Global 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 53 experiments across 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 reduced host infection intensity and lowered mortality after re-exposure, whereas immunisation using Bd-derived natural chemicals reduced infection prevalence. Other immunisation approaches showed no clear effects across species. Immunisation effects also varied across disease progression stages, host life stages, and taxonomic groups. Our findings suggest that live-pathogen immunisation can be an effective strategy for increasing survival during reintroductions. Management actions that supplement immunised individuals or help wild amphibians clear infections may enhance survival in vulnerable populations.

KEYWORDS

antifungal, microbe, metabolites, vaccine, frog

INTRODUCTION

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 cause chytridiomycosis, a disease that can trigger mass mortality events[5,6]. Since the 1970s, the spread of chytridiomycosis has contributed to population declines in more than 500 amphibian species, with at least 90 species potentially extinct due to these pathogens[7]. Consequently, amphibians are now the most imperilled vertebrate class, with more than 40% of species threatened, with chytridiomycosis identified as the major threatening process[7,8].

The challenge for susceptible amphibian species is that, once established in the wild, chytridiomycosis appears virtually impossible to eradicate. Persistence stems not only from the pathogen's ability to infect non-amphibian hosts[9,10] and remain viable in in the envrionment[11], but also from non-susceptible amphibian host species that act as long-term pathogen reservoirs and continual sources of infection for more vulnerable species[12]. As a result, conventional conservation measures, including translocations and reintroductions, often fail because disease-causing pathogens persist in the environment[13-15]. For Bd and Bsal, the only proven management strategy is to remove susceptible individuals from natural habitats and maintain disease-free captive populations[16], a scenario that risks leaving 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.

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[17,18]. For amphibians, successful immunisation strategies that enhance host survival[19-21] could substantially improve conservation outcomes, for example by enabling the 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[22,23].

However, immunisation efforts targeting Bd and Bsal in amphibians have produced mixed outcomes[24,25], 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[24,26], dead pathogens[21,27], pathogen metabolites[28,29], antifungal microbes[30,31], and synthetic anitfungals[32,33], 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 rates, lower pathogen loads in infected individuals, and/or decrease mortality. We evaluated how these outcomes varied across immunisation methods (Table 1) and among different amphibian life stages, origin (captive or wild bred) and taxonomic family (Table 2).

RESULTS

We screened 1,517 studies and identified 38 containing relevant datasets (Figure 1 and S1). 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 Bd[24] or live Bsal[34,35] 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 species[24] but not others[34,35], while prevalence was unaffected by immunisation treatment in all cases. Given the limited number of Bsal experiments, we estimated effect sizes only for studies involving Bd exposure.

Immunisation treatments reduce susceptibility to Bd across amphibians

We quantified differences in infection rates (prevalence), infection intensity, and mortality between immunised and control groups following a live Bd exposure challenge. We used multi-level meta-analytic models that included within-study, between-study and phylogenetic random effects to estimate the overall success of immunisation at reducing disease (Figure 2). Effect sizes for prevalence (k = 90) and mortality (k = 62) were calculated as the natural logarithm of the risk ratio (logRR). Effect sizes for infection intensity (k = 56) were calculated as the natural logarithm of the response ratio (lnRR) and were based only on infected individuals.

Mortality is arguably the most consequential outcome because it links directly to population performance and the potential for long-term persistence. Only one immunisation approach reduced mortality following subsequent live Bd exposure – the live-pathogen immunisation which reduced death rates 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 was also the only approach that 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.

Natural-chemical immunisation was the only approach that significantly reduced prevalence (Figure 3), lowering infection rates by an average of 27% (p = 0.003). While live-pathogen immunisation reduced mortality and infection intensity, it had no clear effect on prevalence (p = 0.21; Figure 3). The remaining immunisation approaches (probiotic, dead-pathogen, synthetic antiparasitic, and other) showed no clear effects on either 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 pathogen load were positively correlated (ρ [CIs] = 0.72 [0.28 to 1.00]), suggesting a reinforcing effect. Immunisation that limits initial infection also suppresses within-host pathogen abundance. 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 pathogen load (ρ [CIs] = –0.32 [–1.00 to 1.00]), 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 S2). The effects of immunisation on prevalence were highly consistent across studies ($I^2 \approx 0\%$), largely because most immunisations did not clearly alter infection rates (Figure S2). 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 ($I^2_{between-study}$ = 46%, $I^2_{within-study}$ = 50%, $I^2_{phylogeny} \approx$ 0%). For mortality, heterogeneity primarily arose from between-study differences with a weak phylogenetic signal ($I^2_{between-study}$ = 21%, $I^2_{within-study} \approx$ 0%, $I^2_{phylogeny}$ = 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 risk by an average of 28% (p = 0.01). In contrast, immunisation effects on infection intensity and mortality were not moderated by life stage (Table S3).

