

Meta-analysis shows that immunisation reduces amphibian susceptibility to the chytrid fungus

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ABSTRACT

Emerging infectious diseases are increasingly impacting vertebrates, sometimes causing severe population declines. Chytridiomycosis, a lethal disease caused by the pathogen *Batrachochytrium dendrobatidis* (Bd), is among the most devastating, responsible for global amphibian collapses and extinctions. Developing strategies that improve host survival in the presence of Bd has therefore become a conservation priority. We conducted a meta-analysis to evaluate whether immunising individuals reduces disease susceptibility. Using 208 effect sizes from 36 studies and 22 species, we compared infection prevalence, infection intensity, and mortality between experimentally immunised and non-immunised individuals subsequently exposed to Bd, representing the first such synthesis for a major emerging wildlife disease. Immunisation using live pathogens significantly reduced host infection intensity and lowered mortality after re-exposure, whereas immunisation using Bd-derived natural chemicals reduced infection prevalence. Other immunisation approaches showed non-significant effects across species. Immunisation effects also varied across disease progression stages, host life stages, and taxonomic groups. Our findings suggest that live-pathogen immunisation could be an effective strategy for increasing survival during reintroductions. Supplementing vulnerable populations with immunised individuals or helping wild amphibians clear infections may thus increase survival.

KEYWORDS

antifungal, microbe, metabolites, vaccine, frog

INTRODUCTION

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The emergence and spread of infectious diseases represent one of the most significant threats to wildlife populations worldwide^{1,2}. While many taxa have been impacted by diseases, amphibians have been hardest hit due to the global expansion of two fungal pathogens, *Batrachochytrium dendrobatidis* (Bd)³ and *B. salamandrivorans* (Bsal)⁴. These pathogens
45 cause chytridiomycosis, a disease that can trigger mass mortality events^{5,6}. Since the 1970s, the spread of Bd has contributed to population declines in more than 500 amphibian species, with at least 90 species potentially extinct due to these pathogens⁷. Consequently, amphibians are now the most imperilled vertebrate class, with more than 40% of species threatened, with chytridiomycosis identified as a major threatening process^{7,8}.

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Once established in the wild, Bd is extremely difficult to eradicate. Persistence stems not only from the pathogen's ability to infect non-amphibian hosts^{9,10}, grow as a saprotroph^{11,12}, and remain viable in the environment¹³, but also from non-susceptible amphibian host species that act as long-term pathogen reservoirs and continual sources of infection for more vulnerable
55 species¹⁴. As a result, conventional conservation measures, including translocations and reintroductions, often fail because disease-causing pathogens persist in the environment¹⁵⁻¹⁷. While small-scale eradication of Bd and Bsal may be possible under specific conditions^{18,19}, in most cases, the only proven management strategy is to remove susceptible individuals from natural habitats and maintain disease-free captive populations²⁰, a scenario that risks leaving
60 many amphibian species extinct in the wild. This reality highlights the urgent need to develop approaches that mitigate disease impacts and enable amphibian hosts to coexist with the pathogens.

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A promising avenue for reducing disease impacts involves stimulating protective immune responses in susceptible species. In many animals, exposure to live-attenuated pathogens, inactivated pathogens, or their derivatives enables immune cells to recognise pathogens, develop immunological memory, and mount faster and more effective responses upon subsequent exposure^{21,22}. For amphibians, successful immunisation strategies that enhance host survival²³⁻²⁵ could substantially improve conservation outcomes, for example by enabling the
70 release of immunised individuals to bolster threatened populations, or by guiding habitat manipulations that help amphibians clear infection naturally, develop immunity under wild conditions, and thus enable long-term population persistence^{26,27}.

However, immunisation efforts targeting Bd in amphibians have produced mixed outcomes^{28,29}, with no clear consensus and considerable uncertainty about their overall effectiveness. This inconsistency likely reflects the wide variety of immunisation approaches that have been trialled (Table 1), including exposure to live pathogens^{28,30}, dead pathogens^{25,31}, pathogen metabolites^{32,33}, antifungal microbes^{34,35}, and synthetic antifungals^{36,37}, as well as the broad diversity of host species on which these methods have been tested.

To resolve debates around immunisation efficacy for combating amphibian fungal pathogens, we conducted a meta-analysis drawing on all available amphibian immunisation studies targeting Bd and Bsal to identify the most effective strategies and to understand the drivers of variation in immunisation success. Synthesising empirical results meta-analytically is a powerful way to identify the most promising strategies for effective immunisation. We focused on how different immunisation approaches influenced subsequent infection prevalence (i.e. the rate of infection), infection intensity when infected, and mortality when immunised individuals were exposed to live Bd or Bsal, anticipating that effective strategies would reduce infection prevalence, lower pathogen loads in infected individuals, and/or decrease mortality. Variation across immunisation methods (Table 1) and among different amphibian life stages, origin (captive or wild bred) and taxonomic family (Table 2) has been suggested to influence infection outcomes following Bd exposure. We therefore evaluated how these methodological and biological factors moderate immunisation effectiveness.

RESULTS

We screened 1,518 studies and identified 38 containing relevant datasets (Figures 1 & 2; Extended Data Figure 1). Together, these studies reported 275 effects from 59 experiments, each comparing outcomes following Bd or Bsal exposure between immunised (treated) and control (untreated) animals under common conditions. Most experiments targeted Bd ($n = 53$) with only six addressing Bsal. All Bsal experiments involved live-pathogen immunisation: exposing animals to either live Bd²⁸ or live Bsal^{38,39} and testing their subsequent susceptibility in a live Bsal challenge. Results from these Bsal experiments were mixed: infection intensity and mortality in immunised individuals were reduced relative to controls in some species²⁸ but not others^{38,39}, while prevalence was unaffected by immunisation

treatment in all cases. Given the limited number of Bsal experiments, we estimated effect sizes only for Bd studies.

Immunisation treatments reduce susceptibility to Bd across amphibians

Mortality is arguably the most consequential outcome because it links directly to population performance and the potential for long-term persistence. Live-pathogen immunisation reduced mortality following subsequent live Bd exposure by an average of 26% ($p = 0.054$; Figure 3). The confidence intervals (CIs) slightly overlapped zero (-45% to $+0.5\%$), although the magnitude of the effect suggests that live-pathogen immunisation can substantially improve host survival (Table S1). In addition to reducing mortality, live-pathogen immunisation significantly reduced infection intensity (Figure 3), decreasing pathogen loads in infected individuals by an average of 80% ($p = 0.02$), helping to explain reductions in mortality. While live-pathogen immunisation reduced mortality and infection intensity, it had no clear effect on prevalence ($p = 0.21$; Figure 3).

Natural-chemical immunisation was the only approach that significantly reduced prevalence (Figure 3), lowering infection prevalence by an average of 27% ($p = 0.003$). Among infected individuals, natural-chemical immunisation tended to reduce infection intensity by an average of 76%; however, the CIs included zero (-96% to 37%), indicating that the effect may be large for quite a few species but was highly variable across studies ($p = 0.11$).

Synthetic-antiparasitic immunisation reduced mortality risk by an average of 34% (Figure 3), and its CIs were largely skewed towards reduced mortality risk (-60% to $+8\%$). However, this effect was not statistically significant ($p = 0.10$), possibly because it was estimated from only five experiments ($k = 5$). The remaining immunisation approaches (probiotic, dead-pathogen and other) showed no clear effects on prevalence, infection intensity, or mortality (Table S1).

