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Microbiology

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Abstract:	<p>The fungal pathogen <i>Batrachochytrium dendrobatidis</i> has caused declines and extinctions in hundreds of amphibian species across the world. Virulence varies among and within lineages; the Global Panzootic Lineage (GPL) is the most pathogenic, although there is also variation in lethality between GPL isolates. Amphibians have a number of defences against pathogens, and skin products including the microbiota and host peptides have been shown to have considerable influence over disease progression. Here we show the collective skin products (the mucosome) of two amphibian species show significant variation in their ability to inhibit different globally-distributed isolates of GPL. This may in part explain the variation in disease susceptibility of hosts to different strains of <i>Batrachochytrium dendrobatidis</i>. More work is required to identify particular traits associated with mucosomes that confer broad-spectrum inhibition across GPL in order to facilitate the development of prophylaxis and/or treatments for chytridiomycosis in situ.</p>

1 **Amphibian skin defences show variation in ability to inhibit growth of *Batrachochytrium***
2 ***dendrobatidis* isolates from the Global Panzootic Lineage**

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13 Keywords: chytridiomycosis, microbial symbionts, pathogen susceptibility, fungal pathogens, innate
14 defences, mucosome, skin products

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17 Abbreviations:

18 GPL: Global Panzootic Lineage

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41 **Abstract**

42 The fungal pathogen *Batrachochytrium dendrobatidis* has caused declines and extinctions in hundreds
43 of amphibian species across the world. Virulence varies among and within lineages; the Global
44 Panzootic Lineage (GPL) is the most pathogenic, although there is also variation in lethality between
45 GPL isolates. Amphibians have a number of defences against pathogens, and skin products including
46 the microbiota and host peptides have been shown to have considerable influence over disease
47 progression. Here we show the collective skin products (the mucosome) of two amphibian species
48 show significant variation in their ability to inhibit different globally-distributed isolates of GPL. This
49 may in part explain the variation in disease susceptibility of hosts to different strains of
50 *Batrachochytrium dendrobatidis*. More work is required to identify particular traits associated with
51 mucosomes that confer broad-spectrum inhibition across GPL in order to facilitate the development of
52 prophylaxis and/or treatments for chytridiomycosis *in situ*.

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56 **Main article**

57 Although there are a number of emerging infectious diseases that are devastating wildlife populations
58 globally, chytridiomycosis is unique in its ability to infect amphibian hosts across an unprecedented
59 diversity of genera and species within a given class of vertebrates [1]. This disease has been linked to
60 the decline and extinction of hundreds of amphibian species worldwide, and it is the most devastating
61 wildlife disease of vertebrates in recorded history [1]. Amphibian chytridiomycosis is caused by fungal
62 Chytridiomycetes of the genus *Batrachochytrium*, of which two have been identified to date; *B.*
63 *dendrobatidis* and *B. salamandrivorans* [2,3]. Declines from *B. salamandrivorans* are thought to be
64 recent and restricted to salamander populations in Northern Europe, although its' spread to other
65 geographical regions are predicted to cause additional population declines and extinctions [4, 5].
66 *Batrachochytrium dendrobatidis*, on the other hand, has been causing declines across the whole class
67 of amphibians on a worldwide scale since the 1970's [1]. Although there are a number of globally
68 distributed endemic lineages of *B. dendrobatidis* that do not appear to cause mass mortality events
69 within their range, the hypervirulent Global Panzootic Lineage (GPL) continues to cause amphibian
70 declines and extinctions in the Americas, Australia and Europe [1]. In addition, there is variation in the
71 virulence of different GPL isolates for a given host species, however little is known about factors that
72 influence host susceptibility across the genetic and pathogenicity variation exhibited by GPL [6-9].
73 Amphibians, like all vertebrates, have evolved a number of defences to protect them from infectious
74 diseases. Of particular interest are skin-associated products found in the mucus of amphibians, which
75 form the first line of defence on contact with pathogens such as *Batrachochytrium spp.* These
76 products include peptides, lysozymes, alkaloids, antibodies, symbiotic bacteria and bacterial
77 metabolites, and are collectively known as the 'mucosome' [10]. The *in vitro* anti-*B. dendrobatidis*
78 function of the mucosome has been shown to correlate directly with *in vivo* susceptibility and pathogen
79 prevalence across a number of amphibian species [10]. It has previously been shown that individual
80 bacteria isolated from the skin of amphibians show variation in their ability to inhibit across the range
81 of genetic variation shown by GPL [11-13], but whether this is also true for the mucosome has not yet
82 been tested.