Species in different families are known to vary in their responses to immunisation and disease outcomes due to taxa-specific physiology[36] and ecological requirements[37], 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 S4). Hylids are known to be particularly susceptible to Bd and often experience high Bd-induced mortality[7,38,39]. 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 S5).

Immunisation effectiveness did not clearly differ between wild and captive-bred hosts (Figure 4; Table S6) 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 S7). These results indicate that immunisation performs similarly in both wild-bred and captive-bred amphibians, even across studies with differing experimental durations and measurement frequencies. Consequently, 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, or through habitat interventions that facilitate pathogen clearance and could thus generate immunity in wild populations.

Publication bias

Immunisation effects were not predicted by effective sample sizes[40] or publication year[41] of the study (all slopes overlapped zero; all $p > 0.14$; Table S8), 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 reintroductions[22]. Our meta-analysis shows that, on average, prior exposure to live Bd lowers mortality risk and pathogen intensity in subsequently infected individuals, whereas exposure to natural chemicals reduces infection prevalence but not mortality or pathogen intensity. These two 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 chemical reduces susceptibility to Bd, while exposure to dead Bd had no effect

Live-pathogen immunisation reduced infection intensity and mortality upon re-exposure. 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[17,18]. In addition to live-pathogen immunisation, we also found evidence that natural-chemical immunisation can reduce infection prevalence. Most natural-chemical immunisation studies used Bd metabolites (cell-free by-products of pathogen cultures) to induce resistance[28,29,42]. Together, these findings highlight that pathogen-related immunisation is an effective strategy for reducing susceptibility to Bd.

Interestingly, we detected no clear effect of dead-pathogen immunisation, consistent with epidemiological evidence that non-infectious vaccines generally confer weaker protection than live vaccines[18]. What remains unresolved, however, is why Bd metabolites were effective. One explanation is that Bd metabolites function more like a drug than a vaccine, directly inhibiting or killing pathogens without engaging host immunity. However, this seems unlikely given that synthetic antiparasitics (antifungals or pesticides) showed no detectable effects in our analysis. A second possibility is that inhibitory compounds produced by Bd, such as methylthioadenosine[43] and spermidine[44], stimulate host immune responses. These compounds originate from the walls of Bd zoosporangia rather than from zoospore cells[45]. This distinction is important because dead-Bd immunisation typically exposes hosts to only purified dead zoospores that lack zoosponrangia[21,27,46,47]. In contrast, Bd metabolites are usually prepared by filtering culture media containing both zoosporangia and zoospores[29]. 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 zoosporangia[48], but not when exposed solely to purified dead zoospores[47,49].

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). We might therefore expect positive correlations among effects on prevalence, infection intensity, and mortality. A key question, then, is why live-pathogen immunisation reduced morality but not prevalence, whereas natural-chemical immunisation reduced prevalence but not mortality. Overall, while we found that prevalence and 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[50,51]: (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 (see the diverse relationships between Bd resistance and tolerance in 35 amphibian species[52]). In our meta-analysis, prevalence and infection intensity are indicators of resistance, whereas mortality reflects tolerance[51,52]. 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[12,53]. 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

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 mouthparts[54], and infections in this region rarely cause mortality[38,51]. 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 uncommon[51], infected tadpoles often die during or shortly after metamorphosis[55], when keratin appears across the entire skin surface and Bd can spread more extensively[54]. Tadpoles can also act as important reservoirs that maintain Bd in a population and increase disease transmission[56]. Evidence that immunisation reduces infection rates 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[57,58].

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. This gap is important to address, especially given our finding that immunisation significantly reduces infection prevalence in tadpoles. Tadpole immunisation also offers several practical advantages: (1) a single adult breeding pair can 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[51,59], 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, likely because ecological differences make some groups more suspectable to Bd. Immunisation was particularly effective at reducing prevalence in Hylidae. A previous meta-analysis also identified that the superfamily Hyloidea were particularly susceptible to Bd[38], suggesting that immunisation may be more effective in 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. The family Bufonidae have also been identified as highly susceptible to Bd[7,38,60]. We found no clear evidence that this family was more susceptible in our meta-analysis, although the effects were derived from just three 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.