Immunisation effects were correlated between prevalence and infection intensity

Our results above show that different immunisation methods can influence infection dynamics at different stages of disease progression, from the probability of becoming infected to the intensity of infection and ultimately mortality (Figure 3). To examine whether the effects of immunisation on one disease stage were associated with compensatory or reinforcing effects

at other stages, we fitted multivariate models that assessed correlations among effect sizes across the stages of disease progression within experiments. The effects of immunisation on prevalence and infection intensity were positively correlated (ρ [CIs] = 0.72 [0.28 to 1.00]; Table S2) because immunisation that limits initial infection can also suppress within-host pathogen abundance, whereas immunisation that accelerates pathogen clearance can, in turn, reduce prevalence. In contrast, there was no clear correlation between effects on mortality and those on prevalence (ρ [CIs] = -0.88 [-1.00 to 0.44]) or infection intensity (ρ [CIs] = -0.32 [-1.00 to 1.00]) (Table S2), suggesting that effects enhancing resistance (e.g., preventing pathogen invasion or growth) did not necessarily affect tolerance (e.g., mortality risk at a given pathogen burden).

Consistent effects on prevalence but more variable effects on infection intensity and mortality

There was evidence that some immunisation methods significantly lowered Bd prevalence, infection intensity, and mortality, but the heterogeneity of effects varied across studies (Table S3). The effects of immunisation on prevalence were highly consistent across studies ($I^2 \approx 0\%$), largely because most immunisations did not clearly alter infection prevalence (Extended Data Figure 2). In contrast, the effects on infection intensity exhibited high heterogeneity across studies ($I^2 = 96\%$) and the effects on mortality exhibited small to moderate heterogeneity ($I^2 = 30\%$).

For infection intensity, most of the heterogeneity was due to between-study and within-study variation ($I^2_{\text{between-study}} = 46\%$, $I^2_{\text{within-study}} = 50\%$, $I^2_{\text{phylogeny}} \approx 0\%$). For mortality, heterogeneity primarily arose from between-study differences with a weak phylogenetic signal ($I^2_{\text{between-study}} = 21\%$, $I^2_{\text{within-study}} \approx 0\%$, $I^2_{\text{phylogeny}} = 9\%$). The substantial contribution of between-study differences in both infection outcomes suggests that ecological and/or methodological differences among studies were key drivers of variation in immunisation effects.

Immunisation effectiveness varies by life stage and family, with stronger effects in more susceptible groups

In addition to immunisation method, we found that two host features, life stage and taxonomic group, moderated treatment effectiveness. Overall, immunisation was more effective at lowering prevalence in larval amphibians relative to other life stages (Figure 4), reducing larval infection prevalence by an average of 28% ($p = 0.01$). In contrast, immunisation effects on infection intensity and mortality were not moderated by life stage (Table S4).

Because several immunisation methods have been trialled in juveniles but not adults or larvae (Table S5), analyses of immunisation methods were restricted to juveniles. Live-pathogen immunisation significantly reduced juvenile mortality by an average of 35% ($p = 0.03$) and reduced juvenile infection intensity by an average of 81% ($p = 0.01$), whereas natural-chemical immunisation reduced juvenile prevalence by an average of 46% ($p = 0.06$), with the CIs slightly overlapping zero (-71% to $+3\%$) (Table S6). Juvenile-specific effects of natural-chemical and live-pathogen immunisations are consistent with their effects across life stages (Figure 3). In contrast, synthetic-antiparasitic immunisation tended to *increase* infection intensity of juveniles (Table S6), but this trend was based on only a few effect sizes ($k = 6$; $p = 0.06$). This uncertainty highlights the need for further investigation of synthetic-antiparasitic immunisation.

Species in different families are known to vary in their responses to immunisation and disease outcomes due to taxa-specific physiology⁴⁰ and ecological requirements⁴¹, which may not be captured by phylogenetic relatedness when few taxa are included in an analysis. Indeed, we found that immunisation effects varied by amphibian family, reducing infection risk in Hylidae by an average of 9% ($p = 0.03$), but with no clear reduction in other families (Figure 4; Table S7). Hylids are known to be particularly susceptible to Bd and often experience high Bd-induced mortality^{7,42,43}. To test whether immunisation provides greater benefits in more susceptible groups, we conducted a meta-regression examining the moderating effect of inherent susceptibility, which we measured using the mortality rate of the control group, on immunisation effects. As predicted, immunisation reduced mortality more strongly in groups with higher inherent susceptibility (higher control-group mortality; slope [CIs] = -0.01 [-0.012 to -0.0001], $p = 0.046$), suggesting that more susceptible species or populations gained greater survival benefits from immunisation. In contrast, inherent susceptibility did not strongly predict immunisation effects on prevalence or infection intensity (both slopes overlapped zero, both $p > 0.21$; Table S8).

Immunisation effectiveness did not clearly differ between wild and captive-bred hosts (Figure 4; Table S9) and was not influenced by methodological variation, including the interval between immunisation and live Bd challenge, the interval between challenge and trait measurement, or overall measurement effort (Table 1 and S10).

Publication bias

Immunisation effects were not predicted by effective sample sizes⁴⁴ or publication year⁴⁵ of the study (all slopes overlapped zero; all $p > 0.14$; Table S11), suggesting no evidence of publication bias.

DISCUSSION

Identifying approaches that reduce Bd-induced morbidity and mortality is essential for safeguarding threatened amphibians and advancing global conservation efforts. Because Bd persists in wild populations, improving individual survival is widely viewed as a critical first step toward preventing extinctions and enabling successful reintroductions²⁶. Our meta-analysis shows that, on average, prior exposure to live Bd lowers mortality risk and infection intensity in subsequently infected individuals, whereas prior exposure to natural chemicals reduces prevalence and infection intensity. In addition, prior exposure to synthetic antiparasitics tended to reduce mortality risk. These three approaches offer promise for immunising animals prior to translocation or population supplementation, potentially bolstering amphibian conservation initiatives globally.

Exposure to live Bd or its natural chemicals reduced susceptibility to Bd, whereas exposure to dead Bd had no effect

Live-pathogen immunisation reduced infection intensity and mortality upon re-exposure to Bd. This outcome is consistent with classical vaccination theory, in which exposure to a live pathogen stimulates adaptive immunity and lessens the severity of later infections^{21,22}.

Interestingly, some experiments used low-virulence Bd strains for immunisation without imposing a clearance treatment (such as heat or antifungals) because infection with these Bd strains did not cause any clinical symptoms^{28,46}. These low-virulence Bd strains could remain transmissible⁴⁶ and be spread naturally in untreated individuals, potentially accelerating the

establishment of herd immunity⁴⁷. Notably, however, exposure to low-virulence Bd strains may still be lethal to some individuals²⁸ and potentially increase the risk of co-infection with other pathogens⁴⁸. Immunisation using low-virulence Bd strains without clearance hence requires further assessment.

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In addition to live-pathogen immunisation, we also found evidence that natural-chemical immunisation can reduce infection prevalence and infection intensity. Most natural-chemical immunisation studies used Bd metabolites (cell-free by-products of pathogen cultures) to induce resistance^{32,33,49}. Together, these findings highlight that pathogen-related immunisation is an effective strategy for reducing susceptibility to Bd.

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We detected no clear effect of dead-pathogen immunisation, consistent with epidemiological evidence that non-infectious vaccines generally confer weaker protection than live vaccines²². What remains unresolved, however, is why Bd metabolites were effective. A possibility is that inhibitory compounds produced by Bd, such as methylthioadenosine⁵⁰ and spermidine⁵¹, stimulate host immune responses. These compounds originate from the walls of Bd zoosporangia rather than from zoospore cells⁵². This distinction is important because dead-Bd immunisation typically exposes hosts to only purified dead zoospores that lack zoosporangia^{25,31,53,54}. In contrast, Bd metabolites are usually prepared by filtering culture media containing both zoosporangia and zoospores³³. The presence of zoosporangia may therefore underlie the stronger protective effects seen for metabolite-based immunisation. Consistent with this explanation, antibody production has been detected when frogs were treated with mixtures containing both dead zoospores and zoosporangia⁵⁵, but not when exposed solely to purified dead zoospores^{54,56}.

