Convergent biosynthesis of psilocybin in an ectomycorrhizal lineage: is the psychoactive end-product the selected trait?

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Abstract.

The fungivore-deterrence hypothesis, that psilocybin evolved as a chemical defence against arthropod fungivores via 5-HT receptor agonism, has become the working consensus in fungal chemical ecology, despite resting on a phylogenomic pattern of horizontal gene transfer among saprotrophs and remarkably little direct experimental evidence. Recent biochemistry shows that the ectomycorrhizal *Inocybe corydalina* assembles psilocybin through a convergently evolved, non-homologous *ips* cluster whose branched pathway yields baeocystin, not psilocybin, as the primary end-product. We argue that psilocybin's psychoactivity at vertebrate 5-HT2A receptors is plausibly incidental, with selection most likely acting on the injury-triggered polymerized indoloquinoid end-state of the blueing reaction (with psilocybin functioning as its stable storage precursor) and only secondarily on the monomeric congeners baeocystin or aeruginascin. We propose a five-tier comparative experimental program to adjudicate among these alternatives.

Highlights

- The fungivore-deterrence hypothesis for psilocybin's adaptive function rests on a robust phylogenomic pattern of horizontal gene transfer among saprotrophic Agaricales [1] but has only just begun to be tested experimentally [2], and no controlled feeding assay has yet used purified compound at defined concentrations against any arthropod fungivore.
- Bradshaw et al. [3], in the field's most recent peer-reviewed phylogenomic synthesis, state explicitly that “psilocybin-containing mushrooms regularly have living insect larvae in them and they can be reared to adults,” directly inconsistent with simple deterrence.
- The recent biochemical demonstration that *Inocybe corydalina* assembles psilocybin via a non-homologous, branched *ips* cluster [4] reveals not only convergent invention in an ectomycorrhizal lineage but also that baeocystin, not psilocybin, accumulates as a primary end-product of the branched pathway, with overall psilocybin titres approximately 10- to 60-fold lower than in high-titer *Psilocybe* species.
- Together with Lenz et al.'s [5, 6] demonstration that psilocin oxidatively oligomerizes on tissue injury into indoloquinoid pigments via the conserved PsiP/PsiL cascade, these observations support a structural reframing: psilocybin may function as a stable storage precursor for an injury-triggered polymeric defence (a damage-activated chemical-defence architecture analogous to the glucosinolate/myrosinase system in plants [7]), with the polymer end-state, not the monomer's vertebrate 5-HT2A pharmacology, as the most likely target of selection, and the monomeric congeners baeocystin and aeruginascin as alternative candidates.
- We propose a five-tier comparative experimental program with protocol-level specifications and explicit quantitative predictions for four falsifiable hypotheses, designed to adjudicate among them in paired saprotroph and ECM systems.

Glossary

Inocybaceae. Globally distributed family of ectomycorrhizal mushroom-forming fungi in the Agaricales. As currently circumscribed [8], it comprises seven genera: *Inocybe sensu stricto*, *Inosperma*, *Mallocybe*, *Nothocybe*, *Pseudosperma*, *Auritella*, and *Tubariomyces*, and associates with at least 23 plant families.

Ectomycorrhizal (ECM). A symbiotic association in which fungal hyphae sheathe plant root tips and form a Hartig net between epidermal/cortical cells, exchanging mineral nutrients (chiefly N and P) for plant-derived photosynthate. ECM symbioses are obligate for most temperate and boreal forest trees.

Psilocybin. 4-Phosphoryloxy-N,N-dimethyltryptamine. A tryptamine-derived fungal natural product whose dephosphorylated congener psilocin is a high-affinity serotonin (5-HT) receptor agonist, particularly at 5-HT2A.

Baeocystin. 4-Phosphoryloxy-N-methyltryptamine. The mono-methylated congener of psilocybin and the dominant tryptamine end-product of the *ips* cluster in *Inocybe corydalina* [4, 9], present at 2- to 13-fold higher concentrations than psilocybin in this species.

Aeruginascin. 4-Phosphoryloxy-N,N,N-trimethyltryptamine. Quaternary trimethylammonium tryptamine restricted largely to psilocybin-producing Inocybaceae [10, 11], reaching up to 0.3% dry weight in *I. aeruginascens*; structurally analogous to the toad alkaloid bufotenidine.

Muscarine. A trimethylammonium quaternary alkaloid that activates muscarinic acetylcholine receptors. Plesiomorphic for the *Inocybe* + *Nothocybe* + *Pseudosperma* clade [12].

Blueing reaction. Oxidative dephosphorylation of psilocybin to psilocin followed by quinoid coupling and oligomerization to indoloquinoid pigments described by Lenz et al. [5, 6]; the resulting polymer is the diagnostic colour change of injured psilocybin-containing fungi. The polymer end-state has been hypothesized to play a defensive role through three candidate mechanisms (wound-sealing, tannin-like protein damage with ROS generation, direct invertebrate deterrence), see Appendix for the case for each.

***psi* cluster.** The five-gene biosynthetic cluster (*psiD, psiK, psiM, psiH, psiT*) characterized in *Psilocybe cubensis* [13] and shown to be horizontally transferred among saprotrophic Agaricales [1].

***ips* cluster.** The five-gene cluster recently identified in *Inocybe corydalina* (*ipsD, ipsK, ipsM1, ipsM2, ipsH*) that catalyzes psilocybin biosynthesis through a different reaction sequence and via non-homologous enzymes [4, 14].

Fungivore-deterrence hypothesis. The proposal that psilocybin's adaptive function is to interfere with the nervous systems of arthropod fungivores via 5-HT receptor agonism, reducing basidiome consumption [1].

Basidiome. The spore-producing fruiting body of a basidiomycete (synonymous with “mushroom” or “carpophore”).

Horizontal gene transfer (HGT). The non-vertical transmission of genetic material between organisms, here between fungal lineages, often inferred from phylogenetic incongruence between gene trees and species trees.

Ancestral state reconstruction. A class of phylogenetic comparative methods that infer character states at internal nodes of a tree, typically under continuous-time Markov models [15, 16].

Introduction

Psilocybin is the closest thing to a model system that fungal chemical ecology has. The compound is a tractable single small molecule with a thoroughly characterized biosynthetic pathway [13], a known pharmacological mode of action through 5-HT receptor agonism [17], and a phylogenomic distribution that has, in the past decade, been resolved at unusually high resolution for a fungal natural product [1, 3, 4, 12].

Reynolds et al. [1] established that the *psi* cluster has moved horizontally among three saprotrophic Agaricales genera, *Psilocybe*, *Gymnopilus*, and *Panaeolus*, and speculated that psilocybin and related neuroactive compounds in dung and late wood-decay niches may have evolved to influence the behaviour of co-occurring mycophagous and wood-eating invertebrates. Subsequent work added *Pluteus* [14] and *Pholiotina* [3] as additional HGT-recipient genera. Bradshaw et al. [3] dated the origin of the cluster in *Psilocybe* to approximately 67 mya (concurrent with the K-Pg boundary), with subsequent HGT acquisitions in other genera ranging from approximately 40 mya (*Panaeolus subbalteatus*) to approximately 9 mya (*Gymnopilus junonius*). The behaviour-modification framework, hardened in popularization to a deterrence framework, has in the eight years since Reynolds et al. [1] become the field's working consensus.

We argue that this consensus rests on weaker empirical foundations than has been appreciated, and that the recent biochemical characterization of an alternative biosynthetic pathway in an ectomycorrhizal lineage forces a structural rethink. Four observations motivate the argument. First, despite decades of attribution, the deterrence hypothesis has only just begun to be tested in controlled feeding assays against any fungivore: Matthews Nicholass et al. [2], in the first published experimental test, exposed *Drosophila melanogaster* larvae to methanolic extracts of *Psilocybe cubensis* basidiomes and detected effects on development and locomotion, but, critically, found that mutants with ~90% reduced 5-HT2A receptor expression exhibited heightened sensitivity to the extracts rather than the reduced sensitivity predicted by a strictly receptor-mediated mechanism, indicating that whatever the active compound is in the extract, its effect is not mediated by the canonical psychedelic receptor in the manner the deterrence hypothesis requires. No published feeding assay has used purified psilocybin (or its congeners baeocystin, aeruginascin, or norbaeocystin) at defined concentrations against any mycetophilid or sciarid larva. Second, Bradshaw et al. [3], in the field's most recent peer-reviewed phylogenomic synthesis, state plainly that “psilocybin-containing mushrooms regularly have living insect larvae in them and they can be reared to adults,” citing the Awan et al. [14] demonstration that Sciaridae fly larvae can be reared to adults from *Psilocybe cyanescens* fruiting bodies (Awan et al. also independently identified *Exechia fusca* (Mycetophilidae) DNA in those fruiting bodies). Third, Schäfer et al. [4] have now shown experimentally that *Inocybe corydalina* assembles psilocybin through a convergently evolved, non-homologous *ips* cluster: not horizontally acquired from saprotrophs, but independently invented in an obligate ectomycorrhizal lineage where dung and wood-decay niches are absent. Fourth, and most consequentially, the *ips* pathway is branched: it produces baeocystin as a primary end-product at concentrations approximately 2- to 13-fold higher than psilocybin in *I. corydalina* basidiomes [9], and Lenz et al. [5, 6] have shown that psilocin polymerizes oxidatively into indoloquinoid pigments on tissue injury, proposing that these oligomers may add a layer of defensive bioactivity beyond the receptor-mediated effects of monomeric psilocin. Bradshaw et al. [3], citing the comprehensive review by Meyer & Slot [18], characterize the ecological role of psilocybin as one where “speculation reigns supreme” and empirical studies remain lacking.

Taken together, these observations suggest a structural reframing: psilocybin's psychoactive properties, the property that gives the molecule its place in the cultural and pharmacological literature, may be incidental to the trait actually under selection. Selection on the psilocybin pathway may operate on baeocystin or aeruginascin (psilocybin's congeners), on pathway-level redox handling, or on the polymerized indoloquinoid end-state of the blueing reaction [5], with psychoactivity at vertebrate 5-HT receptors being a side-effect of conserved tryptamine chemistry rather than the trait itself. This is a stronger claim than “the deterrence hypothesis is untested,” and the empirical record does not currently distinguish between them.

The Inocybaceae case, with its convergent invention, branched pathway, and concentration asymmetry, is the natural experiment that would let the field begin to distinguish them. In what follows, we lay out the empirical state of play, articulate four falsifiable alternative hypotheses for psilocybin's function in ectomycorrhizal *Inocybe* (including the audacious one that psilocybin is not the selected trait), and propose a coordinated comparative experimental program designed to treat saprotroph and ECM systems as paired tests.

