

1 **FORUM-ARTICLE**

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3 **Resisting antimicrobial resistance: Lessons from fungus farming ants.**

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5 *Ayush Pathak*¹, *Steve Kett*², *Massimiliano Marvasi*^{3*}

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7 ¹ Department of Life Sciences, Imperial College London, London. UK.

8 ² Department of Natural Sciences, Middlesex University London, London. UK.

9 ³ Department of Biology. University of Florence, Italy.

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11 * Corresponding Author: Massimiliano Marvasi. Department of Biology. University of
12 Florence. Via Madonna del Piano 6, Sesto Fiorentino, Florence. Tel. +39 055 4574749. Italy.
13 email: massimiliano.marvasi@unifi.it.

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16 **ABSTRACT**

17 Attine ants use antimicrobials produced by commensal bacteria to inhibit parasites on their
18 fungal gardens. However, in this agricultural system, antimicrobial use does not lead to
19 overwhelming resistance, as is typical in clinical settings. Mixtures of continually-evolving
20 antimicrobial variants could support this dynamic.

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23 **Symbiotic antiparasite defence strategies.**

24 In an antimicrobial-mediated evolutionary arms race, the synthesis and release of
25 antimicrobials evolves in one group of organisms and their competitor organisms counter this

26 by evolving antimicrobial resistance. Within such a milieu of intense selection and counter-
27 selection, not only will participating organisms evolve, but so too will the antimicrobials
28 themselves.

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30 Fungus-growing ants (tribe: *Attini*) have cultivated specific fungi as food for 60 million years
31 [1]. Their co-evolutionary interdependence is so refined that, for many attine species, their
32 fungal cultivars are not found outside this symbiotic association [1, 2]. The fungi need
33 specific microclimates and nutrition provided by the ants and, in turn, constitute the ants' sole
34 food source. Other fungi, of the genus *Escovopsis*, can invade the cultivated fungus and,
35 because the ants rely entirely on cultivated fungus for food, this parasitism is detrimental to
36 the ants [2]. To counteract these parasitic fungi, ants have evolved multiple strategies. One
37 is a tripartite mutualistic relationship, within which the ants host antimicrobial-producing
38 bacteria on their bodies to protect their fungal cultivar. Many of these bacteria have
39 coevolved with their hosts, producing antimicrobials to inhibit the parasitic fungi whilst in
40 return the ants provide them with nutrition and a microclimate suitable for growth [3]. The
41 parasitic fungi compete with the ant-associated bacteria, as both depend upon the same
42 fundamental source from which they directly, or indirectly, derive nutrition (BOX 1).

43 Thus, a question arises: Why are these antimicrobials still effective in controlling the parasitic
44 fungi even after millions of years, whilst pharmaceutical antimicrobials are rendered
45 ineffective within a few decades?

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47 **Antimicrobial heterogeneity through the evolution of gene clusters.**

48 To control the parasitic *Escovopsis*, the ants house antimicrobial-producing strains of
49 *Pseudonocardia* and *Streptomyces* bacteria on their cuticle. *Streptomyces* strains inhabiting
50 attine clades are acquired via environmental sampling and are, thus, not associated with