Precise measurement of infection outcomes provides a better understanding of immunisation effects

We separated immunisation effects on prevalence and infection intensity to clarify how immunisation influences different stages of disease progression. To do this, 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[19,61-64]. 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 S9). 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 S9), 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.

Measuring mortality in laboratory studies can be logistically challenging because ethical guidelines often require treating or curing sick amphibians. Similarly, it is often recommended to use nonlethal pathogen doses to avoid rapid deaths[38]. In our meta-analysis, many immunisation studies (e.g.[21,65,66]) reported zero mortality ($k = 27$). The absence of deaths may indicate that animals were naturally tolerant, or that post-immunisation live Bd challenges were not sufficiently virulent to cause mortality even in control groups. We excluded these studies from our mortality analyses because we could not estimate immunisation effects on mortality when no deaths occurred. Future work aiming to evaluate immunisation effects on mortality – the most consequential disease outcome – should carefully consider the Bd dose or strain used in challenge experiments to ensure that mortality can be meaningfully assessed.

CONCLUSION

Our meta-analysis shows that immunisation through prior exposure to live Bd or natural chemicals (mostly Bd metabolites) can reduce disease susceptibility in amphibians subsequently challenged with Bd. Our study provides the first synthesis of immunisation efficacy for a major emerging wildlife disease. The results indicate that immunisation approaches hold considerable promise for practical conservation applications against Bd and potentially other emerging infectious disease (e.g., white-nose syndrome, snake fungal disease). Immunisation could enhance survival during reintroductions or population supplementation efforts involving Bd susceptible species. In addition, habitat interventions, such as providing thermal refuges or creating salted ponds, that help individuals clear infections could similarly increase immunity and improve survival in wild populations, potentially enabling susceptible species to persist with the pathogen.

METHODS

We conducted a systematic review and meta-analysis following, as closely as possible, the PRISMA-EcoEvo guidelines[67]. A checklist is provided as a supplementary file. For our full PRISMA diagram detailing our search and selection criteria see Figure S1.

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 developed included "chytridiomycosis" (OR synonyms) AND "amphibian" (OR synonyms) AND terms for individual-based interventions (e.g. immunisation, vaccine) (see *Supplementary Material* for full details). To validate our search string, we generated a list of ten gold-standard papers[19-21,25,26,28,29,42,47,68] 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 reviews[69-72] using Web of Science and Scopus in May 2025. All of the above details are provided in the supplementary material. We identified 3,538 records and removed 2,053 duplicates using the R package *litserchr*[73], the website *Rayyan*[74] as well as manual checks (Figure S1). The remaining 1,485 records included 45 PhD and 26 MSc theses, from which we identified an additional 31 papers and 1 PhD thesis (total 1,517 records).

Inclusion criteria

For the 1,517 records, we first screened the title and abstract and then their full text. To be included, the study needed to meet all 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 susceptibility[48] and (b) studies aiming to mitigating 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.
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.
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

rate[75]), because treatment effects cannot be reliably assessed without knowing the infected states of non-recaptured animals.

Data collection and classification

We identified 38 eligible studies (Figure S1; 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 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 temperatures[61], using four different samples of animals, yielded two experiments.

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)[76]. There were no eligible studies on metamorphs. We extracted the life stage both at immunisation and at 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.

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.

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 materials. If raw data were available, we extracted the necessary data using the R package *tidyverse*[77] for calculation of summary statistics. If figures were used, we used the R package *ShinyDigitise*[78] to extract values. The data source is listed in the supplementary files–metadata.

SEs and 95% CIs were first converted to SDs before calculating effect statistics. For studies reporting $\log_{10}$-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 al[79]:

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

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

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 S10). 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 and mortality, we converted percentages into counts of events ('infection' for prevalence; 'death' for mortality) and non-events. We then calculated the log risk ratio (logRR), which compares the risk (probability) of an event in the treatment compared to the control group[80]:

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

We calculated sampling variance, $v(\log \mathrm{RR})$, 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 cells[81]. We excluded experiments with non-events: that is, no infections for prevalence ($k = 2$) and no deaths for mortality ($k = 27$). These experiments were excluded because there were no Bd-caused outcomes to be compared.

