Mitigating amphibian chytridiomycoses in nature

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Summary

Amphibians across the planet face the threat of population decline and extirpation caused by the disease chytridiomycosis. Despite consensus that the fungal pathogens responsible for the disease are conservation issues, strategies to mitigate their impacts in the natural world are, at best, nascent. Reducing risk associated with the movement of amphibians, non-amphibian vectors and other sources of infection remains the first line of defence and a primary objective when mitigating the threat of disease in wildlife. Amphibian-associated chytridiomycete fungi and chytridiomycosis are already widespread, though, and we therefore focus on discussing options for mitigating the threats once disease emergence has occurred in wild amphibian populations. All strategies have shortcomings that need to be overcome before implementation, including stronger efforts towards understanding and addressing ethical and legal considerations. Even if these issues can be dealt with, all currently available approaches, or those under discussion, are unlikely to yield the desired conservation outcome of disease mitigation. The decision process for establishing mitigation strategies requires integrated thinking that assesses disease mitigation options critically and embeds them within more comprehensive strategies for the conservation of amphibian populations, communities and ecosystems.

Introduction

We are confronting an expanding array of pathogenic fungi that cause extensive mortality, demographic decline, and extirpations in livestock, crop, and wildlife hosts[1]. Developing strategies to limit the spread and impact of these pathogens is a priority that crosses the boundaries of politics, economics, science and health, and falls within the remit of the medical, veterinary, agricultural, and conservation sciences. Despite the increasing range of animal and plant taxa threatened by fungal pathogens, conservation science has not advanced disease mitigation in nature as a priority. This shortcoming has no better example than research on amphibian-associated chytridiomycete fungi. Our recognition of the threat posed by the global and regional emergences of the chytrid *Batrachochytrium dendrobatidis* (hereafter, *Bd*), has spurred significant advances in understanding the biology of the fungus and the dynamics of chytridiomycosis since the disease was first identified nearly twenty years ago[2]. Similarly, we have gained important insights into the European emergence of another chytrid fungus, *Batrachochytrium salamandrivorans* (*Bsal*)[3]. Unfortunately, the development of field interventions for disease management has lagged far behind and managing amphibian health in nature remains a largely unexplored topic[4-6]. Because applied conservation always operates under enormous financial constraints, it is important to critically assess the viability of conservation strategies before significant investment, which has rarely been done for strategies for controlling chytridiomycosis in wild amphibians[6-8]. Here we assess some of the commonly proposed approaches to control the spread and impact of amphibian chytridiomycosis in the field. We assume that an ideal strategy will be; i) safe, legal, and ethical; ii) effective and reliable; iii) transferrable across host species, communities, and environments, iv) relatively simple to implement; and v) cost-effective.

Countering disease-driven amphibian declines should consist of a multifaceted approach adapted to the stages of pathogen emergence (pre-arrival, invasion front, epidemic, established)[9]. Current approaches include prevention and short term solutions (e.g. *ex situ* breeding programmes,

cryopreservation) but long term, *in situ*, sustainable solutions are required if the goal of amphibian conservation is to be attained. This implies neutralizing the disease threat in wild populations. Although we do not discuss the prevention of pathogen introduction here in any detail, attempts to do this (e.g. via trade regulations, such as the recent establishment of restrictions on caudate amphibian trade in the USA in response to the emergence of *B. salamandrivorans*, https://federalregister.gov/a/2016-00452) are probably the most effective disease mitigation measure available[9-10]. The international movement of amphibians plays a continuing role in establishing and extending the distribution of amphibian-associated chytrids and other pathogens), but the control of chytridiomycosis and other purely wildlife diseases is largely overlooked in commercial trade[3,11-13]. The World Organisation for Animal Health (OIE) is the international body that can regulate this, but even though its remit includes wildlife conservation it has a poor track record in doing so. *Batrachochytrium dendrobatidis* has been listed by the OIE but enforcement of chytridiomycosis control in the amphibian trade has not been implemented by OIE member states[14].