51 specific taxa, but the *Pseudonocardia* are non-randomly associated with attine species and
52 display co-speciation at higher taxonomic levels [4]. Ant-associated *Streptomyces* produce
53 the antimicrobials candicidin and antimycin [5 and references within]. Variants of antimycin
54 produced by *Streptomyces* differ in ultraviolet absorbance profile, liquid chromatography
55 retention time and mass-to-charge (m/z) ratio [5], and are produced by 14 gene clusters
56 (ranging from 15 to 17 genes). The clusters share a region which synthesises the dilactone
57 core common to all antimycin analogues [5, 6]. Therefore, such gene clusters (originating
58 from one ancestral cluster) would support the synthesis of different antimicrobial variants [5,
59 6]. A similar organisation has been identified in the *Pseudonocardia* spp., which produce
60 multiple, structurally-similar antimicrobials such as dentigerumycin, gerumycin A,
61 gerumycin B and gerumycin C, as well as a polyene antifungal, nystatin P1 [4, 7]. The
62 structural similarity of dentigerumycin, gerumycin A, gerumycin B and gerumycin C, along
63 with region-specific similarity of two of the three clusters responsible for their synthesis,
64 suggests they derive from a single ancestral cluster that diversified in response to selection
65 from antimicrobial-resistant organisms (Figure 1). Several regions of the gene clusters
66 producing these antimicrobials are flanked by mobile genetic elements (transposases,
67 integrases, endonucleases), indicating horizontal gene transfers play a role in their
68 recombination [7]. Such mobile genetic elements could also generate variation within the
69 gene cluster of one clonal line via transference in and out of the cluster. Similarly, in the
70 genomes of the *Pseudonocardia* phylotypes Ps1 and Ps2, novel nystatin P1-like compounds
71 are encoded by at least 14 biosynthetic gene clusters sharing multiple common genes [8].
72 Thus, it seems likely that variability in effectiveness of variants of antimycin produced by
73 *Streptomyces* and variants of nystatin and dentigerumycin produced by *Pseudonocardia* is a
74 result of constant variation in the gene clusters producing them.

75 *Escovopsis* has evolved countermeasures; *in vitro* tests suggesting it can develop resistance to
76 antimicrobials produced by *Pseudonocardia*, even if the majority of wild populations remain
77 susceptible to them [9]. *Escovopsis* is an obligate parasite of the fungus cultivar. This habitat
78 specificity exerts further selection pressure on the parasite to develop resistance to
79 antimicrobials produced by the ant-associated bacteria. Furthermore, recent discovery of two
80 specialised secondary metabolites produced by *Escovopsis* has offered new insight regarding
81 antimicrobial-mediated antagonism between parasite and mutualist triumvirate [2]. Both
82 metabolites inhibit *Pseudonocardia* growth and one, shearinine D, degrades ants' *Escovopsis*
83 weeding efficiency and, at high concentrations, is lethal to them. It may well be that a similar
84 pattern of gene cluster evolution might be present in the *Escovopsis* as described in
85 *Pseudonocardia* and *Streptomyces*.

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87 **Comparing arthropod and human strategies of antimicrobial usage.**

88 If modification of antimicrobial-synthesising gene clusters is amplified in the presence of the
89 parasite, the diversity of bacterially-synthesised antimicrobials suggests that there is
90 continuous selection pressure on the bacteria to evolve new variants. They have coevolved
91 with ants that provide their nutrition and microclimate, so the ants' continuing existence is
92 necessary for their survival. In this co-evolutionary arms race, novel bacterial antimicrobial
93 compounds can be formed via novel gene cluster rearrangement or mutations. Novel
94 compounds so generated achieve greater or lesser evolutionary success based upon the
95 *Escovopsis* strain antimicrobial susceptibility. This mechanism is best explicated by Red
96 Queen Dynamics [10], by which in the long term, continual evolution of novel antimicrobial
97 compounds would be encouraged thus preventing sympatric populations of *Escovopsis* from
98 acquiring effective antimicrobial resistance.

99 In the last decades several models and experimental studies based upon them have been
100 developed, lending some support to this hypothesis. Mathematical and clinical trials show
101 that mixing antibiotics results in resistance reduction [11]. Equally, other symbioses of
102 microorganisms with marine invertebrates, insects and plants, have been shown to rely upon
103 antibiotic mixtures diversified by interspecies and intraspecies interactions, and constructed
104 in conjunction with the evolution of biosynthetic gene clusters [12]. The short generation
105 time of bacteria, rapid recombination of clusters plus horizontal gene transfers [9] are further
106 amplified by marked potency variations offered by only slight antimicrobial structural
107 differences [12]. Such mixtures of antibiotics and their derivatives can even reverse antibiotic
108 resistance via molecular (molecular synergy, antagonism, and suppression) and evolutionary
109 interactions (cross-resistance and collateral sensitivity) [11]. Thus, whereas cross-resistance
110 to whole classes of antimicrobial compounds is a feature of clinical antimicrobial
111 applications, intriguingly, there is little evidence of similar effects limiting efficacy of the
112 structurally-similar compounds employed by the attines' symbionts. If cross-resistance is
113 genuinely absent from this system, it would be of great clinical relevance [11 and references
114 within].