For infection intensity, we calculated the log response ratio (lnRR), which represents the log of proportional difference in the means between groups[82]:

$$\mathrm{lnRR}_1 = \ln\left(\frac{m_T}{m_C}\right)$$

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

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,

lnRR_1 and $v(\text{lnRR})_1$ can be biased when sample size are small to moderate[83]. We further corrected for such bias[84]:

$$\text{lnRR}_2 = \ln\left(\frac{m_T}{m_C}\right) + \frac{1}{2}\left(\frac{\text{CV}_T{}^2}{n_C} - \frac{\text{CV}_C{}^2}{n_T}\right)$$

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

lnRR_2 and $v(\text{lnRR})_2$ cannot be estimated when CVs are unavailable. Only few (6 of 71) observations lacked CVs, so we applied the ‘missing-cases' method[84] – using the weighted average CVs from datasets that report CVs to estimate effect sizes and sampling variances for missing cases ($i = 1, 2, ..., K$):

$$\text{lnRR}_3 = \ln\left(\frac{m_T}{m_C}\right) + \frac{1}{2}\left(\frac{\left[\frac{\sum_{i=1}^{K}(n_{Ti}\,CV_{Ti})}{\sum_{i=1}^{K} n_{Ti}}\right]^2}{n_T} - \frac{\left[\frac{\sum_{i=1}^{K}(n_{Ci}\,CV_{Ci})}{\sum_{i=1}^{K} n_{Ci}}\right]^2}{n_C}\right)$$

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

All types of lnRR and v(lnRR) were calculated following the *func.R* script provided by Nakagawa et al[84].

In all calculations, a negative effect size indicates a reduced infection outcome in immunised individuals compared with control individuals. All analyses were conducted in R[85] (version 4.5.1) using the *escalc* function in the *metafor* package (version 4.8-0)[86].

Data analysis

For each outcome, we ran a multi-level meta-analysis (MLMA) using residual maximum likelihood (REML) and *t*-based interference for parameters. We reported both the confidence intervals of effect size estimates, with associated prediction intervals in the supplementary material[87-89]. 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 material[90]. We used the *orchaRd* package (version 2.1.3)[91] 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 phylogeny[92] 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)[93].

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 control[28]). To account for such non-independence[94], we created a sampling (co)variance matrix using the *vcalc* function in the *metafor* package[86], 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. There was not sufficient 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 S4.

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 S7). 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 *metafor* package[86]. 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 effect[40], whereby studies with small sample sizes and significant results are more likely to be published and (2) time-lag bias[41], 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 (Figure S3).

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DATA AVAIABILITY

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).

Factor	Details
Immunisation method	**1. Natural chemical**: Exposure to chemicals produced by live organisms, including skin peptides from resistant frog species[95] or cell-free pathogen compound (Bd metabolites)[28,29,42]. Exposure to Bd metabolites often involves repeated dosing[28,29,42]. The immunity stimulated by this approach is unclear. **2. Live pathogen**: Exposure to live fungus, either followed by a clearance procedure[19,20,25,26,66,96] or not (in cases without clinical signs[24,34,68], or when individuals are given time to self-cure[35]). This approach aims to stimulate adaptive immunity. **3. Probiotic:** Exposure to antifungal microbes[30,31,61,64,97,98]. Before adding microbes, amphibians are often washed in antimicrobial agents[30,61,64,97] or natural media[31,98]. This washing step aims to remove existing bacteria to increase successful colonisation of the introduced microbes and also minimise unintended bacterial interactions. Probiotics are expected to enhance innate immunity[99,100], whereas recent studies suggest their defences to specific pathogens[101], aligning with adaptive immunity. **4. Dead pathogen**: Exposure to killed fungus[21,27,46,47], commonly administered through repeated doses[21,27,46]. This approach aims to stimulate adaptive immunity. **5. Synthetic antiparasitic**: Exposure to synthetic antifungals (e.g., itraconazole[102] or terbinafine[32]) or pesticides (e.g., lvermectin[103]). 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)[104] or exposing amphibians to metyrapone (an inhibitor of corticosterone synthesis that may reduce stress-related immunosuppression and thus stabilise microbiota)[105]. 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[20,24,26,32], 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*	The chytrid fungus infects keratinized tissues, which are restricted to the mouthparts of tadpoles and then extend to the entire skin after metamorphosis[51,54]. Therefore, tadpoles are typically more disease-resistant than post-metamorphic stages, with metamorphs or newly metamorphosed juveniles the most susceptible[38]. 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[106,107].
Host origin*	Captive amphibians have reduced contact with natural substrates and other species, and their microbial communities often differ from those of wild conspecifics[108]. Whether these differences translate into different responses to Bd infection or to immunisation is unknown.
Host taxonomy*	Taxa-specific biology (e.g., physiology[36], habitat[37] and behaviour[109]) could influence susceptibility to the chytrid fungus and may drive species-specific responses to immunisation[24].
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[110]. In contrast, females usually increase breeding success by investing in soma that promote fecundity[111]. Amphibians have sex-specific reproductive strategies[112], where males are often be more susceptible to Bd than females[113,114]. It is currently unclear, however, whether immunisation benefits males and females differently.
Pathogen features	Chytrid virulence varies with genotype, phenotype and passage history[29,60]. 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 *in vitro* passage[115].