The deterrence hypothesis: phylogenomic strength, empirical weakness

The phylogenomic case for HGT of the *psi* cluster is robust. Reynolds et al. [1] identified homologous *psi* clusters in *Psilocybe*, *Gymnopilus*, and *Panaeolus*, demonstrated significant incongruence between gene trees and species trees using the Approximately Unbiased (AU) topology test as implemented in Consel, and inferred that the cluster has moved repeatedly between distantly related saprotrophic Agaricales. Awan et al. [14] (preprint) extended this to *Pluteus salicinus* and noted alternative gene orders consistent with a circular intermediate. Bradshaw et al. [3] used shotgun sequencing of fungarium specimens to obtain 71 metagenomes and analyzed 2,983 single-copy gene families, applying weighted Kishino–Hasegawa, weighted Shimodaira–Hasegawa, expected likelihood weights, and AU topology tests to confirm HGT; they dated the origin of psilocybin biosynthesis in *Psilocybe* to approximately 67 mya, with subsequent HGT acquisitions ranging from approximately 40 mya (*Panaeolus subbalteatus*) to approximately 9 mya (*Gymnopilus junonius*), and identified two distinct gene orders within *Psilocybe* itself, which they interpret as an ancient gene-cluster rearrangement consistent with the circular-intermediate model proposed by Awan et al. This work builds on earlier *Psilocybe sensu lato* phylogenetic and trait-evolution analyses by Ramírez-Cruz et al. [19], the saprotroph-clade parallel to the Inocybaceae trait-mapping in Kosentka et al. [12]. The persistence of *psi* across HGT and the patchy phylogenetic distribution together imply selection on the cluster in recipient lineages [20, 21], a pattern that demands an adaptive explanation.

The deterrence interpretation, however, was carefully framed by Reynolds et al. [1] as speculation: their original paper used the verb “we speculate” in proposing arthropod fungivores as the selective agent, with the comparative-genomic data offered as motivation rather than as test. That caveat has migrated into the secondary literature as established theory, despite the lack of corresponding direct experimental work. The mechanistic story is plausible on its face. Arthropod 5-HT receptors are deeply conserved with their vertebrate orthologues [22, 23], and pharmacological work in *Drosophila* has shown that LSD elicits measurable behavioural responses through serotonin receptor activation [24], while 5-HT2A signalling modulates larval feeding [25] and serotonin modulates feeding and gut motility in the honeybee [26]. Insects that specialize on chemically defended mushrooms, *Drosophila* species that tolerate amatoxins [27] or muscimol/ibotenic acid [28], represent a coevolutionary backdrop against which psilocybin could plausibly have been selected. But mechanistic plausibility is not validation.

What is striking, given the prominence of the hypothesis, is how thin the direct empirical record actually is. Until very recently, the peer-reviewed literature contained no controlled feeding assays of psilocybin or psilocin against any fungivore taxon: Diptera (Mycetophilidae, Sciaridae, Drosophilidae), Collembola, Acari, nematodes. The only published experimental test, Matthews Nicholass et al. [2], used methanolic extracts of *Psilocybe cubensis* basidiomes (rather than purified compounds) on *Drosophila melanogaster* larvae and detected effects on development and locomotion, but found that mutants with ~90% reduced 5-HT2A receptor expression exhibited heightened sensitivity to the extracts rather than the reduced

sensitivity predicted by a strictly receptor-mediated mechanism, a result that weakens rather than strengthens the canonical 5-HT2A-mediated deterrence model. No published dose-response study has paired purified psilocybin with neutral substrate against a target fungivore. No published functional or binding study has used cloned arthropod 5-HT receptors with psilocin or psilocybin. The mechanistic foundation rests on inference from vertebrate pharmacology and from a small number of pharmacologically-induced behavioural changes in insects exposed to other tryptamines.

The single published direct datum is inconsistent with deterrence. Awan et al. [14], while assembling the *Psilocybe cyanescens* genome from wild-collected basidiomes, recovered fungus-gnat DNA in their sequencing reads and identified *Exechia fusca* (Mycetophilidae) as one of the contaminating species; in a parallel rearing experiment from co-collected *P. cyanescens* fruiting bodies they observed 4–5 dipteran larvae emerging per jar, with one adult fly per jar successfully reared and identified as belonging to the family Sciaridae (dark-winged fungus gnats). The broader fungivore-basidiome literature provides quantitative context for how unsurprising this should be. Põldmaa et al. [29] systematically reared 37 mycetophilid species from 100 boreal mushroom species (460 fruitbodies) and found that approximately 80% of fungal species and 74% of fruitbodies were infested. Bruns [30] documented diverse fungivore guilds on Boletales basidiomes; mycetophilids show measurable host preference at the genus level despite an overall polyphagous pattern [31]. The expectation that fleshy basidiomes, including those of psilocybin producers, support reproductive populations of mycetophilid and sciarid larvae is the empirical baseline, not the anomaly. A secondary metabolite that obligate fungivores can complete development inside is not behaving as a deterrent in the simple sense at the concentrations encountered.

Bradshaw et al. [3] state plainly that “experimental evidence for the ecological role of psilocybin is largely lacking and speculation reigns supreme” (citing Meyer & Slot's review), and that “anecdotes and personal observations confirm that psilocybin-containing mushrooms regularly have living insect larvae in them and they can be reared to adults.” Meyer and Slot [18], in the most thorough review of the question to date, write that “no precise ecological roles of psilocybin have been experimentally determined,” and treat the deterrence framework as one among several unfalsified hypotheses, alongside spore-dispersal manipulation, nitrogen storage, and mycorrhizosphere/microbial-competition framings. Slot and Hoffmeister [32], in their recent *Current Biology* synthesis, frame the same set of possibilities as “may include deterring predators or facilitating spore dispersal,” the cautious reading of a still-untested literature. The phylogenomic pattern is real; the deterrence interpretation of that pattern is not validated.

The *Inocybe corydalina* convergent invention

Five Inocybaceae species, *Inocybe corydalina*, *I. aeruginascens*, *I. haemacta*, *I. coelestium*, and *I. tricolor*, are reliably reported to contain psilocybin and related tryptamines [9, 12, 33]. Aeruginascin (N,N,N-trimethyl-4-phosphoryloxytryptamine), a quaternary congener of psilocybin, was discovered by Gartz [11, 34] in *I. aeruginascens* at concentrations of the same order of magnitude as psilocybin and baeocystin, and was structurally elucidated as the trimethylammonium analogue of psilocybin by Jensen et al. [10]. Kosentka et al. [12] mapped psilocybin and muscarine onto a densely sampled single-locus (nuclear ribosomal LSU) Inocybaceae chronogram of ~500 species: muscarine is a shared-derived trait of the *Inocybe* + *Nothocybe* + *Pseudosperma* inclusive clade, whose common ancestor they date to approximately 60 mya, with several recent losses; psilocybin-producing taxa are nested inside muscarine-loss subclades, with at least two inferred independent transitions to psilocybin production roughly 10–20 mya. Statistical constraint

analyses in [12] firmly rejected a single origin of muscarine in the family; for psilocybin, the chronogram supports at least two independent gains, though the comparative test for psilocybin is correspondingly weaker.

The mechanistic question, whether the *Inocybe* psilocybin pathway is the same machinery as in *Psilocybe*, was opened by Awan et al. [14], who reported that the *I. corydalina* genome assembly contains no homologue of the canonical *psi* cluster but instead carries five colocalized candidate genes encoding a different aromatic amino-acid decarboxylase, kinase, P450 monooxygenase, and pair of methyltransferases. Schäfer et al. [4] closed the question. They expressed the *ips* genes recombinantly in *Escherichia coli*, characterized IpsD, IpsK, IpsM1, and IpsM2 in product-formation assays, and analyzed the insoluble monooxygenase IpsH in silico. Their central finding is that "none of the reactions intrinsic to the [psilocybin] pathway in *Psilocybe* species takes place in *I. corydalina*." The two pathways converge on 4-hydroxytryptamine as a shared intermediate but proceed through different reaction orders, and the *Inocybe* pathway is branched, yielding baeocystin as a second end-product. The enzymes are distantly related at best; the Ips methyltransferases, for example, are not orthologous to PsiM. Schäfer et al. conclude that "mushrooms recruited distantly or entirely unrelated enzymes to evolve the metabolic capacity for [psilocybin] biosynthesis twice independently."

This finding reorders the explanatory burden. The Reynolds et al. [1] hypothesis was constructed for a saprotroph natural history. The presumed selective agents were mycophagous and wood-eating invertebrates encountered in dung and late wood-decay niches; the presumed mechanism of cluster movement was HGT among co-occupants of those niches; the presumed selective context was a substrate matrix shared with insect competitors of the mycelium. The Inocybaceae are obligate ectomycorrhizal symbionts of at least 23 plant families [8]: their carbon source is their tree partner, their substrate is forest soil and root tips rather than dung or wood, and the *ips* cluster is non-homologous to *psi* and was therefore not acquired from a *Psilocybe*-type saprotroph donor (its evolutionary origin remains unknown [4]). Multiple links in the saprotroph deterrence chain, the carbon-source assumption, the substrate-matrix argument for substrate-sharing fungivores, and the HGT-vector framework, break in the *Inocybe* case. Selection has independently assembled a psilocybin biosynthetic pathway in a niche where the central ecological elements of the original adaptive story do not apply.

We caution that the inference of two independent transitions [12] is a lower bound and is not yet on a rigorous comparative footing. The Kosentka et al. [12] phylogenetic scaffold was based solely on nuclear ribosomal large subunit (LSU) data and pre-dated the Matheny et al. [8] generic revision (which split the family into seven genera) and the substantial European multilocus and barcoding work of Bandini, Esteve-Raventós, and colleagues [35–37]. Ascertainment bias is plausible: psychoactive *Inocybe* taxa have been preferentially screened, and absence of detection in unscreened taxa cannot be equated with absence of production. Most importantly, with only ~2 inferred transitions, the phylogenetic comparative framework cannot deliver the kind of correlated-evolution claim that the muscarine-loss-then-psilocybin-gain pattern superficially invites, see Maddison and FitzJohn [15] on the phylogenetic-pseudoreplication problem and Boyko and Beaulieu [16] on the hidden-rates Markov model correction.

The chemistry asymmetry

The chemical evidence from Schäfer et al. [4] and from quantitative UHPLC-MS/MS profiling [9, 33] sharpens the puzzle further, in a direction that the existing literature has not foregrounded. The *ips* pathway is branched. The IpsM1/IpsM2 methyltransferase pair, together with the IpsK kinase, generates baeocystin as a primary end-product, not solely as an intermediate to psilocybin [4, 38]. In *I. corydalina* basidiomes, baeocystin (0.50–0.98 mg/g dry weight) is approximately 2- to 13-fold more abundant than psilocybin (0.08–0.28 mg/g), and aeruginascin (0.02–0.37 mg/g) reaches comparable levels to psilocybin [9]. Gartz [34] reported aeruginascin in *I. aeruginascens* at concentrations of the same order of magnitude as psilocybin and baeocystin in that species. Across the comparison, psilocybin concentrations in *Inocybe* (maximum ~0.28 mg/g) are approximately 10- to 60-fold lower than in high-titer *Psilocybe* species such as *P. cyanescens* (2.3–13.8 mg/g), *P. semilanceata* (1.3–11.4 mg/g), *P. serbica* var. *bohemica* (1.6–15.5 mg/g), or *P. azurescens* (up to 17.8 mg/g) [3, 9, 39]. Two readings of this asymmetry are possible. The first, the standard reading in the existing literature, treats the lower titres in *Inocybe* as evidence of weaker or relaxed selection on psilocybin in the ECM context. The second, which we develop in detail in Hypothesis 4 below, treats psilocybin as an incidental coproduct of the branched pathway, with selection acting on baeocystin or aeruginascin and psilocybin emerging as a side-product of the methyltransferase chemistry whose psychoactive properties at vertebrate 5-HT2A are incidental to function. Critically, the existing literature does not distinguish between these readings: no published feeding assay, antimicrobial test, or signalling assay has used baeocystin or aeruginascin as the test compound. The field has assumed that the molecule with the most prominent vertebrate pharmacology is the molecule under selection, and the assumption has not been examined.

