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

40 **INTRODUCTION**
41
42 The emergence and spread of infectious diseases represent one of the most significant threats
43 to wildlife populations worldwide[1,2]. While many taxa have been impacted by diseases,
44 amphibians have been hardest hit due to the global expansion of two fungal pathogens,
45 *Batrachochytrium dendrobatidis* (Bd)[3] and *B. salamandrivorans* (Bsal)[4]. These pathogens
46 cause chytridiomycosis, a disease that can trigger mass mortality events[5,6]. Since the 1970s, the
47 spread of chytridiomycosis has contributed to population declines in more than 500 amphibian
48 species, with at least 90 species potentially extinct due to these pathogens[7]. Consequently,
49 amphibians are now the most imperilled vertebrate class, with more than 40% of species
50 threatened, with chytridiomycosis identified as the major threatening process[7,8].
51
52 The challenge for susceptible amphibian species is that, once established in the wild,
53 chytridiomycosis appears virtually impossible to eradicate. Persistence stems not only from the
54 pathogen's ability to infect non-amphibian hosts[9,10] and remain viable in in the envrionment[11],
55 but also from non-susceptible amphibian host species that act as long-term pathogen reservoirs
56 and continual sources of infection for more vulnerable species[12]. As a result, conventional
57 conservation measures, including translocations and reintroductions, often fail because
58 disease-causing pathogens persist in the environment[13-15]. For Bd and Bsal, the only proven
59 management strategy is to remove susceptible individuals from natural habitats and maintain
60 disease-free captive populations[16], a scenario that risks leaving many amphibian species extinct
61 in the wild. This reality highlights the urgent need to develop approaches that mitigate disease
62 impacts and enable amphibian hosts to coexist with the pathogens.
63
64 A promising avenue for reducing disease impacts involves stimulating protective immune
65 responses in susceptible species. In many animals, exposure to live-attenuated pathogens,
66 inactivated pathogens, or their derivatives enables immune cells to recognise pathogens,
67 develop immunological memory, and mount faster and more effective responses upon
68 subsequent exposure[17,18]. For amphibians, successful immunisation strategies that enhance host
69 survival[19-21] could substantially improve conservation outcomes, for example by enabling the
70 release of immunised individuals to bolster threatened populations, or by guiding habitat
71 manipulations that help amphibians clear infection naturally, develop immunity under wild
72 conditions, and thus enable long-term population persistence[22,23].
73
74 However, immunisation efforts targeting Bd and Bsal in amphibians have produced mixed
75 outcomes[24,25], with no clear consensus and considerable uncertainty about their overall
76 effectiveness. This inconsistency likely reflects the wide variety of immunisation approaches
77 that have been trialled (Table 1), including exposure to live pathogens[24,26], dead pathogens[21,27],
78 pathogen metabolites[28,29], antifungal microbes[30,31], and synthetic anitfungals[32,33], as well as the
79 broad diversity of host species on which these methods have been tested.
80
81 To resolve debates around immunisation efficacy for combating amphibian fungal pathogens,
82 we conducted a meta-analysis drawing on all available amphibian immunisation studies
83 targeting Bd and Bsal to identify the most effective strategies and to understand the drivers of
84 variation in immunisation success. Synthesising empirical results meta-analytically is a
85 powerful way to identify the most promising strategies for effective immunisation. We focused
86 on how different immunisation approaches influenced subsequent infection prevalence (i.e. the
87 rate of infection), infection intensity when infected, and mortality when immunised individuals
88 were exposed to live Bd or Bsal, anticipating that effective strategies would reduce infection
89 rates, lower pathogen loads in infected individuals, and/or decrease mortality. We evaluated

90 how these outcomes varied across immunisation methods (Table 1) and among different
91 amphibian life stages, origin (captive or wild bred) and taxonomic family (Table 2).
92
93 **RESULTS**
94
95 We screened 1,517 studies and identified 38 containing relevant datasets (Figure 1 and S1).
96 Together, these studies reported 275 effects from 59 experiments, each comparing outcomes
97 following Bd or Bsal exposure between immunised (treated) and control (untreated) animals
98 under common conditions. Most experiments targeted Bd (n = 53) with only six addressing
99 Bsal. All Bsal experiments involved live-pathogen immunisation: exposing animals to either
100 live Bd[24] or live Bsal[34,35] and testing their subsequent susceptibility in a live Bsal challenge.
101 Results from these Bsal experiments were mixed: infection intensity and mortality in
102 immunised individuals were reduced relative to controls in some species[24] but not others[34,35],
103 while prevalence was unaffected by immunisation treatment in all cases. Given the limited
104 number of Bsal experiments, we estimated effect sizes only for studies involving Bd
105 exposure.
106
107 ***Immunisation treatments reduce susceptibility to Bd across amphibians***
108
109 We quantified differences in infection rates (prevalence), infection intensity, and mortality
110 between immunised and control groups following a live Bd exposure challenge. We used multi-
111 level meta-analytic models that included within-study, between-study and phylogenetic
112 random effects to estimate the overall success of immunisation at reducing disease (Figure 2).
113 Effect sizes for prevalence (k = 90) and mortality (k = 62) were calculated as the natural
114 logarithm of the risk ratio (logRR). Effect sizes for infection intensity (k = 56) were calculated
115 as the natural logarithm of the response ratio (lnRR) and were based only on infected
116 individuals.
117
118 Mortality is arguably the most consequential outcome because it links directly to population
119 performance and the potential for long-term persistence. Only one immunisation approach
120 reduced mortality following subsequent live Bd exposure – the live-pathogen immunisation
121 which reduced death rates by an average of 26% (p = 0.054; Figure 3). The confidence intervals
122 (CIs) slightly overlapped zero (–45% to +0.5%), although the magnitude of the effect suggests
123 that live-pathogen immunisation can substantially improve host survival (Table S1). In addition
124 to reducing mortality, live-pathogen immunisation was also the only approach that reduced
125 infection intensity (Figure 3), decreasing pathogen loads in infected individuals by an average
126 of 80% (p = 0.02), helping to explain reductions in mortality.
127
128 Natural-chemical immunisation was the only approach that significantly reduced prevalence
129 (Figure 3), lowering infection rates by an average of 27% (p = 0.003). While live-pathogen
130 immunisation reduced mortality and infection intensity, it had no clear effect on prevalence (p
131 = 0.21; Figure 3). The remaining immunisation approaches (probiotic, dead-pathogen,
132 synthetic antiparasitic, and other) showed no clear effects on either prevalence, infection
133 intensity, or mortality (Table S1).
134
135 ***Immunisation effects were correlated between prevalence and infection intensity***
136
137 Our results above show that different immunisation methods can influence infection dynamics
138 at different stages of disease progression, from the probability of becoming infected to the
139 intensity of infection and ultimately mortality (Figure 3). To examine whether the effects of