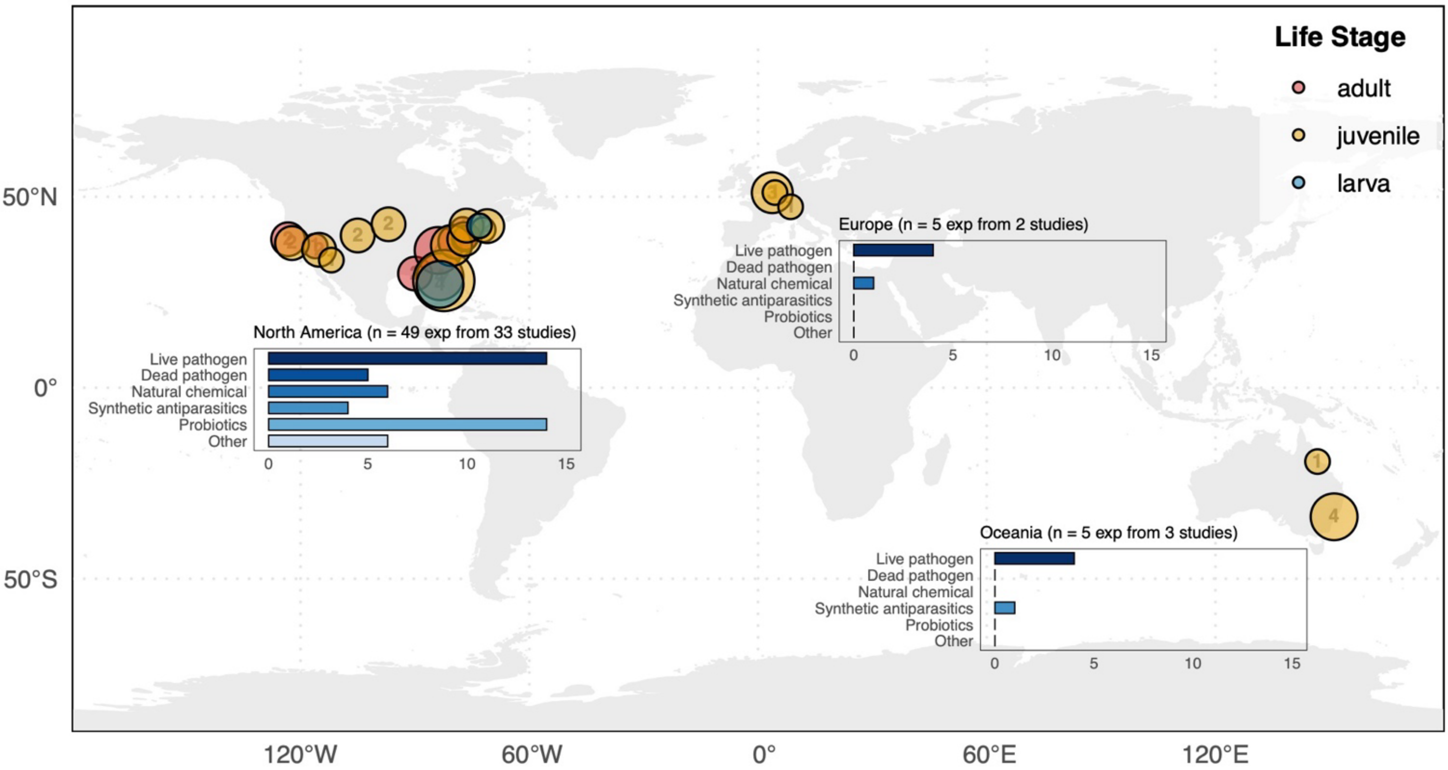

Figure 1. Geographic locations of eligible studies (both Bd and Bsal). There are usually multiple independent experiments in a study, so counts represent the number of experiments. Bubble size indicates the number of experiments per location, and colour represents the life stage. Histograms show the number of experiments for each immunisation approach on each continent. Experiments that tested multiple approaches were counted separately for each approach.