A third ecological context: entomopathogens and lichens

A third ecological context for psilocybin production has been documented outside of the mushroom-forming Agaricales entirely. Boyce et al. [40] reported psilocybin in *Massospora platypediae* and *M. levispora* (which infect annual cicadas and likely represent a single biological species), and the amphetamine-like alkaloid cathinone in the related *M. cicadina*, which infects 13- and 17-year periodical cicadas; all three are obligate entomopathogens in the Entomophthoromycotina, a phylogenetically distant lineage from the Agaricales. The biosynthetic genes have not been identified, but Schäfer et al. [4] themselves flag *Massospora* as a possible third independent origin of psilocybin biosynthesis, and the first instance outside the mushrooms. The Boyce et al. [40] work is particularly significant for our argument: the cicada pathogens use psilocybin in a behaviour-modifying context (host manipulation), and the chemistry occurs in a lineage in which a related species produces cathinone instead of psilocybin, suggesting that the production of psychoactive alkaloids in *Massospora* reflects selection on host behaviour modification rather than fungivore deterrence per se. *Massospora* also offers a sharp cross-system test of our central thesis. If selection acts on the same molecule across saprotroph, ECM, and entomopathogen contexts, as Hypothesis 1 implicitly assumes, psilocybin should be the dominant tryptamine in *Massospora* spore masses, with baeocystin and aeruginascin at the trace levels seen in *Psilocybe* [9, 41]. If selection is context-dependent and the molecular target varies with ecological setting, as Hypothesis 4 implies, *Massospora* should show a tryptamine profile distinguishable from both *Psilocybe* and *Inocybe*, and the active molecule for cicada manipulation may not be psilocybin at all. Boyce et al. [40] did not quantify the full tryptamine profile of *Massospora* fungal plug and spore-mass material at the resolution required; targeted UHPLC-MS/MS quantification of psilocybin, psilocin, baeocystin, norbaeocystin, aeruginascin, and (where detectable) polymer pigments in *M. platypediae* and *M. levispora* is a high-value, low-cost falsifier. A second, more

preliminary lichen-symbiotic context has been proposed: Schmull et al. [42] described the lichenized basidiomycete *Dictyonema huaorani* from Amazonian Ecuador, with reported psilocybin content based on ethnobotanical use among the Waorani people and confirmed by LC-MS analysis. Schmull et al. themselves note that they were "unable to use pure reference compounds" and that their identification, from a single small sample, could not be made conclusively; the report should be treated as preliminary pending re-analysis with authentic standards.

The methodological implication is significant. The field's screens for psilocybin-producing fungi rely heavily on detection of *psi* cluster homologs in genome assemblies [1, 3, 43]. *Inocybe* was discovered through chemistry first [33], then through enzymology [4, 14]. *Massospora* was discovered through chemistry of cicada manipulation. *Dictyonema* was discovered through ethnobotany. Convergent inventions whose enzymes share no sequence homology with PsiD/H/K/M will be invisible to homology-based screens, and the count of psilocybin-producing lineages is almost certainly an undercount.

Box 1. Convergent biosynthetic invention is probably underdetected

The Schäfer et al. [4] demonstration that *Inocybe corydalina* and *Psilocybe* arrived at psilocybin biosynthesis through entirely non-homologous enzymatic routes is methodologically as significant as it is biologically. The standard approach to identifying psilocybin-producing fungi, and to inferring the phylogenetic distribution of the trait, has been to search genome assemblies for homologs of the canonical *psi* cluster genes [1, 3, 41, 43]. This approach is blind to convergent invention. The *ips* cluster shares no individual enzymatic step with *psi* [4]; the IpsM methyltransferases are not orthologs of PsiM. A homology screen run in 2018 on *I. corydalina* would have returned no hits and concluded the species lacks psilocybin biosynthesis, yet the chemistry was reported in 1985 [33] and was later confirmed by independent UHPLC-MS/MS analysis [9].

The methodological prescription is to complement homology-based screens with logic-based ones: hidden Markov model searches that target the reaction architecture of psilocybin biosynthesis (decarboxylation → 4-hydroxylation → 4-OH-tryptamine kinase → SAM-dependent methyltransferase) rather than specific protein sequences. Pathway-architecture screens have been developed for siderophore biosynthesis, terpene cyclases, and ribosomally-synthesized peptides through tools such as antiSMASH [44], with the broader literature on BGC discovery and evolution reviewed in [20, 21]; their application to tryptamine biosynthesis would likely identify additional convergent inventions of psilocybin (or related psiloid) production in lineages currently missed because their enzymes belong to different sequence families. We predict that systematic application of such screens to fungal genomes will identify a substantially larger and more ecologically diverse set of psiloid-producing lineages than is currently recognized, and that several will lie outside the saprotroph/ECM dichotomy that defines the existing literature. *Massospora* [40] and *Dictyonema huaorani* [42] are early indications that the count is incomplete; a sequenced *Massospora* genome with confirmed biosynthetic genes would be the next major step.

Four falsifiable alternative hypotheses

We articulate four hypotheses for psilocybin's adaptive function in ectomycorrhizal Inocybaceae, ranging from grounded to essentially unstudied, each falsifiable.

Hypothesis 1: Basidiome-restricted deterrence against the mycetophilid/sciarid larval community.

Even in ECM contexts, basidiomes are colonized by fungivorous Diptera larvae. Põldmaa et al. [29, 31] showed in systematic boreal forest sampling that fungivorous mycetophilids and bolitophilids commonly colonize Agaricales basidiomes (37 fungus gnat species reared from 100 fungal hosts; 74% of fruitbodies infested) and that host specialization, while modest, is not negligible. Bruns [30] documented diverse fungivore guilds on Boletales basidiomes. Direct rearing observations of fungivores from Inocybaceae basidiomes have been published: Kitabayashi et al. [45] reported the springtail *Morulina alata* feeding on *Inocybe fastigiata* (= *Pseudosperma rimosum*); and the Jakovlev [46] review of fungal hosts of mycetophilids documents that *Rymosia batava* (Mycetophilidae) has been reared from multiple *Inocybe* species including the psilocybin-producer *I. aeruginascens* (Dely-Draskovits 1974, in Hungary; Jakovlev 1995, in Russian Karelia). The latter observation, mycetophilid larvae successfully completing development inside the basidiomes of a psilocybin-producing *Inocybe*, is direct evidence that the deterrence hypothesis, as applied to the chemistry-relevant Inocybaceae specifically, is not supported. Under this hypothesis, psilocybin is selected to interfere with the basidiome-feeding larval community, with selection acting on the time window during which the basidiome is ephemerally available. **Quantitative predictions:** (i) *Lycoriella ingenua* [47] development time on psilocybin-positive *Inocybe* basidiome material should be elevated by ≥30% relative to muscarine-positive non-psilocybin congeners under matched conditions; (ii) psilocybin-spiked artificial diet at 0.001–1% w/w should reproduce a measurable dose-response effect within this concentration range; (iii) *ips* gene expression should be elevated in basidiome relative to mycelium. The empirical foundation is the established mycetophilid-rearing literature [29–31, 45, 46]; the empirical gap is that no controlled feeding bioassay or dose-response study has been published for any of the five primary psilocybin-producing *Inocybe* species.

Hypothesis 2: Mycorrhizosphere chemistry, microbial competition, or host signalling.

ECM root tips and the surrounding rhizosphere are chemically active. *Laccaria bicolor* releases sesquiterpenes including (−)-thujopsene that reprogramme host root architecture [48] and secretes effector proteins such as MiSSP7 that interact with poplar JAZ6 and modulate jasmonate signalling [49, 50]. The Martin et al. [51] *Laccaria* genome and subsequent ECM genome-comparative work [52, 53] have established that ectomycorrhizal basidiomycetes deploy a substantial chemical and proteinaceous toolkit at the root interface [54]. Under this hypothesis, psilocybin or its intermediates act in the mycorrhizosphere, modulating microbial competitors, inhibiting fungivorous nematodes, or signalling to host or microbiome partners. We emphasize that this hypothesis is essentially unstudied. There are no published measurements of psilocybin in *Inocybe* mycelium, root tips, or soil, no published in vitro assays of psilocybin against ECM-relevant microbes, and no in planta tests. **Quantitative predictions:** (i) psilocybin or baeocystin should be detectable in *I. corydalina* mycelium, root tips, and rhizosphere soil above ~0.1 nM by UHPLC-MS/MS [9]; (ii) rhizosphere bacterial 16S diversity should differ measurably between psilocybin-producing and muscarine-positive non-producing congeners under matched host trees, with effect sizes detectable at $n \approx 20$ replicates per treatment; (iii) in vitro antimicrobial assays at 0.1–10 µM against ECM-relevant *Pseudomonas* and *Burkholderia* mycorrhization-helper bacteria [54] should show ≥10% growth inhibition for the hypothesis to gain traction. A polar zwitterionic structure is, a priori, a poor template for a broad-spectrum antimicrobial, which sets the expected effect size low; this is a reason to look quantitatively rather than a reason not to look.

Hypothesis 3: Defensive-vacuum substitution.

The Kosentka et al. [12] pattern, psilocybin presence concentrated in muscarine-loss subclades, is consistent with replacement chemistry: loss of muscarine creates a selective vacuum into which alternative defensive metabolites can be recruited. The fungal precedent for defensive-metabolite class shifts is well documented, *Aspergillus* aflatoxin/sterigmatocystin cluster gain and loss [55–57], *Fusarium* trichothecene cluster turnover [58, 59], Clavicipitaceae alkaloid loci [60], and the broader pattern of biosynthetic gene cluster birth, evolution, and death described by Rokas et al. [20]. Lind et al. [61] documented within-species BGC polymorphism in *Aspergillus*, and Khaldi et al. [62] documented HGT of the ACE1 secondary metabolite gene cluster from a *Magnaporthe*-like ancestor into *Aspergillus clavatus*. Spiteller [63] reviewed chemical defence strategies of higher fungi as the broader context. Under this hypothesis, the temporal sequence (muscarine loss preceding psilocybin gain) and the lineage-specific repetition of the pattern are the testable claims. **Quantitative prediction:** A Pagel [64] four-state correlated-evolution test on the joint trait states {Mus–Psi–, Mus–Psi+, Mus+Psi–, Mus+Psi+} should reject the independent model of evolution by likelihood-ratio test with ΔAICc > 4, with the path-dependence hypothesis specifically encoded by constraining q34 (psilocybin gain in a muscarine-positive background) to zero and contrasting q12 (psilocybin gain in a muscarine-absent background) against the independent-model rate. Critically, this rejection must also survive the Boyko–Beaulieu [16] hidden-rates Markov correction implemented in fitCorrelationTest (corHMM): if the HMM-corrected dependent model is no longer favoured over the rate-heterogeneous independent model by ΔAICc > 4, the path-dependence claim should be downgraded to "consistent with rate heterogeneity but not statistically distinguishable from it." These thresholds prospectively address the Maddison–FitzJohn [15] pseudoreplication critique and the unreplicated-burst pathology. The hypothesis cannot be advanced confidently until the Inocybaceae phylogeny is reanalyzed on the Matheny [8] generic framework with multilocus barcoding from [35–37], and until correlated-evolution tests are run with hidden-rates extensions [16, 65] and HiSSE-style trait-dependent diversification controls [66–68].