140 immunisation on one disease stage were associated with compensatory or reinforcing effects
141 at other stages, we fitted multivariate models that assessed correlations among effect sizes
142 across the stages of disease progression within experiments. The effects of immunisation on
143 prevalence and pathogen load were positively correlated (ρ [CIs] = 0.72 [0.28 to 1.00]),
144 suggesting a reinforcing effect. Immunisation that limits initial infection also suppresses
145 within-host pathogen abundance. In contrast, there was no clear correlation between effects on
146 mortality and those on prevalence (ρ [CIs] = –0.88 [–1.00 to 0.44]) or pathogen load (ρ [CIs]
147 = –0.32 [–1.00 to 1.00]), suggesting that effects enhancing resistance (e.g., preventing pathogen
148 invasion or growth) did not necessarily affect tolerance (e.g., mortality risk at a given pathogen
149 burden).
150
151 ***Consistent effects on prevalence but more variable effects on infection intensity and***
152 ***mortality***
153
154 There was evidence that some immunisation methods significantly lowered Bd prevalence,
155 infection intensity, and mortality, but the heterogeneity of effects varied across studies (Table
156 S2). The effects of immunisation on prevalence were highly consistent across studies ($I^2 \approx 0\%$),
157 largely because most immunisations did not clearly alter infection rates (Figure S2). In contrast,
158 the effects on infection intensity exhibited high heterogeneity across studies ($I^2 = 96\%$) and the
159 effects on mortality exhibited small to moderate heterogeneity ($I^2 = 30\%$).
160
161 For infection intensity, most of the heterogeneity was due to between-study and within-study
162 variation ($I^2_{between-study}$ = 46%, $I^2_{within-study}$ = 50%, $I^2_{phylogeny} \approx$ 0%). For mortality,
163 heterogeneity primarily arose from between-study differences with a weak phylogenetic signal
164 ($I^2_{between-study}$ = 21%, $I^2_{within-study} \approx$ 0%, $I^2_{phylogeny}$ = 9%). The substantial contribution of
165 between-study differences in both infection outcomes suggests that ecological and/or
166 methodological differences among studies were key drivers of variation in immunisation
167 effects.
168
169 ***Immunisation effectiveness varies by life stage and family, with stronger effects in more***
170 ***susceptible groups***
171
172 In addition to immunisation method, we found that two host features, life stage and taxonomic
173 group, moderated treatment effectiveness. Overall, immunisation was more effective at
174 lowering prevalence in larval amphibians relative to other life-stages (Figure 4), reducing larval
175 infection risk by an average of 28% ($p = 0.01$). In contrast, immunisation effects on infection
176 intensity and mortality were not moderated by life stage (Table S3).
177
178 Species in different families are known to vary in their responses to immunisation and disease
179 outcomes due to taxa-specific physiology[36] and ecological requirements[37], which may not be
180 captured by phylogenetic relatedness when few taxa are included in an analysis. Indeed, we
181 found that immunisation effects varied by amphibian family, reducing infection risk in Hylidae
182 by an average of 9% ($p = 0.03$), but with no clear reduction in other families (Figure 4; Table
183 S4). Hylids are known to be particularly susceptible to Bd and often experience high Bd-
184 induced mortality[7,38,39]. To test whether immunisation provides greater benefits in more
185 susceptible groups, we conducted a meta-regression examining the moderating effect of
186 inherent susceptibility, which we measured using the mortality rate of the control group, on
187 immunisation effects. As predicted, immunisation reduced mortality more strongly in groups
188 with higher inherent susceptibility (higher control-group mortality; slope [CIs] = –0.01 [–0.012
189 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

240 unlikely given that synthetic antiparasitics (antifungals or pesticides) showed no detectable
241 effects in our analysis. A second possibility is that inhibitory compounds produced by Bd,
242 such as methylthioadenosine[43] and spermidine[44], stimulate host immune responses. These
243 compounds originate from the walls of Bd zoosporangia rather than from zoospore cells[45].
244 This distinction is important because dead-Bd immunisation typically exposes hosts to only
245 purified dead zoospores that lack zoosponrangia[21,27,46,47]. In contrast, Bd metabolites are
246 usually prepared by filtering culture media containing both zoosporangia and zoospores[29].
247 The presence of zoosporangia may therefore underlie the stronger protective effects seen for
248 metabolite-based immunisation. Consistent with this explanation, antibody production has
249 been detected when frogs were treated with mixtures containing both dead zoospores and
250 zoosporangia[48], but not when exposed solely to purified dead zoospores[47,49].
251
252 ***Effects on mortality were not correlated with effects on prevalence and infection load,***
253 ***suggesting that immunisation enhances tolerance and resistance independently***
254
255 Immunisation that prevents initial infection (lowering prevalence) might, in principle, also
256 limit pathogen growth within hosts (lowering infection intensity) and prevent pathogen loads
257 from reaching lethal thresholds (lowering mortality). We might therefore expect positive
258 correlations among effects on prevalence, infection intensity, and mortality. A key question,
259 then, is why live-pathogen immunisation reduced morality but not prevalence, whereas
260 natural-chemical immunisation reduced prevalence but not mortality. Overall, while we found
261 that prevalence and intensity tended to be positively correlated, neither were strongly
262 correlated with mortality.
263
264 An explanation for these outcomes is that hosts combat pathogens through two distinct
265 mechanisms[50,51]: (1) resistance, which limits pathogen invasion and growth, and (2)
266 tolerance, which reduces damage without necessarily lowering pathogen burden, for example
267 through tissue repair. Because these mechanisms rely on different physiological pathways,
268 resistant amphibians are not necessarily tolerant, and tolerant individuals may not be resistant
269 (see the diverse relationships between Bd resistance and tolerance in 35 amphibian species[52]).
270 In our meta-analysis, prevalence and infection intensity are indicators of resistance, whereas
271 mortality reflects tolerance[51,52]. This framework helps explain why resistance-related
272 outcomes were positively correlated with each other but showed no clear correlation with the
273 tolerance-related outcome. It is well established that some amphibians can maintain high
274 pathogen loads without developing disease or experiencing mortality[12,53]. Such decoupling
275 between pathogen burden and host survival indicates that reductions in prevalence or
276 infection intensity do not necessarily translate into reductions in mortality.
277
278 ***Future conservation efforts need to consider the timing and life stage of immunisation***
279
280 Another factor contributing to variation in immunisation outcomes is host biology. If hosts
281 are naturally tolerant and unlikely to die from infection, immunisation may help them clear
282 infection and lower prevalence while having little effect on mortality risk. Tadpoles are a
283 good example: they are highly tolerant to Bd because the keratinised tissues that Bd infects
284 are restricted to their mouthparts[54], and infections in this region rarely cause mortality[38,51].
285 Low Bd-induced mortality likely explains why larval immunisation was effective at reducing
286 prevalence but did not lower infection intensity. In fact, only one experiment has reported
287 larval deaths following a live Bd challenge (Figure 4).
288