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Exposure to synthetic antiparasitics: a convenient method to reduce susceptibility to Bd

Contrary to vaccination, which aims to stimulate adaptive immunity, exposure to synthetic antiparasitics has typically been used as a sham procedure to control for pathogen clearance following live-pathogen immunisation^{29,46}, or has arisen from interest in the antifungal properties of common pesticides^{37,57}. Reported effects of synthetic-antiparasitic immunisation are mixed^{37,58}, but our meta-analysis revealed a strong overall tendency toward reduced host mortality. Because this approach does not involve pathogen pre-exposure, it likely acts

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through innate mechanisms, such as epidermal trauma that subsequently reduces host susceptibility⁵⁹. Although this effect was not statistically significant and is based on a small sample size, it nevertheless underscores the potential practical value of synthetic antiparasitics. Synthetic drugs are cheaper and more easily transported than live Bd or its natural chemical extracts, which require zoospore quantification and immediate use, making synthetic-antiparasitic immunisation more practical in remote areas³². While synthetic drugs, particularly itraconazole, is the most widely used therapeutic intervention for mitigating Bd⁶⁰, its use is controversial because of potential impacts on animal performance and ecosystems⁵⁷. Our results also suggest caution because synthetic-antiparasitic immunisation tended to increase infection intensity in juveniles, highlighting the need for additional evaluation before broader application.

Effects on mortality were not correlated with effects on prevalence and infection load, suggesting that immunisation enhances tolerance and resistance independently

Immunisation that prevents initial infection (lowering prevalence) might, in principle, also limit pathogen growth within hosts (lowering infection intensity) and prevent pathogen loads from reaching lethal thresholds (lowering mortality). In turn, immunisation that reduces pathogen loads and accelerates clearance might also reduce prevalence. We might therefore expect positive correlations among effects on prevalence, infection intensity, and mortality. A key question, then, is why live-pathogen immunisation reduced mortality but not prevalence, whereas natural-chemical immunisation reduced prevalence but not mortality. Overall, while we found that prevalence and infection intensity tended to be positively correlated, neither were strongly correlated with mortality.

An explanation for these outcomes is that hosts combat pathogens through two distinct mechanisms^{61,62}: (1) resistance, which limits pathogen invasion and growth, and (2) tolerance, which reduces damage without necessarily lowering pathogen burden, for example through tissue repair. Because these mechanisms rely on different physiological pathways, resistant amphibians are not necessarily tolerant, and tolerant individuals may not be resistant (see⁶³ for a discussion of the relationship between Bd resistance and tolerance across 35 amphibian species). In our meta-analysis, prevalence and infection intensity are indicators of resistance, whereas mortality reflects tolerance^{62,63}. This framework helps explain why

resistance-related outcomes were positively correlated with each other but showed no clear correlation with the tolerance-related outcome. It is well established that some amphibians can maintain high pathogen loads without developing disease or experiencing mortality^{14,64}.

310 Such decoupling between pathogen burden and host survival indicates that reductions in prevalence or infection intensity do not necessarily translate into reductions in mortality.

Future conservation efforts need to consider the timing and life stage of immunisation

315 Another factor contributing to variation in immunisation outcomes is host biology. If hosts are naturally tolerant and unlikely to die from infection, immunisation may help them clear infection and lower prevalence while having little effect on mortality risk. Tadpoles are a good example: they are highly tolerant to Bd because the keratinised tissues that Bd infects are restricted to their mouthparts⁶⁵, and infections in this region rarely cause mortality^{42,62}.

320 Low Bd-induced mortality likely explains why larval immunisation was effective at reducing prevalence but did not lower infection intensity. In fact, only one experiment has reported larval deaths following a live Bd challenge (Figure 4).

Although mortality during the tadpole phase is uncommon⁶², infected tadpoles often die
325 during or shortly after metamorphosis⁶⁶, when keratin appears across the entire skin surface and Bd can spread more extensively⁶⁵. Tadpoles can also act as important reservoirs that maintain Bd in a population and increase disease transmission⁶⁷. For example, seasonal changes in Bd prevalence among *Litoria rheocola* tadpoles closely matched those in terrestrial adults, suggesting that infection dynamics are linked across life stages⁶⁸. Evidence
330 that immunisation reduces infection prevalence in tadpoles is therefore encouraging because fewer infected tadpoles should translate to lower mortality after metamorphosis and reduced transmission risk to other individuals. Field studies further support this idea, showing that low Bd prevalence in tadpoles was associated with rapid recruitment and population recovery despite high adult mortality^{69,70}.

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Our results also indicate that immunisation performs similarly in both wild-bred and captive-bred juveniles and adults. This suggests that immunisation can be broadly integrated into conservation strategies, including being applied to captive animals in reintroduction or supplementation programs, benefitting wild individuals via capture-treat-release approaches,
340 or through habitat interventions that facilitate pathogen clearance and could thus generate

immunity in wild populations. In contrast, all tadpole experiments in our meta-analysis were conducted using wild-caught individuals, leaving the immunisation effect of captive-bred tadpoles unknown. Moreover, most Bd experiments (47 of 53) assessed immunisation success within a single life stage, leaving it unclear whether effects induced in tadpoles persist through metamorphosis. These gaps are important to address, especially given our finding that immunisation significantly reduces infection prevalence in tadpoles.

Tadpole immunisation offers several practical advantages of other life stages: (1) a single adult breeding pair can often produce hundreds to thousands of embryos, enabling efficient collection of large cohorts; (2) tadpoles can be reared at high densities on inexpensive plant-based diets, unlike post-metamorphic frogs that require more expensive carnivorous diets; and (3) tadpoles are far more tolerant of Bd infection than post-metamorphic frogs^{62,71}, making them particularly suitable for live-pathogen immunisation. If resistance acquired during the tadpole stage carries over across metamorphosis and results in more disease-resistant adults, tadpole immunisation could be a powerful conservation tool. It would allow large numbers of individuals to be immunised at much lower cost and could substantially increase the number of immunised animals available for reintroduction or supplementation programs.

Immunisation effects differ among taxonomic families and depend on inherent susceptibility

We found that immunisation effects varied among taxonomic families,. With immunisation particularly effective at reducing prevalence in Hylidae. A previous meta-analysis also identified the superfamily Hyloidea as particularly susceptible to Bd⁴², suggesting that immunisation may be more effective in more susceptible species. Consistent with this idea, we found that inherent susceptibility (measured as control-group mortality) significantly predicted immunisation effectiveness: groups with higher baseline mortality experienced greater reductions in mortality following immunisation. Although the family Bufonidae has also been identified as highly susceptible to Bd^{7,42,72}, we found no clear evidence for immunisation effects in this family. However, the effects were derived from only three bufonid species that were mostly immunised using less effective probiotic treatments (Figures 2 & 3). We therefore encourage additional studies testing more effective

immunisation approaches in Bufonidae, particularly live-pathogen and natural-chemical immunisation, to better evaluate their potential in this highly susceptible family.

CONCLUSION

Our meta-analysis shows that immunisation through prior exposure to live Bd, its natural chemicals (primarily Bd metabolites), or potentially synthetic antiparasitics can reduce disease susceptibility in amphibians subsequently challenged with Bd. The results indicate that immunisation could enhance survival during reintroductions or population supplementation involving Bd-susceptible species. Habitat-based interventions, such as providing thermal refuges²⁴ or creating salted ponds⁷³, that facilitate infection clearance could similarly increase immunity and improve survival in wild populations, potentially enabling susceptible species to coexist with Bd. In contrast, evidence for immunisation against Bsal remains limited and mixed^{28,38,39,74}, and is insufficient to evaluate its general effectiveness across species. We also found that immunised larvae have lower infection prevalence, highlighting the potential to increase population-level resistance by releasing large numbers of immunised tadpoles. A key unresolved question is whether the benefits of larval immunisation persist through metamorphosis. If resistance endures, larval immunisation could be an efficient way to produce large cohorts of disease-resistant adults, thereby improving the feasibility and effectiveness of reintroduction and supplementation programs. Together, our study provides the first synthesis of immunisation efficacy for a major emerging wildlife disease. Immunisation holds considerable promise for practical conservation applications against Bd and potentially other emerging infectious diseases, such as white-nose syndrome and snake fungal disease.