Hypothesis 4: Psilocybin is an incidental coproduct of selection on a different metabolite or chemical end-state.

This is the audacious hypothesis we develop in this paper. Hypothesis 3 frames the muscarine-loss/psilocybin-gain pattern as a defensive-vacuum substitution; Hypothesis 4 asks the next question: which molecule in the recruited pathway is the actual target of selection? The two hypotheses are complementary rather than competing: H3 specifies the macroevolutionary context in which recruitment occurs, H4 specifies the molecular target of the selection that retains the recruited pathway. The branched architecture of the *ips* pathway [4] is critical here: the IpsM1/IpsM2/IpsK system generates baeocystin as a primary end-product (formed when IpsK phosphorylates the monomethyl intermediate before further methylation), not solely as an intermediate to psilocybin, and psilocybin is the minor end-product in *I. corydalina* basidiomes [9]. The pathway is therefore not optimized for psilocybin production. **Falsification criterion.** Hypothesis 4 is falsified if purified psilocybin, at *Inocybe*-realistic concentrations (0.001–0.1% w/w basidiome equivalent), against any tier-1 fungivore, produces a clean dose-dependent deterrence effect that is rescued by a 5-HT2A antagonist. A positive result on this canonical receptor-mediated test would directly support Hypothesis 1 over Hypothesis 4, regardless of the bioactivity profiles of baeocystin, aeruginascin, or polymer end-states. Three sub-hypotheses follow. We develop (4a) and (4b) for completeness but consider (4c), selection on the polymerized indoloquinoid end-state of the blueing reaction, to be the strongest version of the structural reframing on independent structural, comparative, and

energetic grounds, and we develop the case for it accordingly.

(4a) Selection acts on baeocystin, the mono-methylated and 2- to 13-fold more abundant congener of psilocybin in *I. corydalina*. Psilocybin emerges as a side-product of further methylation (by IpsM2) followed by IpsK phosphorylation. The chemical-ecological literature has not tested baeocystin's bioactivity against any fungivore.

(4b) Selection acts on aeruginascin, the quaternary trimethylated tryptamine restricted to psilocybin-producing Inocybaceae and reported by Gartz [34] in *I. aeruginascens* at concentrations of the same order of magnitude as psilocybin and baeocystin in that species (UHPLC-MS/MS quantification of *I. corydalina* fungarium specimens detects aeruginascin at 0.02–0.37 mg/g dry weight, comparable to psilocybin in that species [9]). Aeruginascin is structurally related to bufotenidine (5-HTQ), a quaternary trimethyltryptamine known from toad parotoid secretions; the permanent positive charge of the quaternary ammonium prevents passive membrane permeation and substantially alters the receptor-binding profile relative to monomeric psilocybin/psilocin (the dephosphorylated metabolite 4-hydroxy-N,N,N-trimethyltryptamine has been shown to bind 5-HT1A, 5-HT2A, and 5-HT2B but not 5-HT3 [69]), with quite different mechanistic implications for any invertebrate-targeting role. Aeruginascin's distribution is striking: it is essentially restricted to the psilocybin-positive Inocybaceae subclade and is detectable only at trace levels (three orders of magnitude lower than psilocybin) in some *Psilocybe* species [41], raising the possibility that aeruginascin, not psilocybin, is the lineage-specific innovation that selection has been acting on, with psilocybin as the conserved tryptamine precursor.

(4c) Selection acts on the oxidatively polymerized end-state of psilocin. Lenz et al. demonstrated that psilocin polymerizes via a quinoid intermediate into indoloquinoid oligomers, with coupling occurring preferentially through C-5 to yield psilocyl 3- to 13-mers [5]; the 7,7′-coupled dimer was subsequently identified as the major blue chromophore [6]. The polymer chemistry is triggered by tissue injury (the iconic “blueing reaction”) via a two-step PsiP/PsiL enzymatic cascade, and the same authors propose that the phosphate ester of psilocybin serves a reversible protective function relative to the oligomer end-state. Under (4c), psilocybin is a stable, water-soluble, transportable precursor that polymerizes only on basidiome injury, and selection acts on a putative defensive function of the polymer. Three biochemically plausible mechanisms have been advanced in the literature for how such a polymer could function defensively: wound-sealing (a structural defence at the injury site, analogous to fungal melanin [70, 71]), tannin-like protein damage and reactive-oxygen-species generation in fungivore gut tissue (the formal proposal of Slot and Hoffmeister [32], supported by the well-established redox-cycling chemistry of quinoid compounds [72, 73]), and direct invertebrate deterrence by the quinoid pigment itself (paralleling the antifeedant role of plant naphthoquinones such as juglone [74, 75]). None of these mechanisms has been experimentally demonstrated for the psilocyl polymer specifically, but each has established precedent in fungal or plant chemical ecology; we develop the case for each in the Appendix.

The most consequential precedent is in-phylum and architecturally identical. *Cortinarius austrovenetus* basidiomes turn red-violet at the bite site when injured by insects: the fungal pigment austrovenetin is oxidized on tissue damage to hypericin, a known photoactive ROS generator [76]. The chemistry is mechanistically parallel to the psilocybin → psilocin → polymer cascade, a stable phenolic precursor accumulates in mature tissue, an injury-triggered enzymatic oxidation generates a reactive end-state, and the end-state (not the precursor) is the species under selection. Spiteller [63], in his review of higher-fungal

chemical defences, catalogued damage-activated chemical defence as a recurring strategy in basidiomycetes, and Siewert [76] extends the framework specifically to basidiomycete pigment chemistry. The polymer-as-selected-target hypothesis we develop here is therefore not novel in its broad architecture, the field has long recognized that some basidiomycete pigments operate as injury-triggered defences whose precursor is the storage form. What is novel is applying the framework specifically to psilocybin, where the cultural and pharmacological prominence of the monomer has anchored the chemical-ecology literature on receptor-mediated mechanisms rather than on the polymer end-state.

The structural-precedent argument: this precursor-to-active-defence architecture has direct precedent in plant damage-activated chemical defences. The most extensively characterized case is the glucosinolate–myrosinase system in Brassicaceae [7]: glucosinolates are stable, water-soluble storage compounds that are hydrolyzed by myrosinase upon tissue damage to release isothiocyanates, the actual herbivore-deterrent active species. The structural analogy is tight: both molecules carry stabilizing chemical modifications, a glucose moiety on glucosinolates, a phosphate ester on psilocybin, that confer water solubility and storage stability; like myrosinase, the PsiP/PsiL cascade is triggered by injury; like isothiocyanates, the polymer end-state is the chemically reactive species with putative defensive activity. Damage-activated chemical defence with stable inactive precursor and injury-triggered conversion to a reactive end-state is one of the best-characterized strategies in plant chemical ecology [7], and the psilocybin/polymer system fits the architecture exactly.

The energy-budget argument: producing 17.8 mg/g psilocybin in *Psilocybe azurescens* basidiomes [77] commits substantial tryptophan-derived metabolic flux to a single secondary metabolite. If the active defensive molecule were the monomer, a metabolically efficient defence would optimize for rapid turnover and tissue-localized concentration, producing psilocin where and when needed, rather than maintaining stable mg/g pools across the entire basidiome. The observed pattern, high-titer stable accumulation across mature basidiome tissue with injury-triggered conversion, is the metabolic signature of a precursor pool, not of a deployed defence. The expense of high-titer stable storage is justifiable only if storage is the function; otherwise the same defensive bioactivity could be achieved at a fraction of the metabolic cost by producing smaller quantities of an actively deployed molecule.

The conservation argument: the injury-triggered conversion to polymer is conserved across all psilocybin-producing lineages investigated. PsiP and PsiL homologues are encoded outside the canonical *psi* cluster [5] but are present and functional in saprotrophic Agaricales linked by HGT [1, 3], in the convergently-invented *ips* system in Inocybaceae [4], and (where investigated) in the entomopathogen *Massospora* [40]. The polymer-forming machinery has therefore been conserved across HGT events and convergent invention more strictly than any individual upstream biosynthetic step, Schäfer et al. [4] showed that the upstream enzymes of *ips* are non-homologous to those of *psi*, yet both pathways feed substrate into a polymer-forming downstream cascade. What is conserved across both modes of pathway acquisition is presumably what is selected for, and what is conserved is the conversion to polymer end-state, not the specific upstream enzymology that generates the precursor.

The biochemical-coincidence argument: the vertebrate pharmacology of monomeric psilocin is, on this reading, biochemically expected rather than indicative of selection. Tryptamines as a chemical class are 5-HT receptor ligands by structural necessity, they are built on the same indolylamine scaffold as serotonin itself [17], and any 4-hydroxylated methylated tryptamine will incidentally bind 5-HT receptors with

measurable affinity. Psilocin's psychedelic effect on mammals is a predictable consequence of structural conservation of 5-HT receptors across the bilaterian phylogeny, not evidence of fungal selection on the receptor-binding profile. The molecule that the chemical-ecology field has anchored on (psilocin/psilocybin) has the most prominent vertebrate pharmacology because of conserved tryptamine biochemistry, not because vertebrate-active pharmacology was the trait under selection in fungi. Under (4c), psychoactivity at 5-HT2A is downstream of a chemical scaffold that selection requires for unrelated reasons, and the cultural prominence of psilocybin in human pharmacology reflects this scaffold coincidence rather than the molecule's adaptive function.

A genuine complication: the entomopathogen context. The cicada pathogen *Massospora* uses psilocybin in a behaviour-modifying context (host manipulation), where polymer formation seems unlikely to be the active mechanism, the cicada is not damaging the fungal plug at the moment of behavioural manipulation, and behavioural manipulation is more naturally explained by a monomer-mediated central nervous system effect [40]. Three readings are possible: (i) *Massospora* deploys psilocybin via a mechanism distinct from the saprotroph/ECM context, in which case (4c) applies in the deterrence contexts but not in entomopathogenesis, and the chemistry has been recruited for two functionally distinct roles; (ii) the active molecule in *Massospora* is something other than psilocybin, the cross-system tryptamine-profile measurement we propose above would settle this, and a non-psilocybin active molecule would actually reinforce the broader argument that psilocybin is not the trait under selection; (iii) the polymer interpretation requires revision in light of the manipulation context, and (4c) needs to be reconciled with the observation that psilocybin-as-monomer has at least one documented adaptive role. We treat this as an honest open question rather than as a defeater for (4c): the strongest version of the deterrence-context argument is consistent with multiple readings of the manipulation context, and the cross-system test directly discriminates among them.