289 Although mortality during the tadpole phase is uncommon[51], infected tadpoles often die
290 during or shortly after metamorphosis[55], when keratin appears across the entire skin surface
291 and Bd can spread more extensively[54]. Tadpoles can also act as important reservoirs that
292 maintain Bd in a population and increase disease transmission[56]. Evidence that immunisation
293 reduces infection rates in tadpoles is therefore encouraging because fewer infected tadpoles
294 should translate to lower mortality after metamorphosis and reduced transmission risk to
295 other individuals. Field studies further support this idea, showing that low Bd prevalence in
296 tadpoles was associated with rapid recruitment and population recovery despite high adult
297 mortality[57,58].
298
299 Most Bd experiments (47 of 53) assessed immunisation success within a single life stage,
300 leaving it unclear whether effects induced in tadpoles persist through metamorphosis. This
301 gap is important to address, especially given our finding that immunisation significantly
302 reduces infection prevalence in tadpoles. Tadpole immunisation also offers several practical
303 advantages: (1) a single adult breeding pair can produce hundreds to thousands of embryos,
304 enabling efficient collection of large cohorts; (2) tadpoles can be reared at high densities on
305 inexpensive plant-based diets, unlike post-metamorphic frogs that require more expensive
306 carnivorous diets; and (3) tadpoles are far more tolerant of Bd infection than post-
307 metamorphic frogs[51,59], making them particularly suitable for live-pathogen immunisation. If
308 resistance acquired during the tadpole stage carries over across metamorphosis and results in
309 more disease-resistant adults, tadpole immunisation could be a powerful conservation tool. It
310 would allow large numbers of individuals to be immunised at much lower cost and could
311 substantially increase the number of immunised animals available for reintroduction or
312 supplementation programs.
313
314 ***Immunisation effects differ among taxonomic families and depend on inherent***
315 ***susceptibility***
316
317 We found that immunisation effects varied among taxonomic families, likely because
318 ecological differences make some groups more suspectable to Bd. Immunisation was
319 particularly effective at reducing prevalence in Hylidae. A previous meta-analysis also
320 identified that the superfamily Hyloidea were particularly susceptible to Bd[38], suggesting that
321 immunisation may be more effective in susceptible species. Consistent with this idea, we
322 found that inherent susceptibility (measured as control-group mortality) significantly
323 predicted immunisation effectiveness: groups with higher baseline mortality experienced
324 greater reductions in mortality following immunisation. The family Bufonidae have also been
325 identified as highly susceptible to Bd[7,38,60]. We found no clear evidence that this family was
326 more susceptible in our meta-analysis, although the effects were derived from just three
327 species that were mostly immunised using less effective probiotic treatments (Figures 2 & 3).
328 We therefore encourage additional studies testing more effective immunisation approaches in
329 Bufonidae, particularly live-pathogen and natural-chemical immunisation, to better evaluate
330 their potential in this highly susceptible family.
331
332 ***Precise measurement of infection outcomes provides a better understanding of***
333 ***immunisation effects***
334
335 We separated immunisation effects on prevalence and infection intensity to clarify how
336 immunisation influences different stages of disease progression. To do this, we calculated
337 infection intensity using only infected individuals (i.e. those with non-zero Bd loads).
338 However, a few studies reported infection intensity while including uninfected

339 individuals[19,61-64]. We excluded these studies from our intensity analyses because they
340 confound effects on prevalence with effects on infection intensity. Indeed, several of our
341 findings changed when these experiments were included (k = 15). For live-pathogen
342 immunisation, the estimated reduction in infection intensity weakened – from an average
343 reduction of 80% (p = 0.02; Figure 3) to 70% (p = 0.04; Table S9). Because live-pathogen
344 immunisation had no clear effect on prevalence, mixing prevalence and intensity outcomes
345 likely weakened the strong underlying effect on infection intensity (Figure 3). For natural-
346 chemical immunisation we saw the opposite: effects on infection intensity shifted from
347 uncertain (p = 0.11; Figure 3) to more clearly negative (p = 0.053; Table S9), likely because
348 strong effects on prevalence inflated the apparent effect on infection intensity (Figure 3).
349 These outcomes highlight the importance of a clear and consistent definition of infection
350 intensity for accurately identifying where immunisation exerts its effects.
351
352 Measuring mortality in laboratory studies can be logistically challenging because ethical
353 guidelines often require treating or curing sick amphibians. Similarly, it is often
354 recommended to use nonlethal pathogen doses to avoid rapid deaths[38]. In our meta-analysis,
355 many immunisation studies (e.g.[21,65,66]) reported zero mortality (k = 27). The absence of
356 deaths may indicate that animals were naturally tolerant, or that post-immunisation live Bd
357 challenges were not sufficiently virulent to cause mortality even in control groups. We
358 excluded these studies from our mortality analyses because we could not estimate
359 immunisation effects on mortality when no deaths occurred. Future work aiming to evaluate
360 immunisation effects on mortality – the most consequential disease outcome – should
361 carefully consider the Bd dose or strain used in challenge experiments to ensure that mortality
362 can be meaningfully assessed.
363
364 **CONCLUSION**
365
366 Our meta-analysis shows that immunisation through prior exposure to live Bd or natural
367 chemicals (mostly Bd metabolites) can reduce disease susceptibility in amphibians
368 subsequently challenged with Bd. Our study provides the first synthesis of immunisation
369 efficacy for a major emerging wildlife disease. The results indicate that immunisation
370 approaches hold considerable promise for practical conservation applications against Bd and
371 potentially other emerging infectious disease (e.g., white-nose syndrome, snake fungal
372 disease). Immunisation could enhance survival during reintroductions or population
373 supplementation efforts involving Bd susceptible species. In addition, habitat interventions,
374 such as providing thermal refuges or creating salted ponds, that help individuals clear
375 infections could similarly increase immunity and improve survival in wild populations,
376 potentially enabling susceptible species to persist with the pathogen.
377
378 **METHODS**
379
380 We conducted a systematic review and meta-analysis following, as closely as possible, the
381 PRISMA-EcoEvo guidelines[67]. A checklist is provided as a supplementary file. For our full
382 PRISMA diagram detailing our search and selection criteria see Figure S1.
383
384 **Literature searches**
385
386 We aimed to retrieve a large representative sample of studies measuring changes in
387 amphibian susceptibility to chytridiomycosis after applying different interventions. We
388 searched publications from ISI Web of Science (Core Collection), Scopus, ProQuest and