Here we review strategies for mitigating amphibian disease following pathogen emergence. These range from minimizing effects on host populations to pathogen eradication. Short term solutions have been discussed in detail or summarized elsewhere and these are considered vital in temporarily preserving amphibian populations at risk[4,6,15,16]. For example, interventions with antifungals during an epidemic can alter infection dynamics and alleviate disease, but in the absence of long term disease management *in situ*, any short term measure is unlikely to result in significant conservation success[17]. We focus on measures that offer the potential for long term chytridiomycosis management *in situ*. *Bd* currently infects hundreds of amphibian species on all continents where amphibians occur (Fig. 1)[18]. Amphibian infections with *Bd* predate the late 20th century identification of lethal chytridiomycosis, and global emergence of the lethal form of the disease at this time was widespread[19,20]. Chytridiomycosis continues to emerge across four continents, precluding its elimination from widespread and complex infected host communities[18]. Instead of focussing on short term solutions, we examine a more pragmatic approach that strives for

long-term, host-pathogen co-existence. An ambitious aim would be to preserve a maximum proportion and diversity of amphibian species across as much of their distributions as possible. This implies that conservation triage will be necessary, accepting the loss of individual populations and even species[21,22]. Indeed, culling of reservoir and superspreader hosts requires consideration (Fig. 2). Irrespective, aims and methods will depend on local conservation priorities and should be defined by local conservation managers[23].

Amphibian chytridiomycosis treatments have been developed for captive populations, but translating these to managing infections in wild amphibian populations and communities is not straightforward. This is because amphibians affected by chytridiomycosis occupy terrestrial, arboreal, aquatic, and subterranean habitats that can overlap in a single landscape. Host population sizes fluctuate enormously, often exhibit highly dynamic spatial dispersion, and are frequently undetectable for much of the year. Therefore, it is not surprising that the number of studies of infection and disease in the wild, and those exploring management of infection in captivity, far outstrip those on *in situ* intervention. We know of few published studies describing the outcomes of attempted mitigation, and only two describing success. Four different strategies to mitigate the impacts of chytridiomycosis in nature have been attempted and published: translocation/reintroduction, augmentation of the host microbiome with probiotics, treatment of individuals with antifungals, and a combination of antifungal treatment with chemical disinfection of the environment[16,17,24-26].

Trialled and tested

Translocations/reintroductions often have strong appeal because they can promote the idea that “something is being done”. They are erroneously perceived to be cost-effective, simple to implement and transferrable. However, without a solid understanding of host-pathogen dynamics and the biology of the host and pathogen in the landscape, translocations/reintroductions have little probability of success. Several attempts have been made to repatriate amphibians affected by

chytridiomycosis in Europe, North America, the Caribbean and Africa but none have led to successful, long-term amphibian re-establishment[4,25,27,28] (but see 29 for evidence of short-term post-release survival). Although the majority of failures have been associated with the re-emergence of lethal chytridiomycosis in the translocated/reintroduced species, the cause behind failure to re-establish in almost every case could not be attributed clearly[25,27] (but see 11). This is important because lethal chytridiomycosis can be a secondary consequence of other threatening processes, which would mean conservation efforts focussed on the fungus could be misdirected[27,28,30]. The inability to unambiguously identify cause demonstrates the relative immaturity of the science of amphibian reintroduction as a means of mitigating chytridiomycosis, falsifies the assumptions of simplicity and transferability and violates the requirement of threat mitigation before reintroduction[31]. It also calls for greater investment in pathological investigations in concert with post release field monitoring. Given our incomplete understanding of *Bd* dynamics and potential for the development of resistance to *Bd* in wild populations, the use of translocations/reintroductions as a research tool is perhaps more appropriate than as a mitigation strategy against *Bd*.