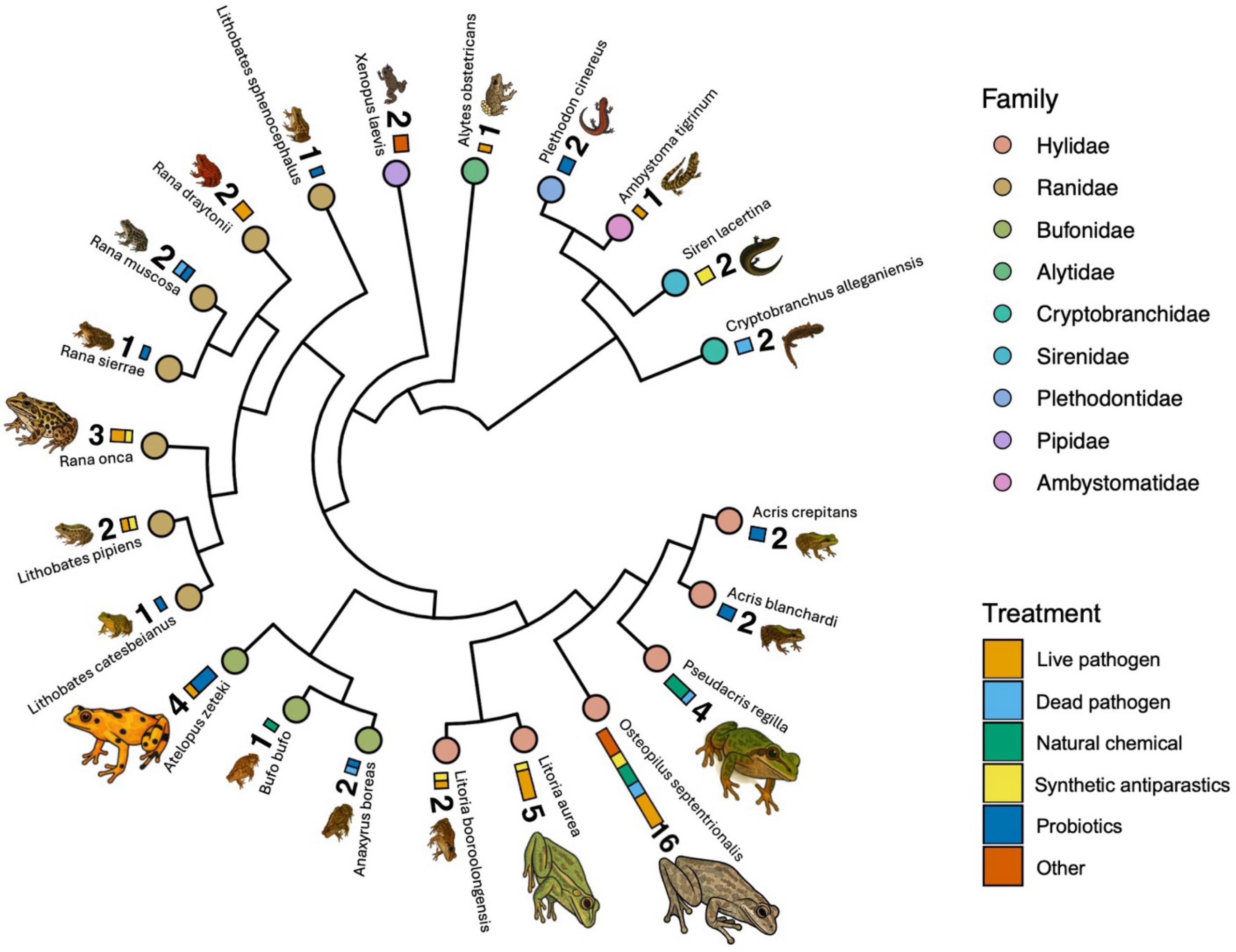

Figure 2. Phylogenetic relatedness among species in the Bd analyses. This tree was pruned according to the recently published phylogeny[92]. Node colours indicate family, and rectangle colours indicate approach types. The sizes of each rectangle and animal silhouette reflect the number of experiments per immunisation approach, with counts indicating the total number of experiments per species. Experiments that tested multiple approaches were counted separately for each approach.