83
84 Here we determine whether mucosomes collected from two host amphibian species show variation in
85 their inhibitory capabilities across a suite of eight globally-distributed *B. dendrobatidis* GPL isolates
86 (Table 1). *Batrachochytrium dendrobatidis* isolates were selected that appear in different parts of the
87 *B. dendrobatidis* GPL phylogenetic tree (O'Hanlon, pers. comm.) and that represent an international
88 distribution, including four isolates from South Africa where the frogs used in the study were collected.
89 Isolates originated from a range of different host species (Table 1) and had been passaged between 7
90 and 12 times. For this study, eight sub-adult African bullfrogs (*Pyxicephalus adspersus*) and eight
91 adult common river frogs (*Amietia delalandii*) were collected from Potchefstroom, North-West
92 Province, South Africa and transported individually in sterile plastic bags to the lab, where mucosomes
93 were immediately collected from each individual according to Woodhams et al. [10]. Briefly, frogs were
94 placed in individual sterile cups and a given volume of sterile water added to each cup according to
95 the surface area of each frog. Animals were held in the cups for one hour, after which the mucosome

96 rinse water was collected and filtered through a 0.22µm sterile filter (Millipore, Ireland) and kept on ice
97 until challenge assays were conducted. Mucosomes were challenged against eight *B. dendrobatidis*
98 GPL isolates using an *in vitro* spectrophotometer assay method adapted from Bell et al. [14],
99 Woodhams et al. [10] and Becker et al. [15]. Three flasks of each *Batrachochytrium dendrobatidis*
100 isolate were grown in 1% tryptone broth at 21°C until maximum zoospore production was observed
101 (~3-4 days; ~1 x 10⁶ zoospores ml⁻¹). The three flasks of each isolate were combined and zoospores
102 separated from sporangia by filtering through 20µm sterile filters (Millipore, Ireland). To conduct the
103 spectrophotometer assays, 50µl of mucosome and 50µl of *B. dendrobatidis* suspension were pipetted
104 into 96 well plates. Each *B. dendrobatidis*-mucosome combination was run with six replicates. Positive
105 controls were included using 50µl sterile water instead of mucosome filtrate. Negative controls were
106 included using 50µl sterile water and 50µl of heat-treated *B. dendrobatidis* for each isolate.

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108 Plate readings were taken every 24 hours for four days using a 492nm filter. Data were transformed
109 using the equation $\ln(\text{OD}/(1-\text{OD}))$, and regression analysis used to gain the slope values for each
110 sample over time. Total *B. dendrobatidis* inhibition was calculated using the following formula;
111 Inhibition (%) = $[1-(\text{slope of sample}/\text{slope of control})] \times 100$, where a positive number represents
112 inhibition of *B. dendrobatidis* growth and a negative number indicates enhanced growth of *B.*
113 *dendrobatidis*. The average inhibition percentage was calculated for each individual sample, and the
114 eight samples acted as replicates for a given host species in subsequent analyses.