METHODS

We conducted a systematic review and meta-analysis following, as closely as possible, the PRISMA-EcoEvo guidelines⁷⁵. A checklist is provided as a supplementary file. For our full PRISMA diagram detailing our search and selection criteria see Extended Data Figure 1.

Literature searches

We aimed to retrieve a large representative sample of studies measuring changes in amphibian susceptibility to chytridiomycosis after applying different interventions. We searched publications from ISI Web of Science (Core Collection), Scopus, ProQuest and ScienceDirect in April 2025. The search string we developed included “chytridiomycosis” (OR synonyms) AND “amphibian” (OR synonyms) AND terms for individual-based interventions (e.g. immunisation, vaccine) (see *Supplementary Information* for full details). To validate our search string, we generated a list of ten gold-standard papers²³⁻

^{25,29,30,32,33,46,49,54} that contained suitable datasets and ensured that our search strings retrieved all ten. To capture recent research, we included grey literature (dissertations, theses, preprints and non-English publications). We focused on empirical data and did not retrieve reviews, book chapters, meeting abstracts, editorials, and corrections. Furthermore, we found all papers citing or cited in four reviews⁷⁶⁻⁷⁹ using Web of Science and Scopus in May 2025. All of the above details are provided in the supplementary information. We identified 3,538 records and removed 2,053 duplicates using the R package *litserchr*⁸⁰, the website *Rayyan*⁸¹ as well as manual checks (Extended Data Figure 1). The remaining 1,485 records included 45 PhD and 26 MSc theses, from which we identified an additional 32 papers and 1 PhD thesis (total 1,518 records).

Inclusion criteria

For the 1,518 records, we first screened the title and abstract and then their full text. To be included, the study needed to meet all of the following inclusion criteria:

- 1. *Be an in vivo experiment on amphibians:*** We excluded non-experimental studies, experimental studies on non-amphibians, and *in vitro* studies on amphibians (e.g. isolated cells, skin secretions).
- 2. *Conduct a treatment aimed at reducing individual-level susceptibility to chytridiomycosis, with a comparable control:*** A comparable control comprises individuals with the same initial infection states, from the same population and subjected to identical procedures (except for the active treatment components). This ensures that observed differences are attributable to the focal treatment rather than variation in initial infection states, populations or handling procedures. Accordingly, we excluded (a) studies aiming to enhance host susceptibility⁵⁵ and (b) studies aiming to mitigate host susceptibility by creating chytrid-unfavourable environments. We excluded studies

identified as (b) because treatment differences can result from reduced chytrid performance rather than increased host resistance.

445 **3. *Have comparable treatment conditions:*** We included studies where untreated and treated individuals were (a) uninfected at the onset of experimental infection to avoid confounding by prior infection, (b) exposed to the same chytrid strain and (c) kept in a common-garden setting during the period of experimental infection.

4. *Present the correct infection outcomes:* We only included studies that reported (a) infection prevalence, (b) infection intensity and/or (c) mortality.

450 **5. *Complete an infection assessment:*** We included studies in which infection outcomes were measured in all individuals, or for a randomly selected subset. We excluded mark-recapture studies that failed to assess released individuals (e.g., those with <27% recapture rate⁸²), because treatment effects cannot be reliably assessed without knowing the infected states of non-recaptured animals.

455 **Data collection and classification**

We identified 38 eligible studies (Extended Data Figure 1; supplementary files–study summary) and classified the number of experiments within each study. An experiment was defined as a comparable pair of animal groups whose disease susceptibility was
460 experimentally reduced (treatment) or left unchanged (control), with all other conditions held constant. For instance, a study testing the effect of probiotics independently at two temperatures⁸³, using four different samples of animals, yielded two experiments.

465 Within each experiment, we recorded the research location (Figure 1), methodological and biological variables (Tables 1 & 2). For taxonomy, species were consolidated into family-level groups to increase sample sizes (Figure 2). For life stage, we categorised them into (a) larva (no forelimbs), (b) metamorph (forelimb emergence to tail resorption), (c) juvenile (tail resorption to sexual maturation) and (d) adult (after sexual maturation)⁸⁴. There were no eligible studies on metamorphs. We extracted the life stage both at immunisation and at
470 subsequent live pathogen challenge to clarify cross-stage effects. However, cross-stage experiments were rare (6 of 53), so these two life stages were largely overlapping. We therefore considered only the life stage at live pathogen challenge (where infection outcomes were measured) for analysis. We recorded the time elapsed between immunisation and live pathogen challenge (Table 1) to clarify effect persistence.

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We focused on treatment differences in prevalence, infection intensity and mortality. Mortality rate was defined as the cumulative proportion of deaths following live pathogen challenge, for which we extracted the final measure closest to the end of the challenge period. In contrast, prevalence and infection intensity could either increase or decrease over time as individuals transition between infection states. For studies that tested prevalence and/or infection intensity multiple times throughout the experiment, we extracted the peak value (indicating the most severe outcome caused by the pathogen). Peak prevalence was defined as the greatest proportion infected at a given time point. Peak infection intensity was calculated by identifying the maximum pathogen loads for each individual across all measures and then averaging individual peak values. A single value was extracted if prevalence and/or intensity was only measured once. To quantify variation in measurement effort, we recorded the total number of trait measurements (Table 1) for prevalence and infection intensity.

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We extracted the mean, measures of error (SD, standard error (SE), 95% CI), sample size, and the time elapsed between live pathogen challenge and outcome measurement (Table 1). Data were extracted from text, tables, figures or supplementary information. If raw data were available, we extracted the necessary data using the R package *tidyverse*⁸⁵ for calculation of summary statistics. If figures were used, we used the R package *ShinyDigitise*⁸⁶ to extract values. The data source is listed in the supplementary files—metadata.

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SEs and 95% CIs were first converted to SDs before calculating effect statistics. For studies reporting log₁₀-scale summaries, we converted the data to natural-log mean (Z) and SD (SD_Z) and then back-transformed these to the raw-scale mean (m) and SD (SD_m) following Higgins et al⁸⁷:

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$$m = \exp\left(Z + \frac{SD_Z^2}{2}\right)$$

$$SD_m = \sqrt{(\exp(SD_Z^2) - 1) \exp(2Z + SD_Z^2)}$$

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Back-transformation can be unreliable when SD_Z is missing or large. For such cases, we approximated $m \approx \exp(Z)$ and considered SD_m as unavailable. We ran a sensitivity analysis

excluding these transformed data ($k = 18$; Table S12). Effects of live-pathogen immunisation still tended to reduce infection intensity when excluding these transformed data (mean [CIs]: 70% [-96% to +1.7%]; $p = 0.052$), consistent with the main conclusion (mean [CIs]: -80% [-95% to -21%]; $p = 0.02$).