A decade ago Harris and collaborators discovered that a subset of bacteria isolated from the skin of living amphibians has the ability to inhibit *Bd* growth *in vitro*[32]. Since then bacteria that inhibit *Bd* have been isolated from amphibians from across the Americas, Africa, Europe and Australia. Field studies of amphibian microbiomes indicate that the bacterial community on amphibian skin changes with amphibian life history stage, with fewer *Bd*-inhibitory species in later life stages, suggesting that targets for field intervention may be age-specific[33]. An expanding research programme is underway to ascertain if resistance to or limitation of infection can be enhanced by augmenting amphibian skin microbiomes with inhibitory bacteria. Encouragingly, a limited, but successful, field trial has been published along with a strategy for the isolation and potential application of probiotics to augment skin microbiomes[24,34]. This strategy outlines the advantages of bioaugmentation, including the use of

local bacterial isolates, and describes the potential for environmental application of bacteria that will interact with an entire amphibian community[34].

Several general issues need to be overcome before probiotics can be considered a viable mitigation strategy. First, the potential risk probiotics pose to ecosystem and public health requires assessment and the practicalities of probiotic development are also largely [unassessed35]. For example, there is little available information regarding the relationship between chytrid growth inhibition *in vitro* and effective inhibition of fungal growth or the development of disease *in vivo*. Experimental efforts using probiotics to control *Campylobacter* in poultry show that the relationship will likely not be straightforward and that some bacteria that are inhibitory are ineffective against pre-existing infections[36,37]. Efficient and persistent host and environmental colonization needs to be established: amphibian skin microbiomes are dynamic and can be unstable and unpredictable, and bacterial community composition changes over the animal's lifetime[3]. Bioaugmentation requires a deeper understanding of bacterial community assembly, stability and permeability, couched in the context of amphibian host community, the skin secretions produced by species members of the community and how these are in turn influenced by environmental heterogeneity[38,39]. Probiotics should also exert their beneficial effect across *Bd* genotypes. It has already been documented that the ability to inhibit one isolate of *Bd* does not translate across different isolates of the globally pandemic lineage[40]. Finally, a probiotic should show characteristics that render it suitable for mass production, including prolonged shelf life. As it stands, we have an unclear understanding of how interactions amongst all these factors will influence the development of effective priobiotic therapies against chytridiomycosis. The research required to gain this understanding will likely to be less cost-effective, implementable and transferrable than that for chemical treatments (see below), and, if animal experiment requirements are extensive and not well-justified, ethically questionable. However, if candidate bacteria can be characterized that meet the required criteria, their application could be far more cost-effective, ethical, and less controversial than chemical treatment.

Antifungals applied directly to susceptible hosts have proved ineffective as a long term strategy for *in situ* chytrid mitigation, as they afford no persistent benefits after treatment is stopped[17,26]. However, in an isolated and structurally simple ecosystem containing a single amphibian host species, antifungal treatments of individuals combined with chemical treatment of the environment did eliminate *Bd* and clearance persisted across years[26]. These findings suggest that the environmental application of fungicides may be a viable, cost-effective, simple to implement, and broadly transferrable strategy for controlling infection in some wild amphibian populations. Environmental treatment might not be applicable to many amphibian communities and species, however, and the environmental application of chemical pesticides has significant ecological, legal, and ethical ramifications. To be effective in the long-term, fungicides may have to be applied on a regular basis, much as they are in agricultural systems. Although any strategy that requires ongoing maintenance and has the potential for collateral impacts might seem untenable, decades of fungicide applications to food crops have had a significant and positive effect on global food yields[41]. The parallel suggests that in the face of the chytridiomycosis crisis environmental treatment with fungicides should be considered as a viable, long term management strategy for wild amphibians threatened by the disease. Very little effort has been expended in investigating existing chemical compounds that are effective against amphibian-associated chytrids or the development of chemical agents that specifically target chytrids, despite the evidence that some chemical pesticides mitigate infection in the aquatic environment without compromising amphibian development and larval survival[42] (but see 43). Although the use of agricultural pesticides is greatly debated, the focal, short term application of antifungals targeted at a reduction of infection prevalence and infection load in specific cases of acute chytridiomycosis-driven amphibian die offs is worth exploring[44]. The application of any such measure should be weighed against its potential negative impacts on biodiversity, ecosystem function, human health, and the potential for amphibian-associated chytrids to develop resistance to these treatments[45]. Advances in our understanding of the virulence factors and cellular components key for chytrid reproduction, growth, and infectivity should inform the

selection of compounds that exhibit multi-modal antifungal action and also guide the development of application strategies[46,47].