115 When comparing the attine model of natural selection and diversification of antimicrobials to
116 antimicrobial usage in clinical settings, the contrast is striking. Humans use diverse
117 antimicrobials, but they are structurally discrete compounds rather than the diverse range of
118 subtle variants utilised by the ants and their mutualists. The humans' strategy is also
119 different; employment of discrete antimicrobials as means of rapid pathogen elimination
120 rather than one facet of a long-term strategy of progressive inhibition. Finally, and brutally,
121 the attines have no ethical constraints: not all individuals involved in this arms race must
122 survive.

123 Bearing this in mind, although all anthropogenic antimicrobials have natural blueprints, it
124 could be that use of structurally-discrete antimicrobials has outlived its usefulness. A new
125 strategy of *in vitro* antimicrobial-mediated arms race simulations would permit evaluation of
126 gene cluster response and emulate an evolutionary approach to antimicrobial generation and
127 utilisation which has served the attines and their allies for 60 million years.

128

129 **BOX 1**

130 **Attine ants, allies, enemies and coevolved crypts.** More derived clades of attine ants such
131 as the leaf-cutting ants (*Atta* spp. and *Acromyrmex* spp.) specialise in the cultivation of
132 *Leucoagaricus* spp. fungi. In this mutualistic association *Leucoagaricus gongylophorus* is an
133 obligate cultivar, and forms the ant colony's dominant food source [1]. The fungus is
134 vertically transmitted from colony to colony by the gynes (female reproductive ants) when
135 they first establish their own colonies. The *Leucoagaricus* cultivar may be parasitised by
136 fungi of the genus *Escovopsis* [2]. In response, the ants house multiple antimicrobial-
137 producing bacteria on their cuticle in coevolved cuticular crypts and specialised exocrine
138 glands. The two Actinobacteria predominantly associated with the ants are *Pseudonocardia*
139 and *Streptomyces* [4]. Bacteria are also carried by the gynes on their mating flights and
140 transmitted to offspring colonies [3]. A phylogenetic rooted-tree reconstruction of all known
141 fungus-growing ants showed that the crypts are specifically evolved to house the
142 Actinobacteria. They are morphologically different and differently located on the body of
143 ancient paleo-attines, more basal attine genera and in the later evolved leaf-cutting attines [3].

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148 **CAPTION**

149 **Figure 1.** Summary of the interactions among symbionts of *Acromyrmex* sp. leaf-cutting ants
150 and microorganisms living in their nest. Ants grow the mutualist (green arrows) fungi
151 *Leucoagaricus* spp on cut leaf fragments to provide their sole food source. The parasitic
152 fungus *Escovopsis* also feeds off *Leucoagaricus* (antagonism, red lines). Mutualistic bacteria,
153 *Pseudonocardia* (Phylum Actinobacteria) live on the ants, fed via subcuticular glands and, in
154 return, provide antimicrobial compounds to kill the parasite *Escovopsis* (antagonism).
155 *Escovopsis* counteracts the defensive mutualists by either evolving resistance to the
156 antimicrobials produced by the *Pseudonocardia* or via producing antimicrobials such as
157 melinacidin and shearinine that inhibit *Pseudonocardia*. The evolution of resistance in
158 *Escovopsis* to antimicrobials produced by *Pseudonocardia* induces selection pressures on the
159 bacterial gene clusters (dotted pale blue arrow), causing the evolution of antimicrobial gene
160 clusters harboured in *Pseudonocardia*, via rearrangement of the gene clusters or via
161 positively selected mutations in the gene clusters, thus inducing synthesis of novel
162 antimicrobial compounds.

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