Effect size calculation

Effect sizes were calculated only for *Bd* datasets. For both prevalence ($k = 90$) and mortality ($k = 62$), we converted percentages into counts of events ('infection' for prevalence; 'death' for mortality) and non-events. We then calculated the natural logarithm of the risk ratio (logRR), which compares the risk (probability) of an event in the treatment compared to the control group⁸⁸:

$$\log RR = \ln \left(\frac{T_{event} / (T_{event} + T_{non-event})}{C_{event} / (C_{event} + C_{non-event})} \right)$$

We calculated sampling variance, $v(\log RR)$, for prevalence and mortality using the following formulas:

$$v(\log RR) = \frac{1}{T_{event}} - \frac{1}{T_{event} + T_{non-event}} + \frac{1}{C_{event}} - \frac{1}{C_{event} + C_{non-event}}$$

Here, T_{event} and C_{event} are the count of events for treatment (immunised) and control groups, respectively, whereas $T_{non-event}$ and $C_{non-event}$ are the count of non-events. Cells with a zero value generate undefined estimates. In the cases with at least one zero-count, we added 0.5 to all cells⁸⁹. We excluded experiments with non-events: that is, no infections for prevalence ($k = 2$) and no deaths for mortality ($k = 27$). The absence of infections or deaths may indicate that animals were naturally resistant or tolerant, or that post-immunisation live *Bd* challenges were not sufficiently virulent to cause disease even in control groups. We excluded these studies because there were no *Bd*-caused outcomes to be compared.

For infection intensity ($k = 56$), we calculated the natural logarithm of the response ratio (lnRR), which represents the log of proportional difference in the means between groups⁹⁰:

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$$\ln RR_1 = \ln \left(\frac{m_T}{m_C} \right)$$

$$v(\ln RR)_1 = \frac{SD_T^2}{n_T m_T^2} + \frac{SD_C^2}{n_C m_C^2} = \frac{CV_T^2}{n_T} + \frac{CV_C^2}{n_C}$$

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where m is the mean, n is the sample size, SD is the standard deviation, and $CV (SD/m)$ is the coefficient of variation for treatment (T) and control (C) groups, respectively. However, $\ln RR_1$ and $v(\ln RR)_1$ can be biased when sample sizes are small to moderate⁹¹. We further corrected for such bias⁹²:

$$\ln RR_2 = \ln \left(\frac{m_T}{m_C} \right) + \frac{1}{2} \left(\frac{CV_T^2}{n_C} - \frac{CV_C^2}{n_T} \right)$$

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$$v(\ln RR)_2 = \frac{CV_T^2}{n_T} + \frac{CV_C^2}{n_C} + \frac{CV_T^4}{2n_T^2} + \frac{CV_C^4}{2n_C^2}$$

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$\ln RR_2$ and $v(\ln RR)_2$ cannot be estimated when CVs are unavailable. Only few (6 of 71) observations lacked CVs, so we applied the ‘missing-cases’ method⁹² – using the weighted average CVs from datasets that report CVs to estimate effect sizes and sampling variances for missing cases ($i = 1, 2, \dots, K$):

$$\ln RR_3 = \ln \left(\frac{m_T}{m_C} \right) + \frac{1}{2} \left(\left[\frac{\sum_{i=1}^K (n_{Ti} CV_{Ti})}{\sum_{i=1}^K n_{Ti}} \right]^2 - \left[\frac{\sum_{i=1}^K (n_{Ci} CV_{Ci})}{\sum_{i=1}^K n_{Ci}} \right]^2 \right)$$

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$$v(\ln RR)_3 = \frac{\left[\frac{\sum_{i=1}^K (n_{Ti} CV_{Ti})}{\sum_{i=1}^K n_{Ti}} \right]^2}{n_T} + \frac{\left[\frac{\sum_{i=1}^K (n_{Ci} CV_{Ci})}{\sum_{i=1}^K n_{Ci}} \right]^2}{n_C} + \frac{\left[\frac{\sum_{i=1}^K (n_{Ti} CV_{Ti})}{\sum_{i=1}^K n_{Ti}} \right]^4}{2n_T^2} + \frac{\left[\frac{\sum_{i=1}^K (n_{Ci} CV_{Ci})}{\sum_{i=1}^K n_{Ci}} \right]^4}{2n_C^2}$$

All types of $\ln RR$ and $v(\ln RR)$ were calculated following the *func.R* script provided by Nakagawa et al⁹².

In all calculations, a negative effect size indicates a reduced infection outcome in immunised individuals compared with control individuals. All analyses were conducted in R⁹³ (version 4.5.1) using the *escalc* function in the *metafor* package (version 4.8-0)⁹⁴.

Data analysis

For each outcome, we ran a multi-level meta-analysis (MLMA) using residual maximum likelihood (REML) and *t*-based inference for parameters. We reported both the confidence intervals of effect size estimates, with associated prediction intervals in the supplementary information⁹⁵⁻⁹⁷. To assess effect heterogeneity, we calculated I^2 , defined as the proportion of total variance among effect sizes unexplained by the known sampling variance, as well as mean-standardised heterogeneity (CVH^2) and variance-mean-standardised heterogeneity (M^2) in the supplementary information⁹⁸. We used the *orchaRd* package (version 2.1.3)⁹⁹ to calculate heterogeneity and visualise results.

We included three random factors in each MLMA: (1) ‘observation ID’ to capture within-study variance beyond the expected from sampling error; (2) ‘study ID’ to account for similar experimental setups within studies; and (3) ‘phylogenetic relatedness’ to account for phylogenetic distances among species. We pruned a recently published amphibian phylogeny¹⁰⁰ to species in our datasets (Figure 2) and generated a corresponding phylogenetic correlation matrix using the *vcv* function in the *ape* package (version 5.8-1)¹⁰¹.

Multiple effect sizes within an experiment were often computed using a shared treatment group (such as ‘high-dose Bd metabolites’ vs a sham control, and ‘low-dose’ vs the same control³²). To account for such non-independence¹⁰², we created a sampling (co)variance matrix using the *vcalc* function in the *metafor* package⁹⁴, and we included this matrix off-diagonals to account for shared-control effects. This sampling (co)variance matrix was then included in our models.

Meta-regression

We tested whether *immunisation method* (6 levels; Table 1), *host life stage* (3 levels: larva, juvenile, adult), *host origin* (2 levels: captive, wild) and *taxonomic family* (9 levels; Figure 2) moderated immunisation effectiveness. Each moderator was analysed separately along with the random factors and correlation matrix described above. Interactions between moderators were not estimated due to small (or absent) sample sizes across the moderator combinations (Table S5). Nevertheless, we tested the moderating effect of immunisation method for juveniles because multiple methods were well represented in this life stage (Table S6).

There were insufficient data on host sex (48 of 53 experiments = 91% missing) and pathogen characteristics [lineage (77% missing), virulence (79% missing) and passage history (85% missing)], so we did not test their moderating effects.

For *host origin*, we used juvenile and adult datasets because only these datasets included both captive-bred and wild-bred individuals; in contrast, larvae were all wild-bred. To test whether *origin* moderated effects on infection intensity, we further restricted the analysis to juvenile datasets because intensity data in adults were mostly derived from wild-bred individuals ($k = 15$ of 16). For *taxonomic family*, we only interpreted results for Hylidae, Ranidae and Bufonidae because the other families had a very small number of effects ($k < 5$; Figure 2). For transparency, results for all families were reported in Table S7.

We mean-centred three methodological variables (Table 1): (1) immunisation-to-challenge interval (0–896 days; mean = 38 days), (2) challenge-to-measurement interval (5–241 days; mean = 43 days) and (3) measurement effort (1–33; mean = 6) and included them as separate moderators in the meta-regressions estimating overall effects. None of these variables predicted immunisation effects (Table S10). These variables were not further considered when testing the moderating effects of immunisation method and host features given the relatively small datasets in our meta-analysis.

To test whether more susceptible amphibians benefit more strongly from immunisation, we included the mortality rate of the control group (i.e., inherent susceptibility) as a moderator in the meta-regressions estimating overall effects.

Infection progression

Experiments often reported multiple outcome measures. To quantify how effects on prevalence, infection intensity and mortality covary within experiments, we ran a multivariate meta-analytic model using the *metafor* package⁹⁴. We considered infection outcome as a moderator with an experiment-level random effect (outcome -1 | experiment ID) to estimate between-experiment effect correlations among outcome measures. Effect size and sampling variance were calculated as in the univariate models. We allowed heterogeneity to differ among the three outcomes by using an unstructured (co)variance matrix for the true effects. We tested overall effect correlations rather than correlations within specific immunisation methods because of limited sample sizes ($n = 29$ experiments reporting both prevalence and infection intensity, $n = 18$ reporting both infection intensity and mortality, $n = 29$ reporting both prevalence and mortality).