389 ScienceDirect in April 2025. The search string developed included "chytridiomycosis" (OR
390 synonyms) AND "amphibian" (OR synonyms) AND terms for individual-based interventions
391 (e.g. immunisation, vaccine) (see *Supplementary Material* for full details). To validate our
392 search string, we generated a list of ten gold-standard papers[19-21,25,26,28,29,42,47,68] that contained
393 suitable datasets and ensured that our search strings retrieved all ten. To capture recent
394 research, we included grey literature (dissertations, theses, preprints and non-English
395 publications). We focused on empirical data and did not retrieve reviews, book chapters,
396 meeting abstracts, editorials, and corrections. Furthermore, we found all papers citing or cited
397 in four reviews[69-72] using Web of Science and Scopus in May 2025. All of the above details
398 are provided in the supplementary material. We identified 3,538 records and removed 2,053
399 duplicates using the R package *litserchr*[73], the website *Rayyan*[74] as well as manual checks
400 (Figure S1). The remaining 1,485 records included 45 PhD and 26 MSc theses, from which
401 we identified an additional 31 papers and 1 PhD thesis (total 1,517 records).
402
403 **Inclusion criteria**
404
405 For the 1,517 records, we first screened the title and abstract and then their full text. To be
406 included, the study needed to meet all the following inclusion criteria:
407
408 1. ***Be an in vivo experiment on amphibians****:* We excluded non-experimental studies,
409 experimental studies on non-amphibians, and *in vitro* studies on amphibians (e.g. isolated
410 cells, skin secretions).
411 2. ***Conduct a treatment aimed at reducing individual-level susceptibility to***
412 ***chytridiomycosis, with a comparable control***: *A comparable control* comprises
413 individuals with the same initial infection states, from the same population and subjected
414 to identical procedures (except for the active treatment components). This ensures that
415 observed differences are attributable to the focal treatment rather than variation in initial
416 infection states, populations or handling procedures. Accordingly, we excluded (a) studies
417 aiming to enhance host susceptibility[48] and (b) studies aiming to mitigating host
418 susceptibility by creating chytrid-unfavourable environments. We excluded studies
419 identified as (b) because treatment differences can result from reduced chytrid
420 performance rather than increased host resistance.
421 3. ***Have comparable treatment conditions***: We included studies where untreated and treated
422 individuals were (a) uninfected at the onset of experimental infection to avoid
423 confounding by prior infection, (b) exposed to the same chytrid strain and (c) kept in a
424 common-garden setting during the period of experimental infection.
425 4. ***Present the correct infection outcomes***: We only included studies that reported (a)
426 infection prevalence, (b) infection intensity and/or (c) mortality.
427 5. ***Complete an infection assessment***: We included studies in which infection outcomes were
428 measured in all individuals, or for a randomly selected subset. We excluded mark-
429 recapture studies that failed to assess released individuals (e.g., those with <27% recapture
430 rate[75]), because treatment effects cannot be reliably assessed without knowing the infected
431 states of non-recaptured animals.
432
433 **Data collection and classification**
434
435 We identified 38 eligible studies (Figure S1; supplementary files–study summary) and
436 classified the number of experiments within each study. An experiment was defined as a
437 comparable pair of animal groups whose disease susceptibility was experimentally reduced
438 (treatment) or left unchanged (control), with all other conditions held constant. For instance, a

439 study testing the effect of probiotics independently at two temperatures[61], using four different
440 samples of animals, yielded two experiments.
441
442 Within each experiment, we recorded the research location (Figure 1), methodological and
443 biological variables (Tables 1 & 2). For taxonomy, species were consolidated into family-
444 level groups to increase sample sizes (Figure 2). For life stage, we categorised them into (a)
445 larva (no forelimbs), (b) metamorph (forelimb emergence to tail resorption), (c) juvenile (tail
446 resorption to sexual maturation) and (d) adult (after sexual maturation)[76]. There were no
447 eligible studies on metamorphs. We extracted the life stage both at immunisation and at
448 subsequent live pathogen challenge to clarify cross-stage effects. However, cross-stage
449 experiments were rare (6 of 53), so these two life stages were largely overlapping. We
450 therefore considered only the life stage at live pathogen challenge (where infection outcomes
451 were measured) for analysis. We recorded the time elapsed between immunisation and live
452 pathogen challenge (Table 1) to clarify effect persistence.
453
454 We focused on treatment differences in prevalence, infection intensity and mortality.
455 Mortality rate was defined as the cumulative proportion of deaths following live pathogen
456 challenge, for which we extracted the final measure closest to the end of the challenge period.
457 In contrast, prevalence and infection intensity could either increase or decrease over time as
458 individuals transition between infection states. For studies that tested prevalence and/or
459 infection intensity multiple times throughout the experiment, we extracted the peak value
460 (indicating the most severe outcome caused by the pathogen). Peak prevalence was defined as
461 the greatest proportion infected at a given time point. Peak infection intensity was calculated
462 by identifying the maximum pathogen loads for each individual across all measures and then
463 averaging individual peak values. A single value was extracted if prevalence and/or intensity
464 was only measured once. To quantify variation in measurement effort, we recorded the total
465 number of trait measurements (Table 1) for prevalence and infection intensity.
466
467 We extracted the mean, measures of error (SD, standard error (SE), 95% CI), sample size,
468 and the time elapsed between live pathogen challenge and outcome measurement (Table 1).
469 Data were extracted from text, tables, figures or supplementary materials. If raw data were
470 available, we extracted the necessary data using the R package *tidyverse*[77] for calculation of
471 summary statistics. If figures were used, we used the R package *ShinyDigitise*[78] to extract
472 values. The data source is listed in the supplementary files–metadata.
473
474 SEs and 95% CIs were first converted to SDs before calculating effect statistics. For studies
475 reporting $\log_{10}$-scale summaries, we converted the data to natural-log mean (Z) and SD (SD_Z)
476 and then back-transformed these to the raw-scale mean (m) and SD (SD_m) following Higgins
477 et al[79]:
478