The fungal chemical-ecology literature has not articulated this storage-form framing for psilocybin, and doing so explicitly recasts the molecule from defensive agent to defensive precursor, an empirical claim, not a metaphor, with quantitative consequences: psilocybin and polymer should accumulate in different developmental and tissue contexts, the bioactivity profile should be displaced from monomer to oligomer, and the conserved injury-triggered conversion across HGT and convergent invention should be the locus of any selection signature rather than the upstream biosynthetic enzymes that vary in identity across lineages.

The unifying claim across all three sub-hypotheses is that the fungal natural-products literature has anchored on psilocybin because it is psychoactive in vertebrates: detectable, culturally prominent, pharmacologically tractable. But psychoactivity at vertebrate 5-HT2A receptors is unlikely to be the selective agent for a metabolite produced by an obligate ECM symbiont in temperate forest soil. Psilocybin in this reading is a stable repository of tryptamine chemistry whose ecological role is realized only on enzymatic conversion to baeocystin or aeruginascin, or on oxidative conversion to polymer. **Quantitative predictions:** (i) baeocystin or aeruginascin should show measurable insect-deterrent or antimicrobial activity at *Inocybe*-realistic concentrations (0.1–10 µM) where psilocybin shows none; (ii) psilocyl oligomer pigments should be detectable in *I. corydalina* mycelium under oxidative stress and should display measurable reactive-oxygen-species generating, antimicrobial, or invertebrate-deterrent activity; (iii) under the Tier 1 fungivory bioassays described below, dose-response effects should be larger for baeocystin or aeruginascin than for psilocybin against *Lycoriella* or *Bradysia* larvae. A null result for psilocybin coupled with positive results for one or more of its congeners would directly support Hypothesis 4 over Hypothesis 1.

A brief note on hypotheses we do not develop in depth here: non-arthropod grazers (slugs, mammals) and spore-dispersal manipulation through behavioural modification of mushroom-visiting animals are flagged in Meyer and Slot [18]. Both are plausible and warrant separate treatment but lack the empirical traction in the Inocybaceae specifically that the four hypotheses above provide.

Outstanding Questions Box 1

- Does any psilocybin-producing fungus, saprotroph or ectomycorrhizal, deter its co-occurring fungivore community in a controlled feeding assay? No published study addresses this directly.
- In direct head-to-head feeding assays at *Inocybe*-realistic concentrations, do baeocystin and aeruginascin deter *Lycoriella ingenua* or *Bradysia* larvae more strongly than psilocybin itself?
- Are arthropod 5-HT receptors functionally responsive to psilocin at biologically realistic basidiome concentrations, or is the inferred mechanistic basis of the deterrence hypothesis unsupported by direct pharmacology?
- What is the tissue-specific distribution of psilocybin, baeocystin, aeruginascin, and Lenz polymer in *Inocybe corydalina*? Mycelium, basidiome, root tip, and rhizosphere soil concentrations are all unmeasured.
- How many independent psilocybin gain events have occurred across the Inocybaceae when the trait is mapped onto a current multilocus phylogeny [8, 35–37]?
- Does the Kosentka [12] correlation between muscarine loss and psilocybin gain survive the Boyko–Beaulieu [16] hidden-rates Markov correction at $\Delta AICc > 4$?
- What is the regulation of *ips*-cluster expression across developmental stages and substrate conditions, and does it differ from *psi*-cluster regulation in *Psilocybe*?

A staged comparative experimental program

The structural problem we face is that the field has accumulated substantial phylogenomic and biochemical infrastructure but has not run the controlled chemical-ecological experiments those frameworks require for interpretation. We propose a five-tier program, framed as paired saprotroph and ECM tests.

Tier 1: Direct fungivory bioassays in saprotrophs and *Inocybe*. The experiment that has not been run, but should have been first, and now extended explicitly to test Hypothesis 4 against Hypothesis 1. *Lycoriella ingenua* (Sciaridae) per Cloonan et al. [47] as the primary fungivore, with *Bradysia impatiens* as a generalist comparator and *Drosophila putrida* or *D. testacea* as drosophilid representatives. Choice and no-choice feeding assays use basidiome tissue from *Psilocybe cubensis* (high psilocybin), *Psilocybe cyanescens* (documented host for Sciaridae larval development [14]), *Inocybe corydalina* (low psilocybin, dominant baeocystin), *Inocybe aeruginascens* (psilocybin + aeruginascin), and matched non-producing controls: *Stropharia rugosoannulata* (closely related to *Psilocybe* but psilocybin-negative; note that *S. aeruginosa* was the species reared alongside *P. cyanescens* in [14] and would be the more direct natural parallel where field-collectible) and *Agaricus bisporus* (no HGT history, well-characterized fungivore host [47]). Dose-response with purified psilocybin, psilocin, baeocystin, aeruginascin, and enzymatically-generated psilocyl polymer mixtures (PsiP/PsiL acting on psilocybin substrate [5, 6]) spiked into neutral basidiome substrate at 0.001–1% w/w spans the natural concentration range of *Inocybe* (~0.01%) through *Psilocybe*

(~1%) by an order of magnitude in each direction. Larval survival, development time, pupation success, and adult emergence are scored; behavioural choice is recorded by larval distribution after defined exposure times. The critical comparison is whether baeocystin, aeruginascin, or polymer mixtures individually replicate or exceed any deterrence effect observed for psilocybin, testing Hypothesis 4 directly against Hypothesis 1. Polymer mixtures are the highest-priority test compound under (4c): if the polymer end-state is the selected molecule, polymer should show the strongest deterrence at injury-realistic concentrations, and the relative effect sizes (polymer > monomers) provide direct discrimination of (4c) from (4a), (4b), and Hypothesis 1. A null result for psilocybin coupled with a positive result for baeocystin, aeruginascin, or polymer would directly support Hypothesis 4, with the relative rankings of the four candidate selected molecules localizing the actual target. A null result for all four monomers coupled with a positive result for polymer specifically would be the cleanest possible support for (4c). A null result for all five test compounds would falsify Hypothesis 1 and most versions of Hypothesis 4, supporting a non-deterrence framing (Hypothesis 2). If a polymer-specific effect is detected, the three candidate mechanisms outlined in the Appendix (wound-sealing, tannin-like protein damage with ROS generation, direct deterrence) make differential follow-up predictions: wound-sealing predicts species-independent deterrence tracking polymer concentration; tannin-like / ROS predicts dose-dependent gut-tissue damage attenuated by antioxidant co-administration and detectable as digestive-protein crosslinking in midgut tissue; direct deterrence predicts a sharp dose-response and effect even in pre-formed (non-injury-context) basidiome material. These are downstream questions contingent on the Tier 1 baseline result, but designing the bioassay to permit later mechanism-discrimination follow-up (preserving tissue, recording feeding behaviour rather than only mortality, including tissue-localization comparisons) is achievable at marginal cost.

Tier 2: Comparative basidiome fungivory surveys within the Inocybaceae. Pair the five primary psilocybin-producing *Inocybe* species (*I. corydalina*, *I. aeruginascens*, *I. haemacta*, *I. coelestium*, *I. tricolor*) with co-occurring muscarine-positive non-psilocybin *Inocybe* under matched host trees and habitats. Quantify fungivore damage (visual scoring), insect emergence (sealed-rearing), and basidiome microbiome composition. The Kosentka [12] phylogenetic scaffold provides the comparative framework; the European working group [8, 35–37] provides the species-level taxonomic infrastructure required to ensure that pairings are matched at appropriate phylogenetic depth. If psilocybin-producing taxa show systematically reduced fungivore loads relative to muscarine-positive controls under matched conditions, basidiome-restricted deterrence is supported in the ECM context.

Tier 3: Mycorrhizosphere chemistry. LC-MS/MS quantification of psilocybin and intermediates in mycelium, root tips, and rhizosphere soil from natural *Inocybe* ECM associations, using the validated UHPLC-MS/MS workflow established for basidiome tryptamines [9] and adapted for low-concentration soil/exudate matrices. Pair chemical quantification with 16S/ITS amplicon sequencing of the rhizosphere microbiome to test whether *Inocybe* root-tip communities differ between psilocybin-producing and non-producing congeners under matched host trees. In vitro activity assays of purified psilocybin against ECM-relevant bacteria (*Pseudomonas*, *Burkholderia* mycorrhization-helper guilds [54]) and competitor fungi establish baseline biological activity at quantified soil concentrations.

Tier 4: Phylogenetic comparative reanalysis with explicit path-dependence parameterization. Reanalyze the Kosentka [12] character matrix on an updated multilocus phylogenetic scaffold (ITS + LSU + RPB2 + TEF1-α), built within the Matheny [8] generic framework using the species-level taxonomic refinements from [35–37]. The path-dependence hypothesis is formally encoded as the Pagel [64] four-state

model with joint trait states {Mus−Psi−, Mus−Psi+, Mus+Psi−, Mus+Psi+} and eight transition rates, with q34 (psilocybin gain in a muscarine-positive background) constrained to zero and q12 (psilocybin gain in a muscarine-absent background) contrasted against the independent-model rate. Implement in BayesTraits Discrete (RJ-MCMC) and corHMM (corDISC) [16, 65]. The required threshold for sustaining the path-dependence claim is rejection of the independent model with ΔAICc > 4 and survival of the Boyko–Beaulieu [16] hidden-rates correction (fitCorrelationTest); failure of the latter prospectively addresses the Maddison–FitzJohn [15] pseudoreplication critique. HiSSE [66] with character-independent diversification (CID-2, CID-4) null models tests whether the pattern reflects trait-dependent diversification versus rate heterogeneity [67]; FiSSE [68] provides a non-parametric robustness check; the threshold model [78, 79] accommodates the possibility that psilocybin presence is a binarized concentration trait. The output is either statistical support for the muscarine-loss-then-psilocybin-gain hypothesis under modern best practice, or its honest downgrading.