Publication bias

We ran additional meta-regressions that included the square root of the inverse of effective sample size and the mean-centred publication year (from 2009 to 2024) as separate moderators to test for (1) small-study effect⁴⁴, whereby studies with small sample sizes and significant results are more likely to be published and (2) time-lag bias⁴⁵, whereby significant findings are published earlier than non-significant ones. We also visually evaluated funnel asymmetry using funnel plots, which showed the residuals of a meta-regression against effect size precision ($1/SE$). While funnel plots do not formally test for publication bias, they are still useful in visualising relationships between effect size and sampling variance. The funnel plots indicated a high-precision effect size for infection intensity (Figure S1). We therefore conducted a sensitivity analysis, re-running the infection intensity meta-regression using the same model structure but excluding this effect size. The main findings remained unchanged (Table S13).

Sensitivity analysis of inconsistent definitions of infection outcomes

For all analyses described above, we calculated infection intensity using only infected individuals (i.e. those with non-zero Bd loads). However, a few studies reported infection intensity while including uninfected individuals^{23,83,103-105}. We excluded these studies from our intensity analyses because they confound effects on prevalence with effects on infection

intensity. Indeed, several of our findings changed when these experiments were included ($k = 15$). For live-pathogen immunisation, the estimated reduction in infection intensity weakened – from an average reduction of 80% ($p = 0.02$; Figure 3) to 70% ($p = 0.04$; Table S14). Because live-pathogen immunisation had no clear effect on prevalence, mixing prevalence and intensity outcomes likely weakened the strong underlying effect on infection intensity (Figure 3). For natural-chemical immunisation we saw the opposite: effects on infection intensity shifted from uncertain ($p = 0.11$; Figure 3) to more clearly negative ($p = 0.053$; Table S14), likely because strong effects on prevalence inflated the apparent effect on infection intensity (Figure 3). These outcomes highlight the importance of a clear and consistent definition of infection intensity for accurately identifying where immunisation exerts its effects.

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DATA AVAILABILITY

Code, data and supplementary files can be downloaded from Zenodo:

<https://doi.org/10.5281/zenodo.20278226>

Table 1. Methodological sources of variation that may affect estimates of immunisation effectiveness in experiments assessing amphibian susceptibility to chytridiomycosis.

Factors likely to alter observed outcomes include the immunisation method with the expected immune pathways involved, and key experimental design features (immunisation-to-challenge interval, challenge-to-measurement interval, and measurement effort).

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Factor	Details
Immunisation method	1. Natural chemical: Exposure to chemicals produced by live organisms, including skin peptides from resistant frog species¹⁰⁶ or cell-free pathogen compound (Bd metabolites)^{32,33,49}. Exposure to Bd metabolites often involves repeated dosing^{32,33,49}. The immunity stimulated by this approach is unclear. 2. Live pathogen: Exposure to live fungus, either followed by a clearance procedure^{23,24,29,30,107,108} or not (in cases without clinical signs^{28,38,46}, or when individuals are given time to self-cure³⁹). Individuals that undergo live-pathogen immunisation are uninfected at the end of this immunisation procedure. This approach aims to stimulate adaptive immunity. 3. Probiotic: Exposure to antifungal microbes^{34,35,83,105,109,110}. Before adding microbes, amphibians are often washed in antimicrobial agents^{34,83,105,109} or natural media^{35,110}. This washing step aims to remove existing bacteria to increase successful colonisation of the introduced microbes and also minimise unintended bacterial interactions. While probiotics are expected to enhance innate immunity^{111,112}, recent studies suggest they may defend against specific pathogens¹¹³, indicating a possible adaptive immunity pathway. 4. Dead pathogen: Exposure to killed fungus^{25,31,53,54}, commonly administered through repeated doses^{25,31,53}. This approach aims to stimulate adaptive immunity. 5. Synthetic antiparasitic: Exposure to synthetic antifungals (e.g., itraconazole⁵⁸ or terbinafine³⁶) or pesticides (e.g., Ivermectin⁵⁷). The immunity stimulated by this approach is unclear. 6. Other: Other approaches, such as enriching skin mast cells (immune cells that mediate neutrophil recruitment and mucin production)¹¹⁴ or exposing amphibians to metyrapone (an inhibitor of corticosterone synthesis that may reduce stress-related immunosuppression and thus stabilise microbiota)¹¹⁵. These two examples are expected to enhance innate immunity.
Immunisation-to-challenge interval	Time elapsed between immunisation and live pathogen challenge. If immunisation-induced protection declines with time, any benefits should be reduced when challenge occurs long after immunisation. In contrast, if protection is long-lasting, immunised individuals should remain less susceptible over longer periods.
Challenge-to-measurement interval	Time elapsed between live pathogen challenge and outcome measurement. Because infection unfolds over time, the stage of disease reached at the point of measurement can strongly influence observed outcomes. Longer intervals allow greater divergence in infection trajectories, potentially magnifying differences among treatment groups depending on how rapidly disease progresses.
Measurement effort	Disease progression involves moving through different infection states and severities ^{24,28,30,36} , generating temporal variation in observed outcomes. Studies with regular measurements (e.g., regular skin swabs) may therefore be more likely to detect treatment differences than studies with low measurement effort (e.g., skin swabbing only at the end of the experiment).

Table 2. Biological factors that may affect estimates of immunisation effectiveness in experiments assessing amphibian susceptibility to chytridiomycosis. Factors marked with an asterisk (*) had sufficient data for quantitative synthesis and were included in the meta-analysis.

Factor	Details
Host life stage*	<i>Batrachochytrium dendrobatidis</i> (Bd) infects keratinized tissues, which are restricted to the mouthparts of tadpoles and then extend to the entire skin after metamorphosis ^{62,65} . Therefore, tadpoles are typically more disease-resistant than post-metamorphic stages, with metamorphs or newly metamorphosed juveniles the most susceptible ⁴² . The increased susceptibility during metamorphosis is also linked to transient immune downregulation associated with organ reorganisation and the incomplete transition from larval to adult immune systems ^{116,117} .
Host origin*	Captive amphibians have reduced contact with natural substrates and other species, and their microbial communities often differ from those of wild conspecifics ¹¹⁸ . Whether these differences translate into different responses to Bd infection or to immunisation is unknown.
Host taxonomy*	Taxa-specific biology (e.g., physiology ⁴⁰ , habitat ⁴¹ and behaviour ¹¹⁹) could influence susceptibility to Bd and may drive species-specific responses to immunisation ²⁸ .
Host sex	Males and females use different strategies to reproduce. Males often increase fertilisation success by investing more in sexual traits (sperm, courtship) that are immunosuppressive ¹²⁰ . In contrast, females usually increase breeding success by investing in soma that promote fecundity ¹²¹ . Amphibians have sex-specific reproductive strategies ¹²² , where males are often more susceptible to Bd than females ^{123,124} . It is currently unclear, however, whether immunisation benefits males and females differently.
Pathogen features	Bd virulence varies with genotype, phenotype and passage history ^{33,72} . For example, the global pandemic Bd lineage is highly virulent and linked with widespread amphibian declines, whereas the impacts of other chytrid lineages remain unclear ⁵ . Virulence also tends to diminish in strains subject to extensive <i>in vitro</i> passage ¹²⁵ .