479 $$m = \exp\left(Z + \frac{{SD_Z}^2}{2}\right)$$

480

481 $$SD_m = \sqrt{\left(\exp\left({SD_Z}^2\right) - 1\right)\exp\left(2Z + {SD_Z}^2\right)}$$

482
483 Back-transformation can be unreliable when SD_Z is missing or large. For such cases, we
484 approximated $m \approx \exp(Z)$ and considered SD_m as unavailable. We ran a sensitivity analysis
485 excluding these transformed data (k = 18; Table S10). Effects of live-pathogen immunisation
486 still tended to reduce infection intensity when excluding these transformed data (mean [CIs]:

487 70% [–96% to +1.7%]; $p = 0.052$), consistent with the main conclusion (mean [CIs]: –80% [–
488 95% to –21%]; $p = 0.02$).
489
490 **Effect size calculation**
491
492 Effect sizes were calculated only for *Bd* datasets. For both prevalence and mortality, we
493 converted percentages into counts of events ('infection' for prevalence; 'death' for mortality)
494 and non-events. We then calculated the log risk ratio (logRR), which compares the risk
495 (probability) of an event in the treatment compared to the control group[80]:
496

497 $$\log \mathrm{RR} = \ln\left(\frac{T_{event} \,/\, (T_{event} + T_{non-event})}{C_{event} \,/\, (C_{event} + C_{non-event})}\right)$$

498
499
500 We calculated sampling variance, $v(\log \mathrm{RR})$, for prevalence and mortality using the following
501 formulas:
502

503 $$v(\log RR) = \frac{1}{T_{event}} - \frac{1}{T_{event} + T_{non-event}} + \frac{1}{C_{event}} - \frac{1}{C_{event} + C_{non-event}}$$

504
505 Here, T_{event} and C_{event} are the count of events for treatment (immunised) and control groups,
506 respectively, whereas $T_{non-event}$ and $C_{non-event}$ are the count of non-events. Cells with a zero
507 value generate undefined estimates. In the cases with at least one zero-count, we added 0.5 to
508 all cells[81]. We excluded experiments with non-events: that is, no infections for prevalence (k
509 $= 2$) and no deaths for mortality ($k = 27$). These experiments were excluded because there
510 were no Bd-caused outcomes to be compared.
511
512 For infection intensity, we calculated the log response ratio (lnRR), which represents the log
513 of proportional difference in the means between groups[82]:
514

515 $$\mathrm{lnRR}_1 = \ln\left(\frac{m_T}{m_C}\right)$$

516

517 $$v(\mathrm{lnRR})_1 = \frac{{SD_T}^2}{n_T\,{m_T}^2} + \frac{{SD_C}^2}{n_C\,{m_C}^2} = \frac{{CV_T}^2}{n_T} + \frac{{CV_C}^2}{n_C}$$

518
519 where m is the mean, n is the sample size, SD is the standard deviation, and CV (SD/m) is the
520 coefficient of variation for treatment (T) and control (C) groups, respectively. However,
521 lnRR_1 and $v(\mathrm{lnRR})_1$ can be biased when sample size are small to moderate[83]. We further
522 corrected for such bias[84]:
523

524 $$\mathrm{lnRR}_2 = \ln\left(\frac{m_T}{m_C}\right) + \frac{1}{2}\left(\frac{{\mathrm{CV}_T}^2}{n_C} - \frac{{\mathrm{CV}_C}^2}{n_T}\right)$$

525

526 $$v(\mathrm{lnRR})_2 = \frac{{CV_T}^2}{n_T} + \frac{{CV_C}^2}{n_C} + \frac{{CV_T}^4}{2{n_T}^2} + \frac{{CV_C}^4}{2{n_C}^2}$$

527
528 lnRR_2 and $v(\mathrm{lnRR})_2$ cannot be estimated when CVs are unavailable. Only few (6 of 71)
529 observations lacked CVs, so we applied the 'missing-cases' method[84] – using the weighted

530 average CVs from datasets that report CVs to estimate effect sizes and sampling variances for
531 missing cases (i = 1, 2, ..., K):
532

533
$$\text{lnRR}_3 = \ln\left(\frac{m_T}{m_C}\right) + \frac{1}{2}\left(\frac{\left[\frac{\sum_{i=1}^{K}(n_{Ti}\,CV_{Ti})}{\sum_{i=1}^{K} n_{Ti}}\right]^2}{n_T} - \frac{\left[\frac{\sum_{i=1}^{K}(n_{Ci}\,CV_{Ci})}{\sum_{i=1}^{K} n_{Ci}}\right]^2}{n_C}\right)$$

534
535

536
$$v(\text{lnRR})_3 = \frac{\left[\frac{\sum_{i=1}^{K}(n_{Ti}\,CV_{Ti})}{\sum_{i=1}^{K} n_{Ti}}\right]^2}{n_T} + \frac{\left[\frac{\sum_{i=1}^{K}(n_{Ti}\,CV_{Ci})}{\sum_{i=1}^{K} n_{Ci}}\right]^2}{n_C}$$

537
$$+ \frac{\left[\frac{\sum_{i=1}^{K}(n_{Ti}\,CV_{Ti})}{\sum_{i=1}^{K} n_{Ti}}\right]^4}{2{n_T}^2} + \frac{\left[\frac{\sum_{i=1}^{K}(n_{Ci}\,CV_{Ci})}{\sum_{i=1}^{K} n_{Ci}}\right]^4}{2{n_C}^2}$$