Horizon-scanning or wishful thinking?

Several mitigation strategies are gaining traction in the literature although they remain untested in real world settings. Evidence is accumulating that at least some species are responding to the emergence of chytridiomycosis through natural selection on immunity[48,49]. As a result, two arguments that incorporate selection into mitigation strategies are being promoted[50]. The first is based on the idea that, given time, natural selection will operate on immunogenetic variation in amphibian populations. To enable this, amphibians need to persist in the face of the pathogen and translocation/repatriation have been proposed as methods to facilitate population persistence during the process of selection. The second strategy is to breed selectively for resistant or tolerant genotypes for release into the wild[51]. Both strategies seek to establish resistant or tolerant populations and are based on the assumption that amphibian host immune responses to chytrids can be selected for and that immune function will be protective in a wild setting.

We can apply the points for and against translocations/reintroductions that we outlined above to the strategy of translocation/repatriation, compounded with the need to understand resistance and tolerance in captive populations before any release could be ethically undertaken. But what about selective breeding? We are aware of a single example where captive selection and subsequent breeding created defined lines that exhibit variation in immunity in an amphibian: the genus *Xenopus*[52,53]. The knowledge base on *Xenopus* captive breeding, cell biology, genetics, and immunity took decades to develop. Advances are being made in comparative immunogenetics that could conceivably guide breeding designs, but this is still a long way from understanding host-species immune responses to chytrids and exploring heritable variation of amphibian immunity with the goal of selective breeding[54]. The elucidation of mechanisms underpinning resistance against *Bd* would greatly facilitate the development of resistance markers that could be used in marker-assisted

selective breeding programmes. The chances of finding any such marker, or a set of markers, are hampered by the context dependent interaction of *Bd* with the amphibian host[55]. Establishing captive colonies upon which selection can be imposed is a non-trivial task and requires extensive investment and resources. Even if assisted selection does produce genotypes that have the ability to resist or tolerate infection with chytrids, there is no guarantee that these abilities will function when transferred to a natural setting. Research has repeatedly shown how environmental variation can dictate the outcome of the amphibian host/chytrid pathogen interaction and the ability to mount innate immune responses to *Bd* can be significantly impaired simply by modifying ambient temperature[30,55-58]. We do not dismiss the possibility that selection might provide conservation benefits, only caution that the current knowledge base indicates significant research is still required before natural and assisted selection can be applied widely to chytrid mitigation. If genetic determinants of host-resistance are identified in multiple amphibian species and new technologies for genetic manipulation prove amenable to immunogenetic modification of susceptible amphibian species, the situation might change, but it will also open up new ethical issues for conservationists[59-61]. Clearly, it is imperative to continue investigating the genetic basis of amphibian resistance and novel means by which it can be augmented.

At least three published studies have investigated whether frogs could be immunized against *Bd*. Systemic injections of killed *Bd* were ineffective at reducing the probability of infection or death[62,63]. In contrast, increasing numbers of exposures to killed *Bd* or live *Bd* culture followed by clearance with antifungals was negatively correlated with strength of infection and positively correlated with survival following subsequent exposure to *Bd*[64]. The authors themselves questioned how their findings might be applied in a conservation setting but noted the potential for priming hosts against infection prior to release to the wild. These findings are contradicted by Hudson et al., where repeated use of antifungals on naturally infected frogs generated no long term benefits once antifungal treatments ceased[17]. Perhaps more importantly, every immunization study to date has focussed on post-metamorphic animals and immunization of pre-metamorphic stages might not be

possible as adaptive immunity is not available to pre-metamorphic stages[65] (but see 53). Amplification of infection is commonly associated with larval stages, with high rates of mortality occurring at metamorphic climax. Controlling infection in amphibian larvae will be a key factor in mitigating impacts of chytridiomycosis because amphibian population growth rates are highly sensitive to survival rates of postmetamorphic juveniles[66-69].