Tier 5: Heterologous expression in *Laccaria bicolor*. The gold-standard mechanistic test of any hypothesis about the *Inocybe* pathway's function is to express the *ips* cluster heterologously in a tractable ECM basidiomycete and characterize the resulting strain: its mycelial growth, root-tip colonization, basidiome production, and rhizosphere chemistry. *Laccaria bicolor* is the ECM workhorse: its genome [51], Agrobacterium-mediated transformation [80], effector biology [49, 50], and root-architecture chemistry [48] are all established. We are honest that this is a long-horizon experiment. The methods stack: Agrobacterium-mediated transformation per Kemppainen et al. [80], hygromycin B selection at 100–150 µg/mL, and, critically, mandatory codon-optimized intron insertion in each transgene because basidiomycete transgenes lacking introns are typically silenced [81]. The *ips* cluster (IpsD, IpsK, IpsM1, IpsM2, IpsH; [4]) requires at minimum two prerequisite method-development steps that have not been validated in *Laccaria* or any ECM basidiomycete: (i) 2A-peptide-mediated polycistronic processing for multi-gene expression, and (ii) functional reconstitution of the IpsH P450 with an appropriate redox partner. The *Hebeloma cylindrosporum* carboxin-resistance (Hc.SdhR) gene [82] provides a usable second selectable marker for stacking experiments. The *psi* cluster has been heterologously expressed in *Aspergillus nidulans* (110 mg/L [83]), *Saccharomyces cerevisiae* (627 mg/L [84]), and *Escherichia coli* (1.16 g/L by feeding [85]), with subsequent yield optimization in tryptophan-catabolism-repressed fungal hosts [86]; the Hudspeth et al. [38] characterization of methyl transfer in psilocybin biosynthesis informs the methyltransferase optimization step, and Blei et al. [87] have demonstrated tryptophan-synthase-enhanced enzymatic synthesis from 4-hydroxyindole. No multi-gene heterologous BGC has been expressed in any ECM basidiomycete in the peer-reviewed literature. Realistic timeline is 24–36 months and requires dedicated method development. It is the right direction; it is not a near-term deliverable.

The decisive joint test. Among the five tiers above, the combination that most cleanly adjudicates Hypothesis 1 against Hypothesis 4 against the vestigial-cluster null, the possibility that the *ips* cluster is under relaxed selection in extant Inocybaceae and on the way to pseudogenization, is expression-context analysis (RNA-seq across pure culture, pre-contact mycelium, root-exudate-exposed mycelium, Hartig net, mature ectomycorrhiza, and basidiome tissues) coupled with signatures-of-selection analysis on the *ips* genes (pairwise dN/dS, RELAX, aBSREL across the available Inocybaceae sequence sample). Structured *ips* expression coupled with low pairwise dN/dS is the signature of functional retention, and the structuring developmental or symbiotic context names the function. Unstructured expression coupled with relaxed-selection signatures is the signature that supports the vestigial-cluster null. The off-diagonal cases,

structured expression but elevated dN/dS (a recently relaxed contextual remnant), or constitutive expression under purifying selection (a constrained but context-independent role), are messier but interpretable. The remaining tiers localize and validate this joint signal: Tier 1 establishes whether the dominant tryptamine has the bioactivity assumed of it, Tier 2 establishes whether basidiome-restricted deterrence is plausible at all, Tier 3 establishes the rhizosphere chemical context, Tier 4 establishes the macroevolutionary background against which any selection signature is interpreted, and Tier 5 mechanistically validates the inferred function. Critical to the falsification logic is that the vestigial-cluster null is epistemically privileged: any underpowered study fails to reject it by default. Power calculations to detect coordinated cluster expression and to detect deviation from dN/dS = 1 are therefore prerequisite to any claim against the null, not optional addenda. We follow this discipline throughout.

A feasibility caveat. Axenic *Inocybe corydalina* × *Quercus robur* synthesis is not on the methodological shelf. The only published axenic *Inocybe* synthesis on record is *I. lacera* × *Populus tremuloides* in peat–vermiculite synthesis tubes [88], with a realistic time-to-Hartig-net of four to six months and no protocol established for *I. corydalina* on any oak host. Expression-context analysis must therefore plan a fallback in parallel with axenic synthesis: field-collected ECM root tips with ITS-confirmed *I. corydalina* identity, harvested from oak stands beneath fruiting basidiomes, accepting the loss of pre-contact and exudate-only developmental stages and the consequent reduction of the gradient to a two-state contrast. The fallback is publishable and answers a restricted version of the question. We flag this not as a deferral but as a constraint that any honest project plan must absorb from day one.

A regulatory note: psilocybin is a Schedule I controlled substance in the United States and equivalent jurisdictions. The experimental program described here, particularly Tiers 1, 3, and 5 involving purified compound work, requires appropriate authorization (DEA Schedule I registration in the U.S., Home Office licensing in the U.K., or jurisdiction-equivalent) at participating institutions. Tier 2 fungivory surveys conducted on intact wild-collected basidiomes operate under the relevant fungus-collection permitting frameworks but do not require Schedule I authorization where no purified compound or extract is generated. Tier 4 is purely computational. We flag this not as an obstacle but as a constraint that bears on which laboratories are positioned to lead each tier.

Discussion

The Inocybaceae case is methodologically valuable beyond the immediate question of psilocybin's function. It reframes how the field should think about HGT-mobilized biosynthetic gene clusters in general. The implicit assumption in much of the BGC-evolution literature has been that a horizontally-transferred cluster carries its ancestral function with it, that, having been selected for in donor lineage A, it is selected for the same reason in recipient lineage B. The ECM *Inocybe* case shows that this assumption can be wrong in two directions. First, the cluster need not be horizontally inherited at all: convergent invention from non-homologous parts is possible, even for a complex multi-step pathway with rare reaction types like the unusual phosphotransfer step [4, 13], and current homology-based screens systematically miss such inventions (Box 1). Second, when convergent invention occurs in an ecologically distinct lineage, the function is not constrained by the ancestral selective regime, and the molecular product may not be the trait under selection at all. The *Inocybe* pathway and the *Psilocybe* pathway converge on the same molecular product, but the products may not be the trait under selection in either case. The branched *ips* pathway [4] produces baeocystin as a primary end-product; the *psi* pathway produces baeocystin as an intermediate. The

Lenz polymer chemistry [5, 6] suggests both pathways may feed into a common downstream end-state via different intermediate routes. The molecular product is the same; the ecological role is an open empirical question; and the trait under selection may be neither.

A second implication concerns how the field treats hypothesis validation in fungal chemical ecology. The Reynolds et al. [1] phylogenomic pattern is real; the deterrence interpretation of that pattern has been adopted, in our reading, more rapidly and more confidently than the empirical record warrants. The pattern of HGT and retention establishes that the *psi* cluster is under selection in recipient lineages [20, 21]. It does not establish what the selective agent is. Distinguishing selection on a metabolite as a deterrent, as a competitor, as a signal, or as a metabolic byproduct that happens to be retained by linkage to a positively selected gene, requires direct experimental work that has not been done. The Inocybaceae case is useful because it exposes this gap. We cannot port the *Psilocybe* explanation across to *Inocybe*, the niche disqualifies it, and the chemistry asymmetry deepens the disqualification. We are forced to ask the experimental questions we should have been asking of the *Psilocybe* system all along.

Disentangling cluster-level from product-level selection

A standard objection to Hypothesis 4 deserves direct engagement: if psilocybin is incidental, why has the *psi* cluster been retained across HGT events spanning ~67 My in saprotrophic Agaricales [3], and convergently invented in the Inocybaceae [4]? The answer requires distinguishing cluster-level retention from product-level selection. Retention of a multi-step biosynthetic cluster under HGT establishes that the cluster as a unit is under positive selection in recipient lineages [20, 21]; it does not identify which intermediate, end-product, or downstream end-state is the actual target of that selection. A cluster encoding the pathway tryptophan $\rightarrow$ . . . $\rightarrow$ baeocystin $\rightarrow$ psilocybin $\rightarrow$ (on injury) polymer is retained equally well whether selection acts on baeocystin, on psilocybin, on the indoloquinoid end-state, or on any subset of these. The phylogenomic signal is necessary but not sufficient for identifying the molecular target.

This distinction is methodologically central beyond the immediate psilocybin case. The BGC-evolution literature has, until now, largely conflated cluster-level retention with product-level function. The Inocybaceae case forces the disambiguation: the same end-product is reached by non-homologous enzymes through different reaction orders [4], so whatever selection is being satisfied is more conserved than the molecular machinery that delivers it. We propose that BGC retention identifies a class of selection but does not individuate the molecular target within that class, and that discriminating among targets within a retained cluster requires four classes of evidence applied in coordinated form: (i) expression-context analysis across developmental and symbiotic states; (ii) signatures-of-selection analysis on individual cluster genes (dN/dS, RELAX, aBSREL) rather than on the cluster as a whole; (iii) direct dose-response bioassays on each metabolite in the pathway, not solely on the canonical end-product; and (iv) where feasible, heterologous reconstruction of partial-pathway intermediates. Tiers 1, 3, 4, and 5 of our experimental program are designed to deliver each of these four classes of evidence, and the framework generalizes immediately to other HGT-mobilized BGCs: aflatoxin/sterigmatocystin, trichothecenes, ergot alkaloids, where cluster-level evolutionary signal has been similarly conflated with product-level selection.

The audacious version of our argument is structural rather than corrective. The field has anchored on the wrong question. The question is not “what does psilocybin deter?” but “is psilocybin even the trait under selection?” Hypothesis 4c, selection on the polymerized indoloquinoid end-state of the blueing reaction, is the strongest version of this reframing, supported by four independent lines of reasoning. First, the

precursor-to-active-defence architecture has direct precedent both in well-characterized plant systems (glucosinolate/myrosinase, cyanogenic glycoside/β-glucosidase [7]) and, critically, in the same fungal phylum: *Cortinarius austrovenetus* basidiomes turn red-violet at insect bite sites because the precursor pigment austrovenetin is oxidized on tissue damage to hypericin, a known photoactive ROS generator [76]. The chemistry is mechanistically parallel to the psilocybin → psilocin → polymer cascade, and Spiteller [63] catalogued damage-activated chemical defence as a recurring strategy in higher fungi. The architectural class is not new; what is new is recognizing that psilocybin belongs to it. Second, the energy-budget logic favours the storage interpretation: high-titer stable accumulation across mature basidiome tissue (up to 17.8 mg/g psilocybin in *P. azurescens* [77]) is the metabolic signature of a precursor pool, not of a deployed defence whose monomer is the active agent. Third, the injury-triggered polymer-forming machinery (PsiP/PsiL, encoded outside the canonical *psi* cluster [5]) is conserved across both modes of pathway acquisition, HGT-mobilized *psi* in saprotrophic Agaricales [1, 3] and the convergently invented *ips* system in Inocybaceae [4], whereas the upstream biosynthetic enzymes are non-homologous between *psi* and *ips*; what is conserved across both modes of acquisition is presumably what is selected for, and what is conserved is the injury-triggered conversion to polymer. Fourth, the vertebrate pharmacology of monomeric psilocin is biochemically expected of any 4-hydroxylated methylated tryptamine [17] and is therefore not, in itself, evidence of selection on receptor-binding properties. Alternative versions of Hypothesis 4 (4a, baeocystin selected; 4b, aeruginascin selected) are weaker but still consistent with the broader structural reframing. Several lines of evidence converge on the broader (4)-class reading. The *ips* pathway's branched output puts baeocystin at higher concentrations than psilocybin in *I. corydalina* [4, 9]. Aeruginascin is essentially restricted to the psilocybin-positive Inocybaceae subclade [9, 34] (with trace detection in some *Psilocybe* species [41]); its dephosphorylated metabolite binds 5-HT1A, 5-HT2A, and 5-HT2B but not 5-HT3 [69], and the quaternary ammonium's restriction on membrane permeation predicts peripheral rather than central pharmacology, with quite different mechanistic implications for any invertebrate-targeting role than psilocin's BBB-permeable, primarily centrally-acting profile. The Lenz polymer chemistry [5, 6] suggests that both *psi* and *ips* pathways may feed into a common downstream end-state, the oxidatively oligomerized indoloquinoid pigment, triggered by tissue injury, via different intermediate routes. Under this reading, the field has been studying the wrong end-product all along. Psilocybin is the molecule that is psychoactive in humans, the molecule that is detectable, the molecule with cultural and pharmacological prominence; but it may be a stable, water-soluble, transportable precursor whose actual ecological function is realized only on enzymatic conversion to baeocystin or aeruginascin, or on oxidative conversion to polymer.