538
539 All types of lnRR and v(lnRR) were calculated following the *func.R* script provided by
540 Nakagawa et al[84].
541
542 In all calculations, a negative effect size indicates a reduced infection outcome in immunised
543 individuals compared with control individuals. All analyses were conducted in R[85] (version
544 4.5.1) using the *escalc* function in the *metafor* package (version 4.8-0)[86].
545
546 **Data analysis**
547
548 For each outcome, we ran a multi-level meta-analysis (MLMA) using residual maximum
549 likelihood (REML) and *t*-based interference for parameters. We reported both the confidence
550 intervals of effect size estimates, with associated prediction intervals in the supplementary
551 material[87-89]. To assess effect heterogeneity, we calculated I^2, defined as the proportion of
552 total variance among effect sizes unexplained by the known sampling variance, as well as
553 mean-standardised heterogeneity (CVH^2) and variance-mean-standardised heterogeneity (M^2)
554 in the supplementary material[90]. We used the *orchaRd* package (version 2.1.3)[91] to calculate
555 heterogeneity and visualise results.
556
557 We included three random factors in each MLMA: (1) ‘observation ID’ to capture within-
558 study variance beyond the expected from sampling error; (2) ‘study ID’ to account for similar
559 experimental setups within studies; and (3) ‘phylogenetic relatedness’ to account for
560 phylogenetic distances among species. We pruned a recently published amphibian
561 phylogeny[92] to species in our datasets (Figure 2) and generated a corresponding phylogenetic
562 correlation matrix using the *vcv* function in the *ape* package (version 5.8-1)[93].
563
564 Multiple effect sizes within an experiment were often computed using a shared treatment
565 group (such as ‘high-dose Bd metabolites’ vs a sham control, and ‘low-dose’ vs the same
566 control[28]). To account for such non-independence[94], we created a sampling (co)variance
567 matrix using the *vcalc* function in the *metafor* package[86], and we included this matrix off-
568 diagonals to account for shared-control effects. This sampling (co)variance matrix was then
569 included in our models.
570

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

621 findings are published earlier than non-significant ones. We also visually evaluated funnel
622 asymmetry using funnel plots, which showed the residuals of a meta-regression against effect
623 size precision (1/SE). While funnel plots do not formally test for publication bias, they are
624 still useful in visualising relationships between effect size and sampling variance (Figure S3).
625
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627
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634
635 **DATA AVAIABILITY**
636
637 Code, data and supplementary files can be downloaded from Zenodo:
638 https://doi.org/10.5281/zenodo.20278226
639

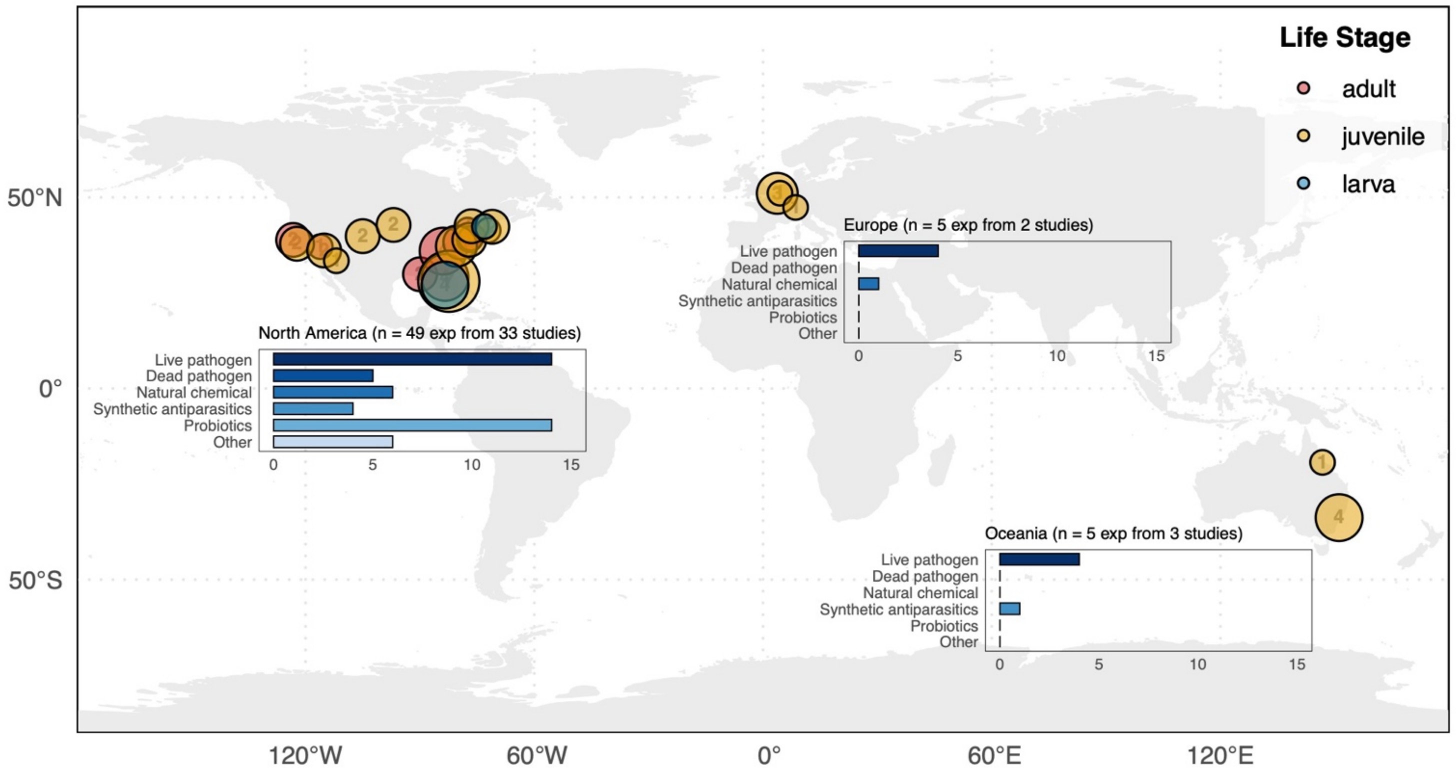
640 **Table 1. Methodological sources of variation that may affect estimates of immunisation**
641 **effectiveness in experiments assessing amphibian susceptibility to chytridiomycosis.**
642 Factors likely to alter observed outcomes include the immunisation method with the expected
643 immune pathways involved, and key experimental design features
644 (immunisation-to-challenge interval, challenge-to-measurement interval, and measurement
645 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., Ivermectin[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).

646 **Table 2. Biological factors that may affect estimates of immunisation effectiveness in**
647 **experiments assessing amphibian susceptibility to chytridiomycosis.** Factors marked with
648 an asterisk (*) had sufficient data for quantitative synthesis and were included in the meta-
649 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].