The ideal vaccine for *in situ* use should elicit a strong protective response across life stages and across species against a broad spectrum of relevant and virulent chytrid genotypes, be safe, and have both its production and administration feasible. Indeed, the research process should engage with the relevant authorities from the outset, as policy applicable to vaccinating free-living wildlife populations also requires development. So far, immunization experiments have been conducted with fairly straightforward and crude fungal preparations. Designing effective vaccines is a time- and money-consuming undertaking, and for diseases in a range of species, fungal vaccines have proved far more difficult to develop than their bacterial and viral counterparts. To date, with few exceptions, potential vaccines against human fungal pathogens are still in preclinical stages of development and very few effective veterinary vaccines are available[70-72]. Although vaccinations currently afford no clear contribution to chytridiomycosis mitigation in wild populations, continued research on vaccines will undoubtedly aid in our understanding of amphibian immunity and host-pathogen interactions, both topics essential for a variety of mitigation strategies including immunization, selection, and bioaugmentation.

Manipulating environments to reduce infectivity or virulence of *Bd* is another strategy that may hold promise. The principle behind this ecological, rather than evolutionary, approach underlies environmental treatments (e.g., see 26), but in practice is accomplished by exploiting environmental variations that reduce chytrid growth and zoospore density and does not require elimination of the pathogen from the environment. The concept follows the recognition that environmental variability can inhibit, as well as exacerbate, the impacts of chytridiomycosis, with evidence of reduced

virulence even in highly susceptible host species[6,73-76]. Refuges from disease, but not necessarily infection, could be created by altering habitats to reinforce environmental factors not conducive to *Bd* growth within the host or zoospore survival outside of it. Habitat management is already integral to most amphibian conservation programmes and often involves repeated efforts to maintain useful habitats (e.g. 77), suggesting that environmental manipulations for the purposes of disease control could have quick uptake by the conservation community, with both concepts and strategies readily transferrable. Interventions could be chemical (e.g. altering salinity); physical (e.g., altering temperatures to not favour chytrid growth and reproduction), or biotic (e.g., promoting the abundance of organisms that consume environmental zoospores)[75,78-80]. These strategies will likely focus, at least initially, on manipulating the aquatic environment, as environmental persistence of *Bd* in water is deemed essential for amphibian decline and extinction scenarios[81,82]. Theory and empirical evidence shows that conservation efforts targeting aquatic life stages that reduce disease-driven losses of newly metamorphosed juveniles should improve recruitment and reduce or reverse the effects of disease-driven decline; additional population models addressing this topic are clearly needed[15, 81,83].

Although environmental manipulations may create pockets of tolerance or resistance, they offer limited opportunities for amphibians with broad geographic ranges and/or disproportionately affected complex communities and habitats. As with environmental disinfection, even in simple settings environmental manipulations must be assessed for their impacts on biodiversity and other ecosystem functions. As with translocations/reintroductions, host ecology must be well-understood before changes to the habitat are undertaken. For now, environmental manipulation might provide long-term refuges for focal species of high conservation concern, but offers no broad scope for chytridiomycosis mitigation.

A focus on disease mitigation may not always be the best way forward because simpler actions might achieve the required results: improving habitat quality might enable losses from

disease at one stage of the amphibian life cycle to be compensated for in gains at other life stages. For example, one might use pond draining to cull predators of amphibian larvae. As a consequence, tadpole survival might increase, leading to increased juvenile recruitment. Even if many juveniles still die of chytridiomycosis, this action might still facilitate population persistence. There is some empirical evidence that this might work and existing theory of harvested and exploited populations might guide such a strategy[5,84].

Single strategies or a marriage of methods?