This reframing generates a different research program. Rather than dose-response feeding assays of psilocybin against fungivores, we should be doing dose-response assays of baeocystin, aeruginascin, and Lenz polymer against fungivores, microbes, and ECM-relevant antagonists, in parallel with psilocybin, not instead of it. Rather than searching genomes for *psi* cluster homologs, we should be using pathway-architecture HMMs (Box 1) to identify convergent inventions whose enzymes belong to different sequence families. Rather than treating psilocybin's psychoactivity as evidence of selection, we should be treating it as a clue to the conserved chemistry that selection has been acting on through different molecular embodiments in different lineages. The Inocybaceae are the natural experiment that exposes this. We propose to take the experiment seriously.

Finally, the Inocybaceae are a tractable model system for chemical-ecological natural experiments. The family's seven-genus framework [8], the muscarine/psilocybin character matrix [12], the European species-level taxonomy [35–37], the convergent biosynthetic pathway [4], and the ECM ecology together provide a comparative structure that few other fungal groups offer at this resolution. We expect that the questions we raise here for psilocybin will arise for other tryptamines (aeruginascin [10, 34]), for muscarine itself (whose adaptive role is also poorly studied), and for the broader chemical ecology of the family. The Inocybaceae are paired naturally with the Strophariaceae and Bolbitiaceae to give the field the controlled comparison, saprotroph versus ECM, *psi* versus *ips*, that other secondary metabolites lack.

What would change our minds

We commit to specific observations that would force us to abandon or substantially weaken the structural reframing. First, a clean dose-response deterrence effect of purified psilocybin at *Inocybe*-realistic concentrations (0.001–0.1% w/w) against any tier-1 fungivore, particularly one rescued by a 5-HT2A-selective antagonist, would directly support Hypothesis 1 over Hypothesis 4. Second, a *Massospora* tryptamine profile dominated by psilocybin (with baeocystin and aeruginascin at the trace levels typical of *Psilocybe*), coupled with evidence that psilocybin is the active molecule in cicada behavioural manipulation, would constitute cross-system evidence that psilocybin, not its congeners, is the selected molecule. Third, demonstrating that baeocystin, aeruginascin, and oligomerized polymer pigments lack measurable bioactivity in tier-1 bioassays at realistic concentrations, with statistical power adequate to detect the relevant effect sizes, would falsify all three sub-hypotheses of Hypothesis 4. Fourth, signatures-of-selection analysis showing strong positive adaptive evolution specifically on IpsM2, the second methyltransferase, required for the di-methylated psilocin/psilocybin end-products but rejected by the alternative branch that yields baeocystin, would distinguish selection on di-methylated tryptamines from selection on the baeocystin branch. Fifth, demonstrating that the PsiP/PsiL polymer-forming machinery is not conserved in convergently evolved psilocybin systems, specifically, that *Inocybe corydalina* and other *ips*-bearing Inocybaceae lack functional homologues of the laccase or phosphatase activities that produce the indoloquinoid end-state, would falsify the conservation argument that motivates (4c) and would force reassignment to (4a), (4b), or back to Hypothesis 1. We frame these as commitments rather than as concessions: a paper that cannot specify what would falsify it is not making an empirical claim.

Conclusion

The Inocybaceae case reframes psilocybin from a model system with a known function to a model system with a contested function, and possibly with a misidentified target of selection. This is more useful for the field, not less. A model system with an established function generates incremental refinements; a model system with a contested function and an open question about which molecule is even the trait under selection generates structural questions and motivates the experimental work that the field has been deferring. The molecule that gives the family its cultural prominence may not be the molecule that gives it its evolutionary persistence.

We conclude with a community call. The five-tier program described above is achievable, but no single laboratory has the regulatory authorization, the host-tree access, the ECM cultivation infrastructure, and the phylogenetic comparative expertise required to run all five tiers. Tiers 1 and 5 require Schedule I authorization. Tier 2 requires multi-site European mycological field access matched to the

Bandini–Esteve-Raventós–Matheny species-level framework [8, 35–37]. Tier 3 requires LC-MS/MS infrastructure validated for tryptamine analysis [9] and adapted to low-concentration mycelium/soil matrices. Tier 4 requires phylogenetic comparative expertise current with the Boyko–Beaulieu hidden-rates literature [16] and the Maddison–FitzJohn pseudoreplication critique [15]. The empirical questions are well-posed, the methods exist, and the regulatory frameworks are navigable. We invite groups with the relevant infrastructure to take up the tiers for which they are positioned, and to engage seriously with the possibility that psilocybin's psychoactivity is incidental rather than adaptive. The Inocybaceae natural experiment will not stay open forever; the regulatory, ecological, and biotechnological pressures on psilocybin research are increasing, and the time to do the basic chemical ecology is now.

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Appendix: Candidate mechanisms by which the indoloquinoid polymer end-state could function as a defence

The polymer-as-selected-target reading of Hypothesis 4c rests on a structural argument, that the system has the architecture of a damage-activated chemical defence, but the specific mechanism by which the polymer end-state harms or deters a fungivore has not been experimentally characterized. Three mechanisms are biochemically plausible, each grounded in established precedent in fungal or plant chemical ecology, and each carrying a distinct experimental signature. We summarise them here for three reasons. First, the choice among them shapes follow-up assay design once Tier 1 has detected (or failed to detect) a polymer-specific deterrent effect. Second, the mechanisms together set up the falsification structure of Hypothesis 4c: a coherent picture of why selection would favour the polymer end-state over the monomer. Third, and most importantly for the paper's argument, the mechanism discussion grounds the claim that polymer-as-target is not speculation but a direct application of established basidiomycete chemical-ecology architecture to a system the field has not yet recognized as belonging to that class.

Mechanism 1: wound-sealing pigment (melanin-analog). The most conservative interpretation is that the polymer functions as a localized structural defence: an injury-triggered insoluble pigment that physically seals damaged tissue, prevents secondary microbial entry, and contributes to mechanical strengthening of the wound site. This is the established function class for fungal melanins [70, 71], which are oxidatively polymerized indoloquinoid or DHN-derived pigments produced by laccase- and tyrosinase-mediated cascades structurally analogous to the PsiP/PsiL system. Direct experimental evidence for the mechanical-strengthening function exists: Mattoon et al. [89] showed that melanized *Cryptococcus neoformans* cells exhibit lower fragmentation rates and greater retained cell area than non-melanized cells under both ultrasonic cavitation and French-press shear stress, the first direct experimental demonstration that fungal melanin per se confers mechanical resistance rather than merely correlating with it. The architectural fit with the Lenz et al. [5, 6] polymer is exact: a stable phenolic precursor (psilocybin) accumulates in mature tissue, an injury-triggered laccase (PsiL) converts it to a reactive intermediate (psilocyl radical), and the radical oxidatively polymerizes into an insoluble indoloquinoid pigment that deposits at the damage site, which is the iconic blueing reaction. The blueing reaction itself, in other words, is the expected phenotype of a wound-sealing mechanism: localized, injury-triggered, insoluble pigment deposition that visibly tracks the spatial extent of tissue damage. Under this interpretation, deterrence of fungivores is a downstream consequence of generic structural defence rather than a specific

receptor-mediated or chemically toxic effect.

Mechanism 2: tannin-like protein damage and reactive-oxygen-species generation in fungivore gut tissue. Slot and Hoffmeister, in their 2025 *Current Biology* synthesis [32], formally propose that "the chromophoric psilocin oligomers formed on mushroom injury bind to and damage proteins of fungivores in a way that is similar to plant tannins," and observe that the existence of dedicated enzymatic machinery accelerating this oligomerization suggests the process is not accidental, a point amplified in our argument by the conservation of the PsiP/PsiL machinery across both HGT-acquired *psi* and convergently invented *ips* systems. They explicitly leave open whether the proteinaceous damage operates through interference with nutrition and digestion, generation of oxidative stress, or some other route, and note that controlled experiments adjudicating among these possibilities are lacking. The plant chemical-ecology literature on tannins clarifies the relationship between these two sub-modes. The classical view that tannins act as protein precipitants reducing digestibility is now understood to apply primarily in vertebrate herbivores; in insects, the contemporary consensus from the Barbehenn–Salminen body of work [90, 91] is that tannin defence operates principally through oxidative stress: tannins oxidize at the high pH of insect midgut lumens, generating semiquinone radicals, hydrogen peroxide, and other reactive oxygen species that damage gut tissue. The chemistry supporting the redox-cycling sub-mechanism is well-established more generally: indoloquinoids of the class produced by the PsiP/PsiL cascade are redox-active, and quinoid compounds with this electronic structure can undergo redox cycling with cellular reductants (NAD(P)H, glutathione, ascorbate), repeatedly accepting and donating single electrons in the presence of molecular oxygen and producing superoxide, hydrogen peroxide, and ultimately hydroxyl radicals on each cycle [72, 73]. Two implications follow for psilocyl polymers in the insect-fungivore context: first, the protein-binding and ROS-generation routes are coupled rather than alternative, protein damage in insect midguts is itself substantially mediated by quinone-derived ROS, and second, the tannin parallel that Slot and Hoffmeister invoke predicts ROS-mediated damage as the operative mode in dipteran and other arthropod fungivores, with vertebrate-style protein precipitation a more relevant route in mammalian mycophagy. The mechanism class has not been demonstrated for psilocyl polymers specifically; we treat it as the most directly literature-attributed of the candidate mechanisms.

Mechanism 3: direct invertebrate deterrence by the indoloquinoid pigment. A third possibility is that the polymer is directly toxic or antifeedant to invertebrates without invoking either structural protection or ROS chemistry. Quinoid pigments in plants are well-characterized invertebrate deterrents: the naphthoquinone juglone, produced by walnut trees (*Juglans* spp.), causes weight reduction, antifeedant activity, deterioration of morphology and sexual development, and reduction in egg hatching across multiple phytophagous arthropod taxa [74, 75], and parallel deterrent effects are documented for plumbagin and other 1,4-naphthoquinones across nematicidal, insecticidal, and acaricidal contexts [74]. Within the basidiomycetes, the architectural argument for selection on quinoid end-states is independently visible: quinone synthetases have evolved twice independently in the phylum, generating the terphenylquinone-derived pigment classes from which boletes and bracket fungi draw their characteristic colours [92]. Two independent enzymatic inventions converging on quinoid pigment chemistry suggest that the chemistry itself, not any single biosynthetic route, is what selection has been acting on; the parallel to the dual *psi*/*ips* architecture is direct. Direct deterrence by the indoloquinoid polymer would not require either melanin-style structural function or systemic ROS toxicity to the fungivore; the compound class itself is sufficient. This mechanism most closely parallels the well-characterized plant quinone defences and is the

simplest of the three.