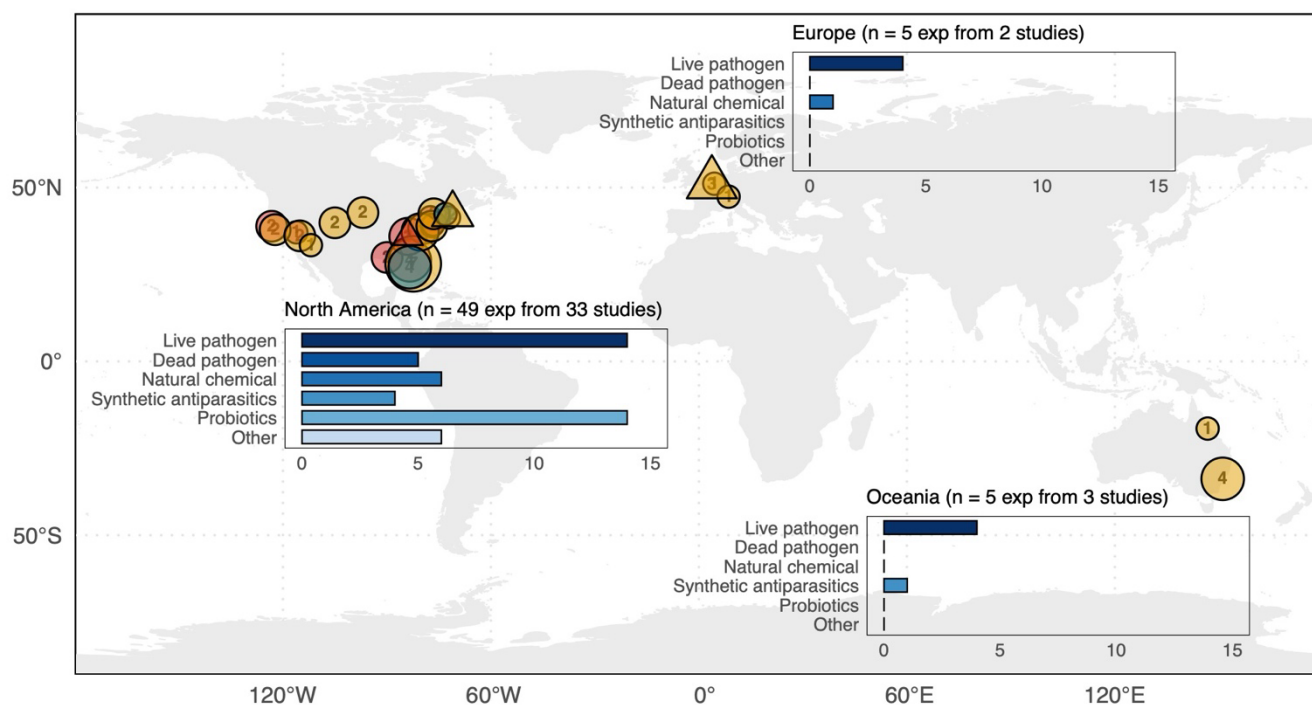


Figure 1. Geographic locations of eligible studies. There are usually multiple independent experiments in a study, so counts represent the number of experiments. Bubble size indicates the number of experiments per location, with the exact count shown within each bubble. Colour represents the life stage (adult = red; juvenile = yellow; larva = blue). Shape represents the pathogen type (Bd = circle; Bsal = triangle). Histograms show the number of experiments for each immunisation approach on each continent. Experiments that tested multiple approaches were counted separately for each approach.

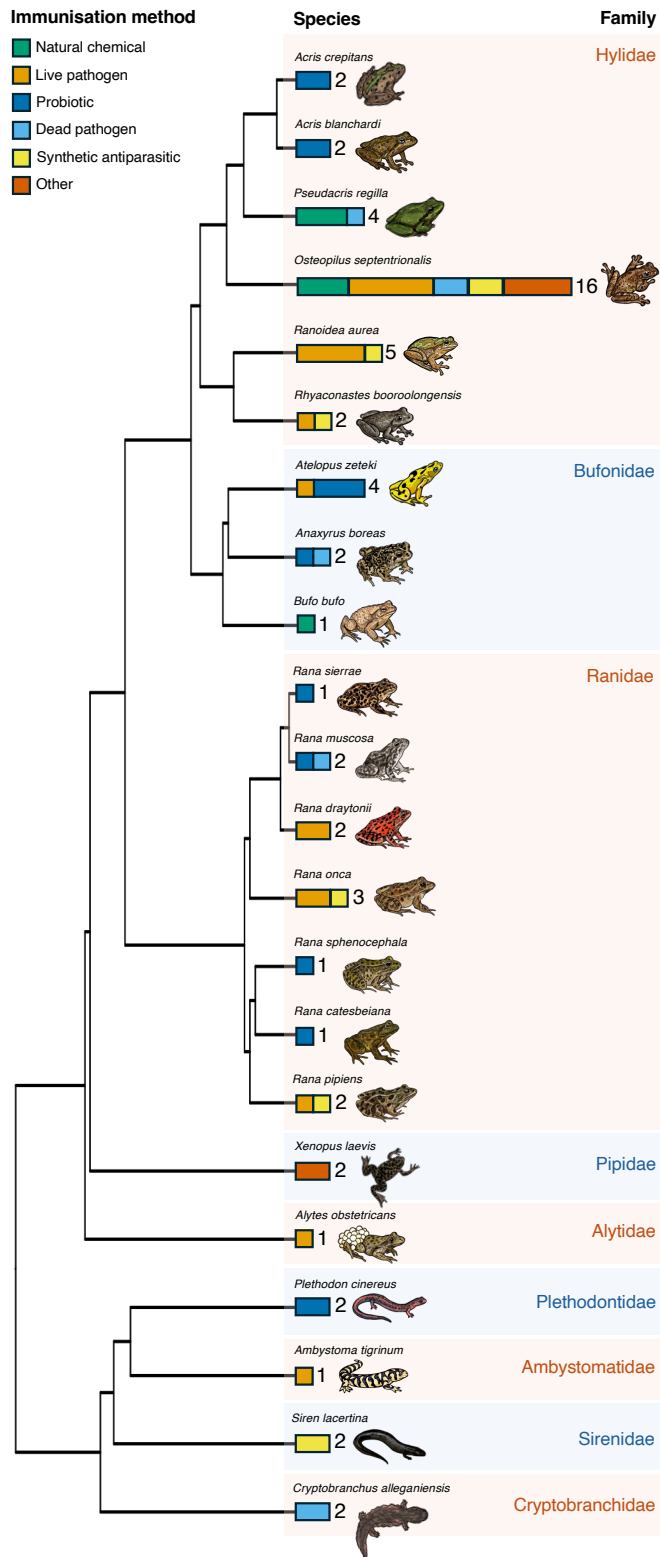


Figure 2. Phylogenetic relationship among species in the Bd analyses. The tree was pruned from a recently published phylogeny¹⁰⁰. Coloured rectangles indicate the immunisation method, with rectangle size proportional to the number of experiments per method. The number beside each species gives its total experiment count. Species are shown by silhouette and grouped by family (brackets). Experiments testing more than one method were counted separately for each method.