Clearly, we do not know how to manage amphibian diseases in the wild and yet conservation managers have to make decisions and manage populations. They cannot wait until we understand amphibian-chytrid host-pathogen biology in great detail; a lack of action because of imperfect information is a management decision[85]. From our review, it is clear that a single strategy is unlikely to achieve the conservation outcome of disease mitigation. Each strategy has pros and cons but by combining methods strategically *in situ* mitigation is likely to have a greater likelihood of success. There are a number of tools to decide which management actions are best or most likely to succeed in the presence of uncertainty. Structured decision making and information analysis can be used to find a best management option and to define the direction of research most likely to illuminate critical uncertainties[86-88]. For example, structured decision making might identify important gaps in our understanding of chytrid epidemiology. These approaches have only recently been used in the context of chytrid mitigation[7,23]. Converse et al. used such an approach to study the effects of translocations in a toad metapopulation and found that efforts to reduce disease spread had weak effects, selection for resistance would increase the number of sites occupied by toads and translocations would speed up species recovery[7].

Shortcomings of individual strategies outlined above may be compensated for by combining two or more strategies. In that sense, our outline of the major alternatives for *Bd* mitigation and the applicability and challenges of each forms a starting template that can inform decision-making

processes. The science of decision making links management options to measurable objectives (e.g. population persistence). Post-management monitoring then determines the outcome of management actions against the objectives and is used to update models for the next round of decision-making. This approach allows real-time assessment of the impact of management alternatives so that management can be rapidly modified to improve outputs[8,89].

For these approaches to work researchers investigating mitigation strategies have to engage in the conservation management process and be willing to alter research programmes based on the outputs of structured decision making and adaptive management exercises. Precedence for this can be found in the literature on chytridiomycosis ecology, evolution and epidemiology and is exemplified by the initial effort to identify chytridiomycosis as the cause of amphibian mass mortality (Berger et al. 1998). Coordinating research and management efforts have already been proposed for Australian amphibian species at risk from chytridiomycosis[6]. Joined-up efforts will require field trials across a more extensive range of settings and amphibian communities than are currently being attempted. It remains to be decided –the authors of this review disagree on this point- at which stage of methods development sufficient knowledge has accumulated to justify field trials.

What must be considered at all stages of the conservation management process, however, are the ethical and legal issues associated with whatever strategies are proposed or adopted. Strategies that are illegal or unethical are inapplicable irrespective of their cost-and field-effectiveness, reliability, transferability, or simplicity. Ethical issues may be identified at any scale. Our example of conservation triage is a knotty ethical question: what is an acceptable format for deciding which species to conserve and which to cull or allow to go extinct? Expending effort on the mitigation of chytridiomycosis should also be subject to ethical consideration, as should any decision to expend highly limited resources available for biodiversity conservation[90].Disease as a conservation issue remains a novel concept for most policy-makers and conservation practitioners, so legal

frameworks may have to be challenged and modified to account for responses to this new and growing threat to amphibian biodiversity. Ethical issues may be difficult to address, but failing to mitigate chytridiomycosis, a disease widely accepted as predominantly driven by human activities, is the least ethical option of all.

Conclusion

Despite decades of research into amphibian-chytrid host-pathogen biology, no effective method to reduce the impact of chytridiomycosis has emerged and been tested broadly in the field. A few case and proof-of-concept studies have produced mixed or limited success at best. A more collaborative approach to chytrid mitigation research is necessary, one that should start with an approach from the family of tools from decision sciences to define the most important research questions. Such exercises to identify those questions should be conducted by interdisciplinary research teams that are working with conservation managers and that can put research outputs into the context of the overall conservation objectives. It is always uncertain how the findings of research undertaken away from the field setting will transfer to the real world, but it is clear from our review that significant *ex situ* research efforts are required for all mitigation methods to ensure that the results of field trials can be fully explained. A lack of *in situ* evidence from chytridiomycosis mitigation efforts, however, indicates that field trials are not yet an objective in many research programmes, despite invoking amphibian conservation as a potential consequence of research discoveries. Clearly, if we are to mitigate chytridiomycosis, research must be focussed on delivering outputs that can be rapidly and critically assessed and, when warranted, implemented in field trials as soon as possible.