Precedent in basidiomycete chemical ecology. Damage-activated pigment chemistry is a recurring theme in mushroom chemical defence [63, 76]. The closest precedent is in *Cortinarius austrovenetus*, where insect bites trigger oxidation of the pigment austrovenetin to hypericin, a known photoactive ROS generator [76]. The chemistry is mechanistically analogous to the psilocybin → psilocin → polymer cascade: a stable phenolic precursor accumulates in mature tissue, an injury-triggered oxidation generates a reactive end-state, and the end-state is the species under selection. Critically, the wound-induced end-state in this system has documented invertebrate toxicity in controlled feeding assay: Samuels and Knox [93] demonstrated that purified hypericin is insecticidal to *Manduca sexta* larvae, a controlled-assay lepidopteran model rather than a natural *Cortinarius* fungivore, with an LD50 of 16 µg/g initial larval fresh weight under standard light conditions, with sublethal doses retarding larval growth in a dose-dependent manner. The relevance is mechanistic: the wound-induced compound class itself has measurable insecticidal bioactivity in controlled exposure, regardless of which specific arthropod taxon is the natural agent of selection. The architectural class is therefore not merely speculative for basidiomycetes: it is established, the wound-induced end-state has measurable defensive bioactivity, and the polymer-as-target framing for psilocybin is a direct application of this established class. *Lepiota americana*, *Lactarius deliciosus*, and *Agaricus xanthoderma* display similar wound-induced pigment chemistry on different chemical scaffolds (a phenoxazone in *Lepiota*, a guaiane sesquiterpene in *Lactarius*, and 4,4′-dihydroxyazobenzene in *Agaricus*) [76], and Spiteller's earlier review [63] catalogued damage-activated chemical defence as a general strategy in higher fungi. The framework we develop for psilocybin is therefore not novel in its broad architecture, the field has long recognized that some basidiomycete pigments operate as injury-triggered defences, and the psilocybin/polymer system is in one respect more strongly conserved than these single-species examples: the precursor-cascade architecture survives both horizontal cluster transfer (in saprotrophic Agaricales) and convergent invention (in Inocybaceae). What is novel here is applying the framework specifically to the psilocybin system, where the cultural and pharmacological prominence of the monomer has anchored the chemical-ecology literature on receptor-mediated mechanisms rather than on the polymer end-state.

Mechanisms may operate jointly. The three mechanisms are not mutually exclusive. A polymer that physically seals the wound site (Mechanism 1) can simultaneously generate ROS in the gut of any fungivore that ingests damaged tissue (Mechanism 2) and provide direct chemical deterrence at the contact level (Mechanism 3). Joint operation is in fact the expectation under H4c, because selection on a polymer end-state with multiple defensive consequences is more easily reconciled with the observed conservation of the PsiP/PsiL machinery across both HGT-acquired *psi* and convergently invented *ips* systems than is selection on any single mode operating in isolation. We frame the three mechanisms separately because they have separable experimental signatures and separable falsification paths, not because the polymer is hypothesized to do exactly one thing.

Why this matters for the experimental program. The three mechanisms differ in their experimental signatures, and Tier 1 of the proposed program (Figure 2) is designed so that an observed polymer-specific deterrent effect can subsequently be assigned to one or more of these modes through a small set of follow-up assays. Wound-sealing predicts that the polymer's defensive activity is localized, physical, and largely independent of fungivore physiology, a polymer-treated wound site should resist invertebrate damage regardless of the species tested, and the deterrent activity should track polymer concentration rather than fungivore-specific receptor or detoxification machinery. The discriminating assay is mechanical removal of

the polymer-coated tissue surface: if abrasion of the wound-site polymer restores fungivore access to underlying tissue, structural sealing is operative. Tannin-like protein damage with ROS generation predicts dose-dependent gut-tissue damage attenuated by antioxidant co-administration, and digestive-protein modification detectable as reduced soluble protein recovery and altered midgut proteomic signatures in fed fungivores; the effect should be amplified in fungivore species with reduced antioxidant capacity. The discriminating assay is co-administration of ascorbate or glutathione precursors with polymer-spiked diet: if antioxidant rescue restores fungivore survival or development, the redox-cycling mode is operative. Direct deterrence predicts a sharp dose-response in feeding assays and a deterrent effect even in pre-formed (non-injury-context) basidiome tissue. The discriminating assay is presentation of pre-polymerized basidiome material at controlled concentrations independent of injury context: if deterrence persists, the compound class itself is sufficient. We treat the choice among Mechanisms 1, 2, and 3 as a downstream question contingent on the Tier 1 result, not as a prerequisite for evaluating Hypothesis 4c. The mechanism question is also of practical relevance for industrial heterologous-expression contexts, where the protective function of the polymer end-state is exactly what would need to be characterized before any field deployment of engineered psilocybin-producing strains.

Outstanding Questions Box 2

- Is psilocybin's psychoactivity at vertebrate 5-HT2A receptors the trait under selection in fungi, or is it incidental to selection on baeocystin, aeruginascin, or polymer end-states?
- Will heterologous expression of the *ips* cluster [4] in *Laccaria bicolor* generate a strain with altered rhizosphere chemistry, root-tip colonization, or fungivore exposure?
- Do the IpsM1 and IpsM2 methyltransferases reflect a gene duplication that allows pathway branching to baeocystin, and is the branched output adaptive or vestigial?
- At *Inocybe*-realistic basidiome concentrations of aeruginascin (0.02–0.37 mg/g dry weight in *I. corydalina* [9]), does it deter fungivores where psilocybin does not, given that its dephosphorylated metabolite binds 5-HT1A, 5-HT2A, and 5-HT2B but not 5-HT3 [69] and is BBB-impermeable through the quaternary ammonium?
- Are there additional convergent psilocybin pathways, in non-Inocybaceae lineages currently missed by genome-based screens, that have escaped detection because they share no homology with the *psi* cluster (Box 1)?
- Do psilocyl oligomers [5, 6] accumulate in *I. corydalina* mycelium under oxidative stress, and which of the three candidate mechanisms (wound-sealing, tannin-like protein damage with ROS generation, direct invertebrate deterrence; see Appendix) best accounts for any defensive activity detected in in vitro assays?
- What is the population genomic signal of selection on the *ips* cluster across European *Inocybe corydalina* populations sampled across host-tree gradients?
- Does the Inocybaceae case generalize: how often, across fungal natural products, has the field misidentified the molecule under selection because it anchored on the molecule with the most prominent vertebrate pharmacology?

Figure Legends

Figure 1. Convergent assembly of psilocybin biosynthesis in the Agaricales, and a chemistry asymmetry that reorders the explanatory burden. Three panels. (A) Phylogenetic backbone showing two ecologically and biosynthetically distinct lineages of psilocybin-producing fungi. Saprotrophic genera (*Psilocybe*, *Gymnopilus*, *Panaeolus*, *Pluteus*, *Pholiotina*) are linked by horizontal transfer of the *psi* cluster (red dashed arrows; based on [1, 3, 14]); the cluster encodes PsiD (decarboxylase), PsiH (P450 monooxygenase), PsiK (kinase), PsiM (methyltransferase), and PsiT (transporter). The ectomycorrhizal genus *Inocybe* (Inocybaceae, blue) carries the non-homologous *ips* cluster (IpsD, IpsH, IpsK, IpsM1, IpsM2) [4, 14], convergently invented rather than horizontally acquired. The cicada pathogen *Massospora* [40] represents a likely third independent origin (biosynthetic genes not yet identified); the lichenized basidiomycete *Dictyonema huaorani* [42] represents a preliminary ecological context. (B) Concentration asymmetry across psilocybin-producing fungi (mg/g dry weight, log scale). Among *Psilocybe*, psilocybin titres span: *P. cubensis* 0.65–3.51 mg/g, *P. semilanceata* 1.28–11.42 mg/g, *P. cyanescens* 2.34–13.81 mg/g, *P. serbica* var. *bohemica* 1.55–15.54 mg/g, with *P. azurescens* reported up to 17.8 mg/g [77]. *I. corydalina* carries psilocybin at ~0.08–0.28 mg/g (approximately 10- to 60-fold lower than high-titer *Psilocybe*), with baeocystin at ~0.50–0.98 mg/g (2- to 13-fold higher than its psilocybin) and aeruginascin at 0.02–0.37 mg/g [9]. (C) Pathway architecture. The *psi* pathway proceeds linearly: tryptophan → tryptamine (PsiD) → 4-hydroxytryptamine (PsiH) → norbaeocystin (PsiK) → baeocystin → psilocybin (iterative PsiM methylation), yielding psilocybin as the main end-product. The *ips* pathway proceeds in a different order: tryptophan → 4-hydroxy-L-tryptophan (IpsH; deduced not tested in vitro), with branching at 4-OH-NMT where IpsK creates baeocystin as a final product (IpsM1/M2 reject phosphorylated substrates).

Figure 2. The proposed staged comparative experimental program. Five-tier paired-test design that adjudicates Hypothesis 1 (deterrence) against Hypothesis 4 (incidental coproduct) and the alternatives. Tier 1 (saprotroph + *Inocybe* fungivory bioassays): controlled feeding assays of *Lycoriella ingenua* [47] and *Bradysia impatiens* larvae on basidiomes from psilocybin-producing *Psilocybe* and *Inocybe* versus dose-matched non-producing controls (*Stropharia rugosoannulata*, *Agaricus bisporus*), with parallel dose-response assays for purified psilocybin, psilocin, baeocystin, and aeruginascin spiked at 0.001–1% w/w. Tier 2 (Inocybaceae basidiome fungivory surveys): paired field surveys of psilocybin-producing and muscarine-positive non-psilocybin *Inocybe* under matched host trees [8, 35, 36]. Tier 3 (mycorrhizosphere chemistry): UHPLC-MS/MS quantification of psilocybin and congeners in *Inocybe* mycelium, root tips, and rhizosphere soil (basidiome workflow [9] adapted for low-concentration matrices) paired with rhizosphere microbiome 16S/ITS amplicon sequencing. Tier 4 (phylogenetic comparative reanalysis): Pagel four-state correlated-evolution test [64] with corHMM hidden-rates correction [16, 65] on an updated multilocus scaffold; required threshold ΔAICc > 4 surviving HMM correction. Tier 5 (heterologous expression in *Laccaria bicolor*): long-horizon (24–36 months) construction of *L. bicolor* strains expressing the *ips* cluster, building on *Aspergillus* [83], *Saccharomyces* [84], and *E. coli* [85] heterologous platforms with mandatory intron insertion [81] and 2A-peptide validation. Each tier is annotated with its primary quantitative prediction, the hypothesis it adjudicates, and its time and regulatory horizon.

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