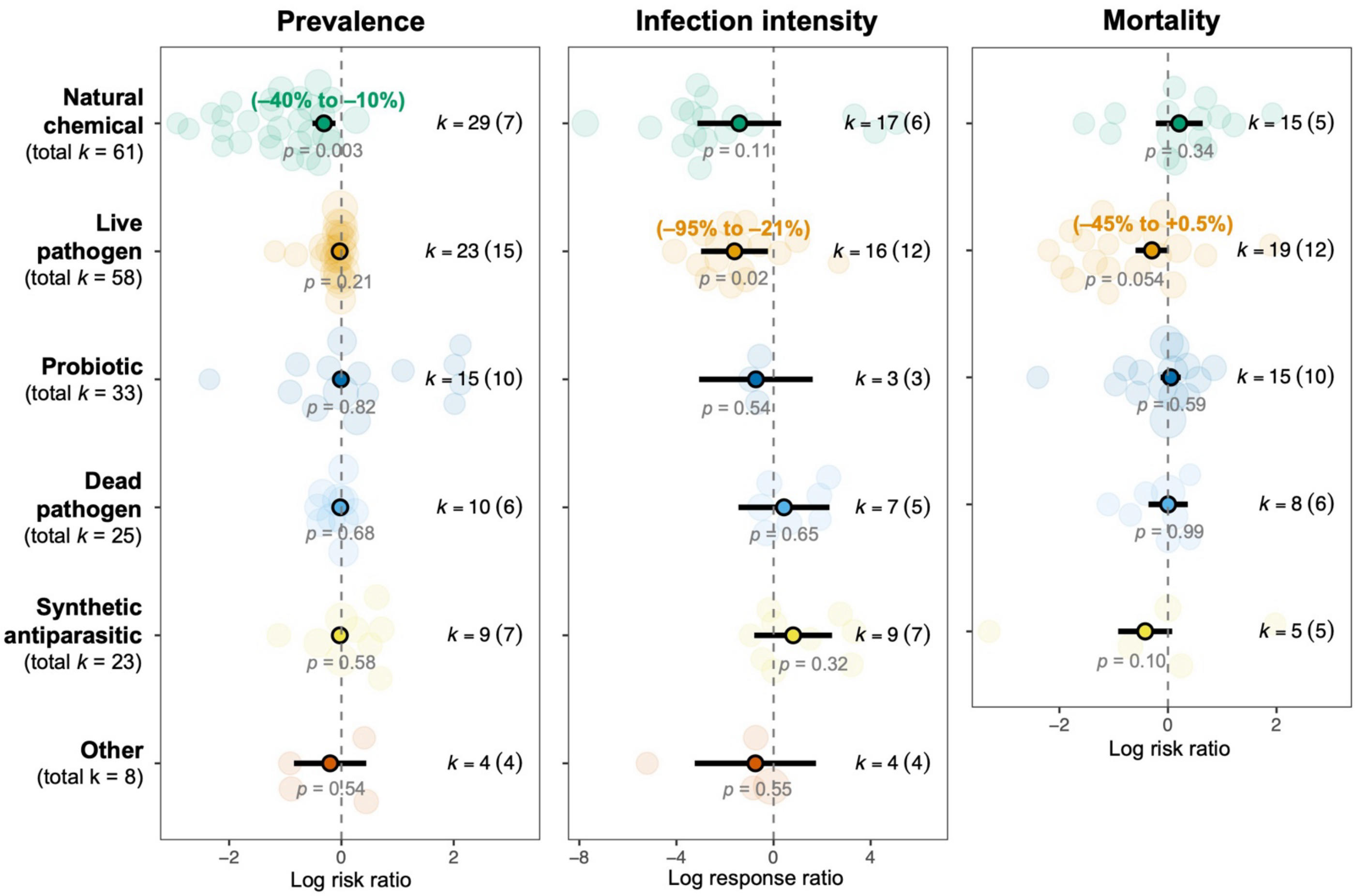

Figure 3. Moderating effects of immunisation method on prevalence, infection intensity and mortality. Darker dots and lines indicate means and 95% confidence intervals, along with *p*-values. Confidence intervals are also presented as percentage changes in outcome measures for specific methods showing 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.