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Figure 1. Examples of lethal chytridiomycosis from Latin America (a) and Europe (b). a) A *Craugastor underwoodi* dead and *in situ* Craugastor sp. killed by lethal chytridiomycosis in Monte Verde, Costa Rica . The isolate derived from this animal in 2008 has served as the source of DNA for qPCR positive controls for two of the authors to this day. B) An *Alytes obstetricans* again dead and *in situ*, found in Peñalara Natural Park, Spain.

Figure 2. The relative impact of culling and antifungal treatment in a simple, single species population paramaterised using data for the Mallorcan midwife toad[91]. (a) Culling of *Alytes* tadpoles, undertaken at point m, results in pathogen elimination. Green line is adult population size, red line is free-swimming zoospore density. (b-c) Population responses after tadpole antifungal treatment and release (b) and culling (c), assuming maintenance of infection in the adult population and keeping model parameters identical across models. Mitigation is undertaken at point m. In (b), mitigation is unsuccessful due to increased host density after antifungal-treated tadpoles are returned to the pond. In (c), pathogen elimination is attributable to more persistent reduction in host density following culling.

Fig 1a

206x137mm (300 x 300 DPI)

Fig 1b

1083x812mm (72 x 72 DPI)

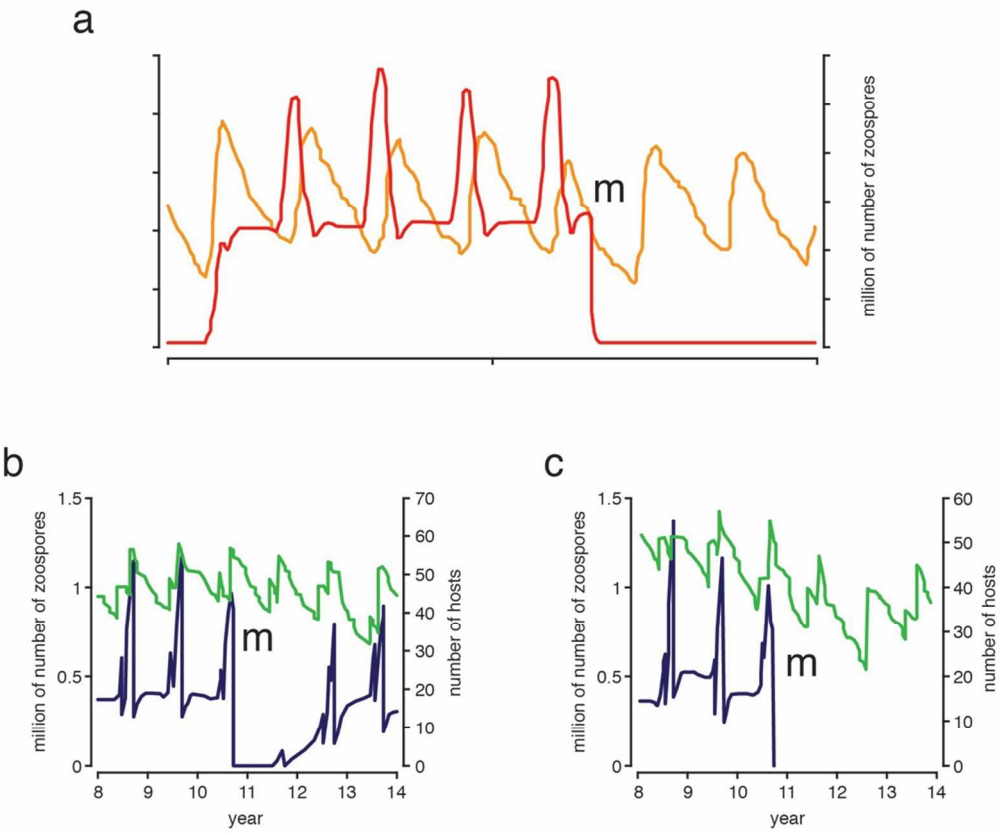
a
m
million of number of zoospores
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Fig 2