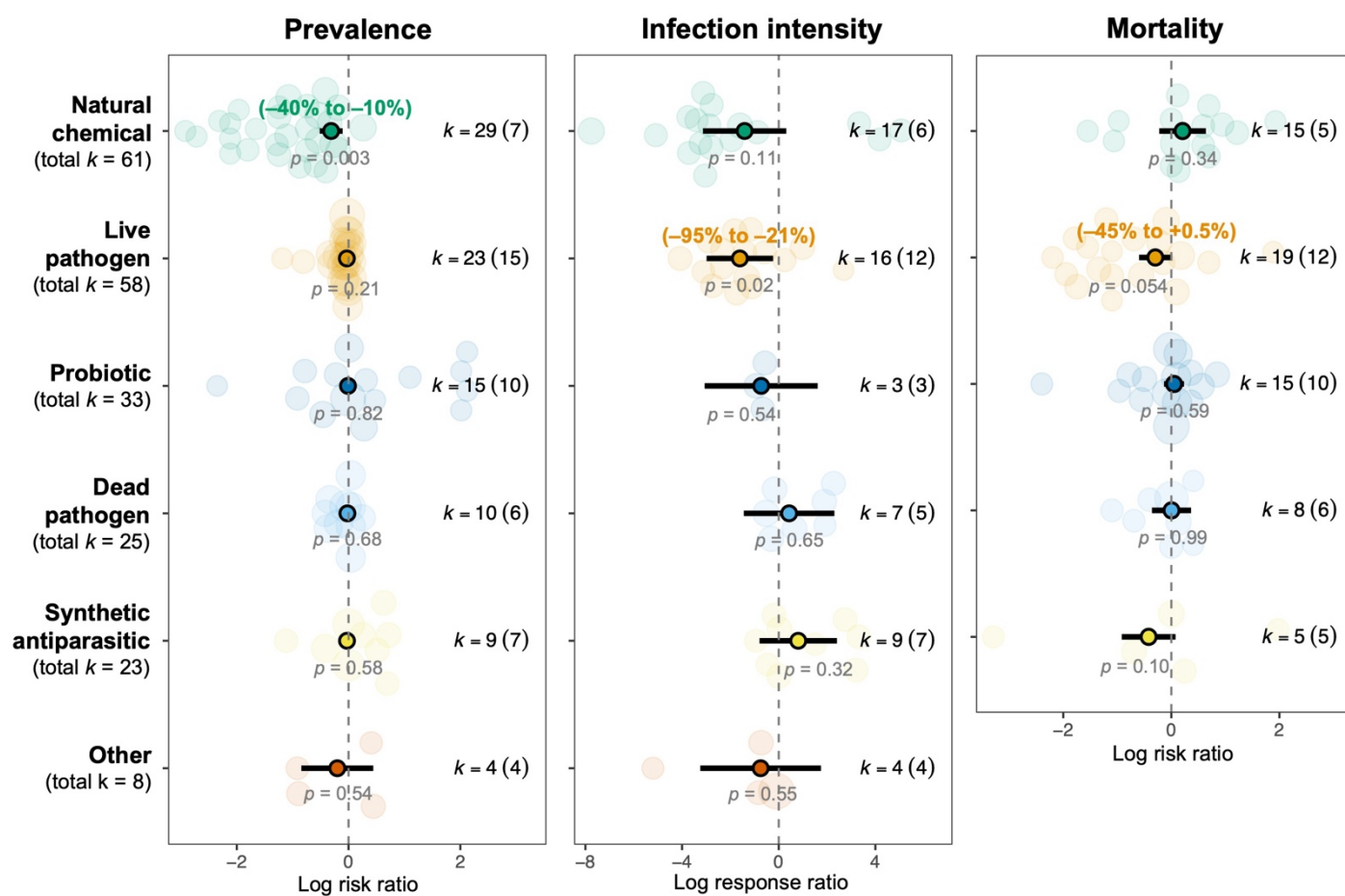


Figure 3. Moderating effects of immunisation method on prevalence, infection intensity and mortality. Darker dots and lines indicate means and 95% confidence intervals, along with p -values. Confidence intervals are also presented as percentage changes in outcome measures for specific methods showing a clear effect. Lighter dots indicate individual effect sizes, and dot sizes reflect the relative precision ($1/SE$) of each effect size. Counts represent the number of effect sizes (k), with the number of experiments in parentheses. Full model outputs (including t -values and prediction intervals) are reported in Table S1.

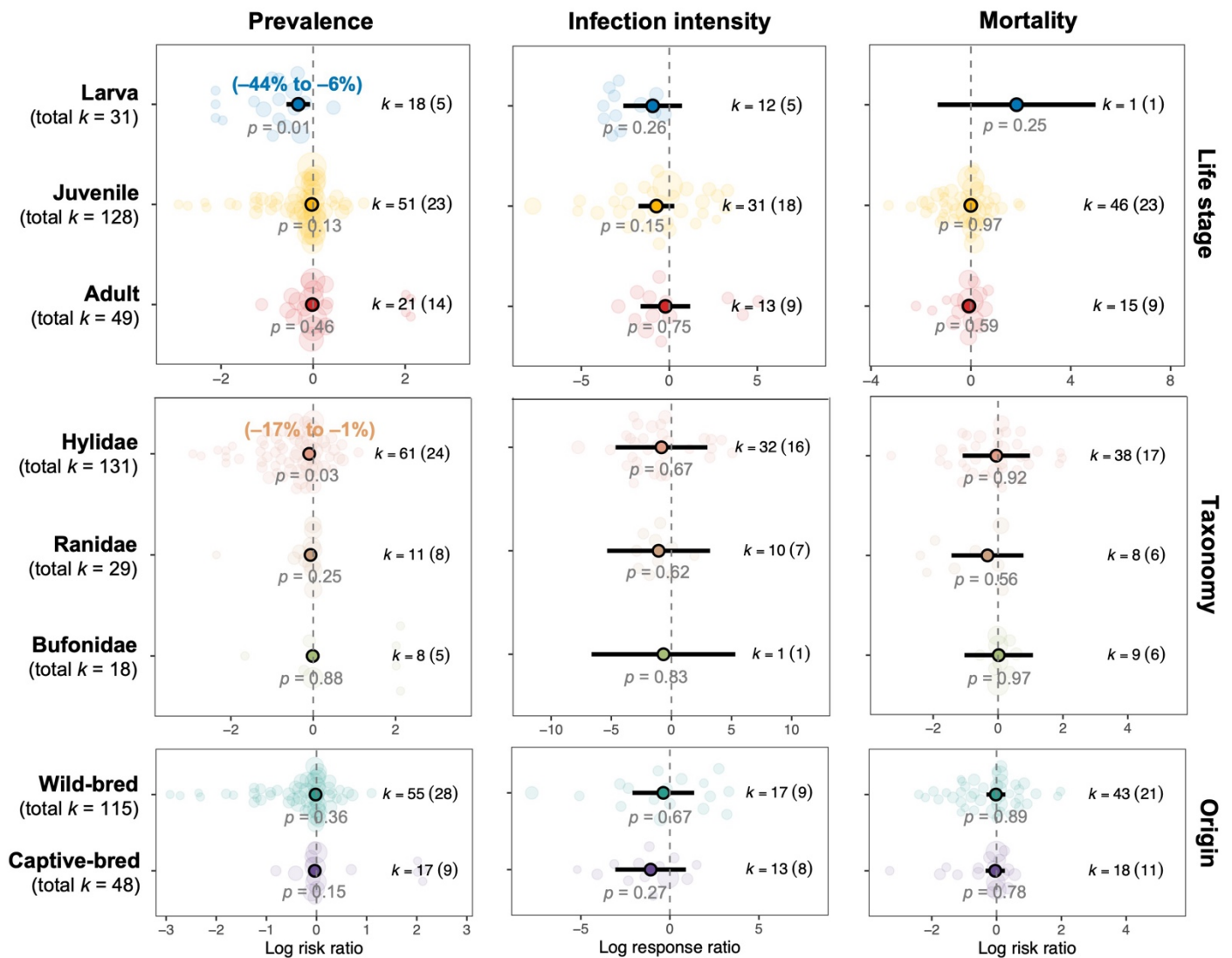


Figure 4. Moderating effects of host feature (life stage, taxonomy, origin) on prevalence, infection intensity and mortality. Darker dots and lines indicate means and 95% confidence intervals, along with p -values. Confidence intervals are further presented as percentage changes in outcome measures for categories showing a significant effect. Lighter dots indicate individual effect sizes, and dot sizes reflect the relative precision ($1/\text{SE}$) of each effect size. Counts represent the number of effect sizes (k), with the number of experiments in parentheses. Full model outputs (including t -values and prediction intervals) are reported in Tables S4, S7, and S9.

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