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116 Overall, most *B. dendrobatidis* isolates were inhibited in the presence of mucosomes from both
117 species (Figure 1). A Mann-Whitney U test indicated significant differences in mucosome inhibition
118 between the two species for the UK1 isolate of *B. dendrobatidis* ($W = 20$, $p = 0.015$), but there were no
119 significant differences between host species for all other isolates (all $p > 0.05$). Almost all *B.*
120 *dendrobatidis* isolates were inhibited when challenged with mucosome from *A. delalandii*, with the
121 exception of two isolates that showed negligible growth or inhibition (South Africa 1a and UK2; Figure
122 1). There were significant differences in *A. delalandii* mucosome inhibition between *B. dendrobatidis*
123 isolates (Kruskall-Wallis chi-squared = 21.686, d.f. = 7, $p = 0.003$) and a Dunn post-hoc analysis
124 indicated significant differences between a number of isolates (Table 2). Almost all isolates were
125 different to 2-4 other isolates, with no discernible relation to geographical origin of isolate. The isolate
126 from Spain was not statistically different to any other *B. dendrobatidis* isolate, with intermediate growth
127 inhibition in comparison to all others (Figure 1; Table 2). As with *A. delalandii*, the growth of most
128 isolates of GPL was inhibited when challenged with mucosome collected from *P. adspersus*, with the
129 exceptions of South Africa 1b (negligible growth or inhibition), South Africa 3 (high level of variation in
130 its response) and UK1, which exhibited very high levels of enhanced growth in the presence of *P.*
131 *adspersus* mucosome (Figure 1). The overall model for differences in growth of *B. dendrobatidis*
132 isolates in the presence of *P. adspersus* mucosome was significant (Kruskall-Wallis chi-squared =
133 21.596, d.f. = 7, $p = 0.003$). The Dunn pairwise comparisons (Table 2) show that UK1 was significantly
134 different to all other isolates of GPL with the exception of South Africa 1b, which was significantly
135 different to the Spain and Sardinia isolates.

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137 Together these results show that the growth of different isolates of *B. dendrobatidis* GPL varies
138 significantly in the presence of amphibian mucosomes, and that there is some variation in mucosome
139 inhibition between host species across the range of isolates. This suggests that the response of the
140 pathogen is linked to traits associated with the host mucosome as well as inherent traits of the various
141 *B. dendrobatidis* isolates. It has previously been shown that individual bacteria isolated from
142 amphibian skin also show variation in their ability to inhibit across a range of *B. dendrobatidis* isolates
143 [11-13], suggesting that the bacteria or their metabolites within the mucosome play a role in
144 determining inhibition of a given isolate of *B. dendrobatidis*. A number of recent studies show that the
145 composition of the bacterial community associated with the skin of amphibians is correlated with
146 infection probability of *B. dendrobatidis* [16-19]. Although the role of the microbiome composition in
147 determining susceptibility across GPL variation has not yet been tested *in vivo*, the *in vitro* data
148 presented here along with that of Antwis et al. [11], Muletz et al. [12] and Bletz et al. [13] indicates
149 strong potential for variation in the response of the host to different isolates of the fungal pathogen,
150 both in terms of changes in the host microbiome and the infection outcome for the host. Other
151 mucosome traits aside from bacteria (e.g. peptides, lysozymes) may also account for the variation in
152 mucosome-pathogen responses in our data. Amphibians show variation in their susceptibility to
153 different isolates of *B. dendrobatidis* [6-9], and the data presented here suggest this may be related to
154 interactions between *B. dendrobatidis* and some aspect(s) of the mucosome defences of amphibians.
155 Additionally, this pathogen has a highly complex genome with widespread aneuploidy [5, 24]; the
156 variation in mucosal inhibition between different *B. dendrobatidis* isolates demonstrated here may be
157 linked to differential phenotypic or genotypic traits associated with these isolates as has been
158 suggested in other studies [6-9].

159

160 Overall, most *B. dendrobatidis* isolates showed reduced growth in the presence of mucosomes from
161 both species (Figure 1). *Amietia delalandii* are not known to be experiencing chytridiomycosis-related
162 declines in the wild although populations are infected with low levels of *B. dendrobatidis* (38.8%
163 prevalence, *B. dendrobatidis* genomic equivalents < 5.0, n = 464; [23]). Infected wild *P. adspersus*
164 have not been found to date (genomic equivalents = 0.0, n = 10; Weldon, unpublished data). The data
165 presented here suggests the mucosomes of both species may play a role in resisting *B. dendrobatidis*
166 infection, although little is known about the defences of these species and there are many other
167 factors that will also influence susceptibility to *B. dendrobatidis*. In addition, it is not known if the
168 individuals used in this study were infected with *B. dendrobatidis*, which may influence the propensity
169 of the mucosome to inhibit the pathogen.