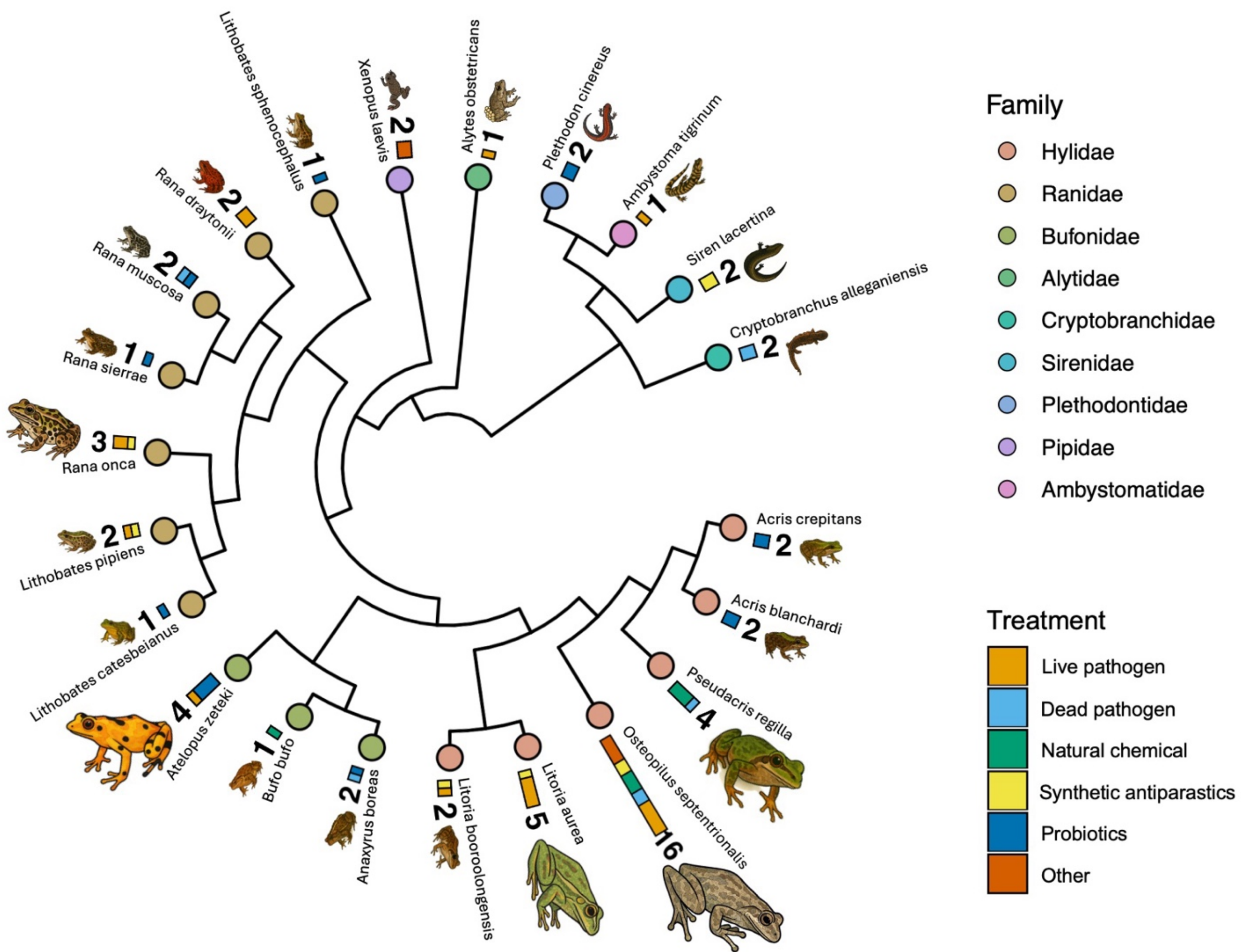
650
651



652

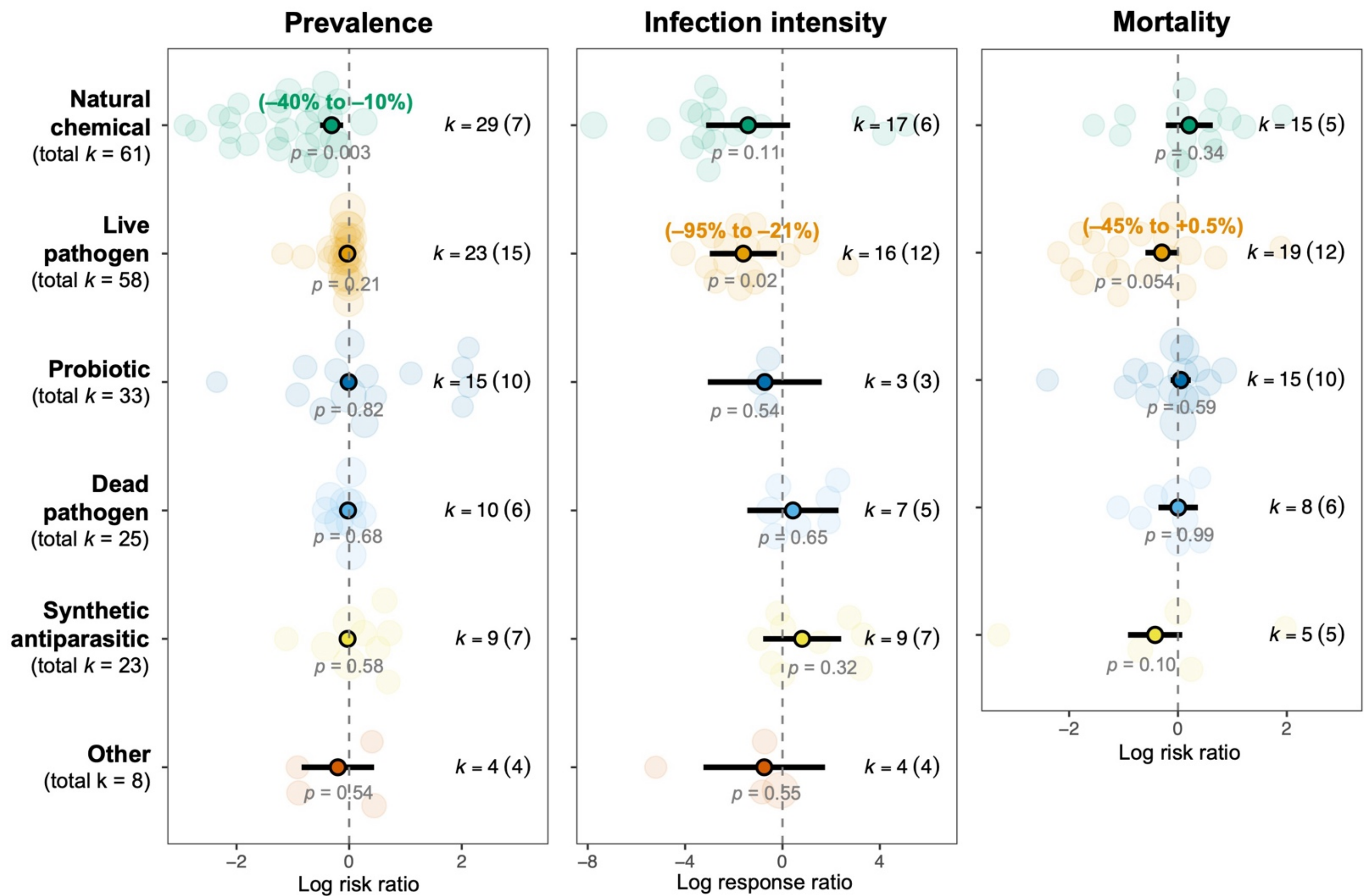
653 **Figure 1. Geographic locations of eligible studies (both Bd and Bsal).** There are usually
654 multiple independent experiments in a study, so counts represent the number of experiments.
655 Bubble size indicates the number of experiments per location, and colour represents the life
656 stage.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

