the "masterful biology lesson" (Martin Shalfie, Nobel Prize 2008) of Wilson and Hastings in "Bioluminescence-Living Lights, Lights for Living" in 2014 [6], there is still a lot to learn about the biochemistry, biology, and ecology of the amazing and yet mysterious luminous mushrooms.

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Evolution: Tinkering within Gene Regulatory Landscapes

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http://dx.doi.org/10.1016/j.cub.2015.02.051

Recently evolved enhancers dominate mammalian gene regulatory landscapes. Mostly exapted from ancestral DNA sequences, many are linked to genes under positive selection. Just as RNA-seq some years ago, unbiased enhancer mapping is on the verge of changing evolutionary research.

> *"Their macromolecules are so alike that regulatory mutations may account for their biological differences."*
>
> Mary-Claire King and Allan Charles Wilson, (1975)

This year marks the 40th anniversary of the landmark paper by Mary-Claire King and Allan Wilson [1] that speculated about the importance of gene regulatory changes versus those in protein-coding sequences during evolution — in this particular case of humans and chimpanzees [1]. Two years later, François Jacob further expanded on some of these ideas in his influential essay 'Evolution and Tinkering' [2,3]. He recognized that evolution via natural selection does not work like an engineer, but like a tinkerer. Instead of redesigning its components, it tinkers by fiddling with the existing bits. During the last decade, some fields, in particular evolutionary developmental biology (evo-devo), reinvigorated the discussion about the relative importance of protein versus regulatory changes