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171 Experimental work may allow for the prediction and/or identification of particular community traits (e.g.
172 high/low abundance of particular bacterial genera) that confer broad-scale inhibition against the wide
173 genetic and virulence variation shown by *B. dendrobatidis*. The current regimes for treating
174 chytridiomycosis are often laborious and have limited transferability to wild populations [20]. However,
175 the potential use of probiotics is increasingly being researched [21, 22], and it may be possible to

176 exploit mucosome traits linked to broad scale inhibition across the variation presented by *B.*
177 *dendrobatidis* in order to develop robust and effective treatments and/or prophylaxis for
178 chytridiomycosis *in situ*. In addition, teasing apart how genomic and transcriptomic factors associated
179 with *Batrachochytrium dendrobatidis* interact with hosts and host-associated mucosomes, and how
180 these factors relate to virulence traits, will provide valuable information about *B. dendrobatidis*
181 epidemiology and ultimately, the mitigation of chytridiomycosis in amphibians.

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194 **Conflicts of interest**

195 There are no conflicts of interest.

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198 **Ethical statement**

199 This study was approved by the Biodiversity and Conservation Ecology Scientific Committee and the
200 Animal Research Ethics Committee (NWU-00013-10-S4) of North-West University, and conducted
201 under research permit 028 NW-11 issued by the Department of Economic Development, Environment,
202 Conservation and Tourism, North West Provincial Government, Republic of South Africa.

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Figure 1

Average (\pm 1 S.E.) inhibition of eight globally distributed isolates of the Global Panzootic Lineage of *Batrachochytrium dendrobatidis* by skin mucosomes collected from two South African host amphibian species. Positive numbers represent inhibition of *B. dendrobatidis* growth and negative numbers indicate enhanced growth of *B. dendrobatidis*. See Table 2 for statistically different pairwise comparisons.

Table 1

Batrachochytrium dendrobatidis isolates used in the study.

Isolate	Archive code	Geographical origin	Host species isolated from
South Africa 1a	MG04	Silver Mine, Western Cape, South Africa	<i>Amietia fuscigula</i>
South Africa 1b	MG06	Silver Mine, Western Cape, South Africa	<i>Amietia fuscigula</i>
South Africa 2	MG08	Magoebaskloof, Limpopo, South Africa	<i>Amietia delalandii</i>
South Africa 3	MG09	Magoebaskloof, Limpopo, South Africa	<i>Hadromophryne natalensis</i>
UK 1	CORN 3.1	Penhale Farm, Cornwall, UK	<i>Ichthyosaurus alpestris</i>
UK 2	SFBC 014	Sellafield, Cumbria, UK	<i>Bufo bufo</i>
Spain	IA 2011	Ibon Acherito, Spain	<i>Alytes obstetricans</i>
Sardinia	MODS 28.1	Mont Olia, Sandinia	<i>Discoglossus sardus</i>

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325 **Table 2**

326 Dunn pairwise comparisons between *Batrachochytrium dendrobatidis* isolate growth in the presence
 327 of *Amietia delalandii* (green) and *Pyxicephalus adspersus* (orange) mucosomes. Results in bold and
 328 with an * indicate a statistically significant result.

329

	South Africa 1a	South Africa 1b	South Africa 2	South Africa 3	UK1	UK2	Spain	Sardinia
South Africa 1a		p=0.412	p=0.033*	p=0.021*	p=0.037*	p=0.444	p=0.168	p=0.038*
South Africa 1b	p=0.131		p=0.067	p=0.038*	p=0.069	p=0.347	p=0.262	p=0.073
South Africa 2	p=0.474	p=0.134		p=0.378	p=0.488	p=0.037*	p=0.240	p=0.495
South Africa 3	p=0.468	p=0.132	p=0.478		p=0.405	p=0.017*	p=0.112	p=0.378
UK1	p=0.016*	p=0.216	p=0.016*	p=0.020*		p=0.029*	p=0.252	p=0.476
UK2	p=0.494	p=0.161	p=0.483	p=0.462	p=0.020*		p=0.109	p=0.030*
Spain	p=0.384	p=0.044*	p=0.373	p=0.434	p=0.007*	p=0.351		p=0.240
Sardinia	p=0.213	p=0.018*	p=0.205	p=0.251	p=0.001*	p=0.175	p=0.382	

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