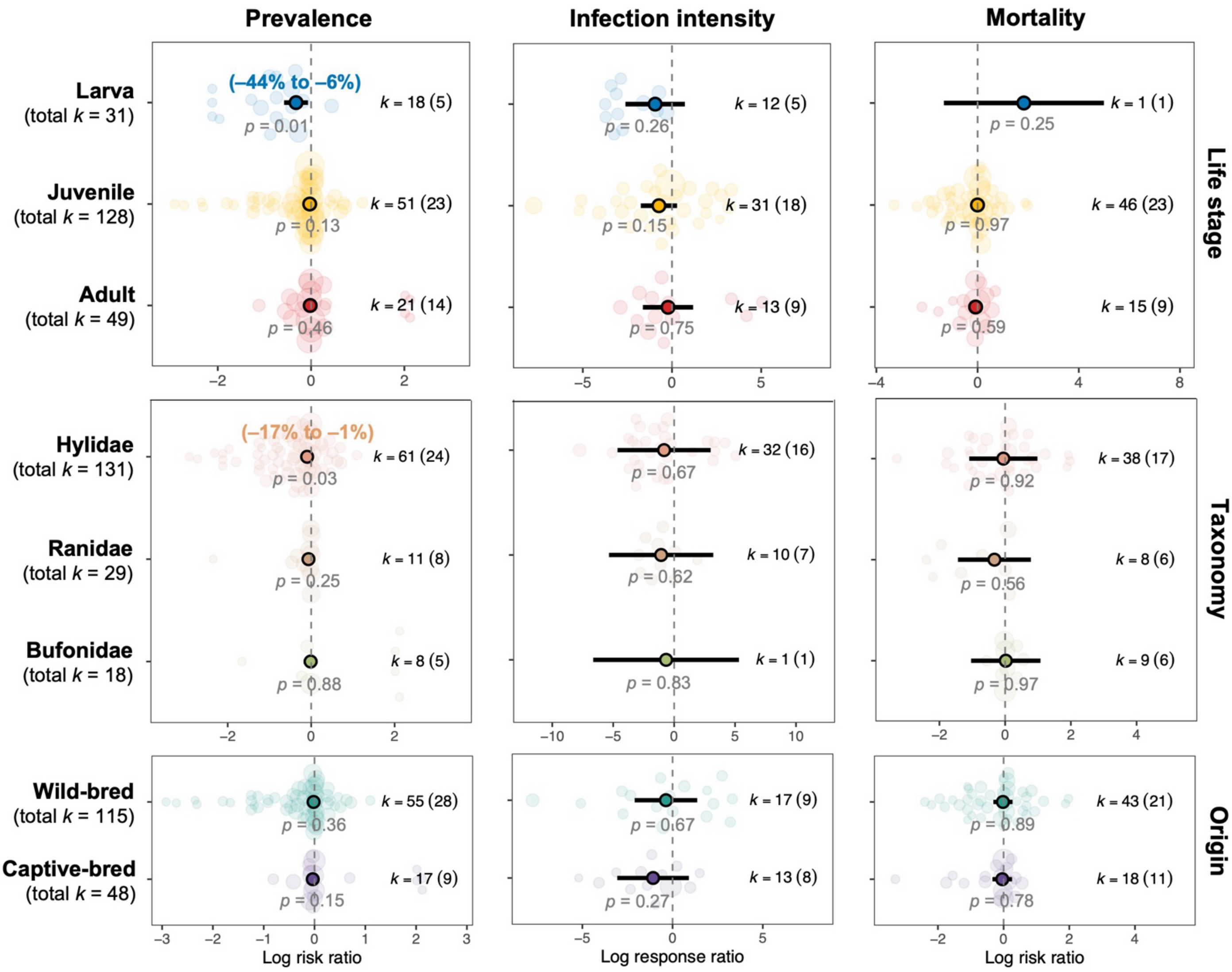

Figure 4. Moderating effects of host feature (life stage, taxonomy, origin) on prevalence, infection intensity and mortality. Darker dots and lines indicate means and 95% confidence intervals, along with *p*-values. Confidence intervals are further presented as percentage changes in outcome measures for categories showing a significant effect. Lighter dots indicate individual effect sizes, and dot sizes reflect the relative precision (1/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 S3, S4, and S6.

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