659

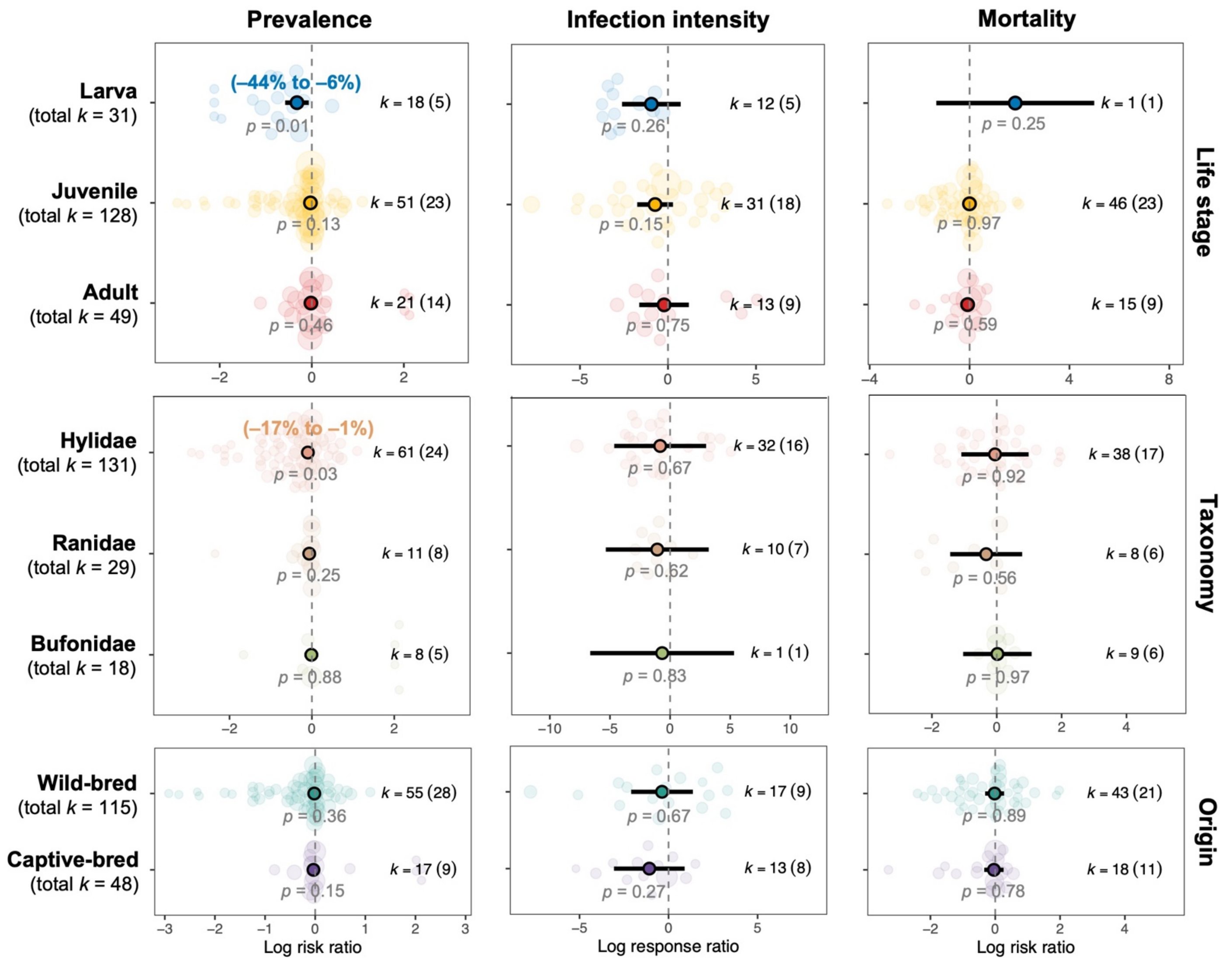


660

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



667
668 **Figure 3. Moderating effects of immunisation method on prevalence, infection intensity**
669 **and mortality.** Darker dots and lines indicate means and 95% confidence intervals, along
670 with p-values. Confidence intervals are also presented as percentage changes in outcome
671 measures for specific methods showing a clear effect. Lighter dots indicate individual effect
672 sizes, and dot sizes reflect the relative precision (1/SE) of each effect size. Counts represent
673 the number of effect sizes (k), with the number of experiments in parentheses. Full model
674 outputs (including t-values and prediction intervals) are reported in Table S1.
675



676

677 **Figure 4. Moderating effects of host feature (life stage, taxonomy, origin) on prevalence,**
678 **infection intensity and mortality.** Darker dots and lines indicate means and 95% confidence
679 intervals, along with *p*-values. Confidence intervals are further presented as percentage
680 changes in outcome measures for categories showing a significant effect. Lighter dots
681 indicate individual effect sizes, and dot sizes reflect the relative precision (1/SE) of each
682 effect size. Counts represent the number of effect sizes (*k*), with the number of experiments in
683 parentheses. Full model outputs (including *t*-values and prediction intervals) are reported in
684 Tables S3, S4, and S6.
685

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