Received: 31 December 2016 | Accepted: 17 October 2017

DOI: 10.1111/1365-2664.13030

RESEARCH ARTICLE

Journal of Applied Ecology BRITISH ECOLOGICAL SOCIETY

Elevated salinity blocks pathogen transmission and improves host survival from the global amphibian chytrid pandemic: Implications for translocations

Simon Clulow | John Gould | Hugh James | Michelle Stockwell | John Clulow | Michael Mahony

School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW, Australia

Correspondence
Simon Clulow
Email: simon.clulow@newcastle.edu.au

Funding information
Port Waratah Coal Services

Handling Editor: Hamish McCallum

Abstract

1. Emerging infectious diseases are one of the greatest threats to global biodiversity. Chytridiomycosis in amphibians is perhaps the most extreme example of this phenomenon known to science. Translocations are increasingly used to fight disease-induced extinctions. However, many programmes fail because disease is still present or subsequently establishes in the translocation environment. There is a need for studies in real-world scenarios to test whether environmental manipulation could improve survival in populations by generating unfavourable environmental conditions for pathogens. Reintroductions of amphibians impacted by chytridiomycosis into environments where the disease persists provide a scenario where this paradigm can be tested.
2. We tested the hypothesis that manipulating environmental salinity in outdoor mesocosms under near-identical environmental conditions, present in a nearby translocation programme for an endangered amphibian, would improve survival and determine the mechanisms involved. One hundred and sixty infected and 288 uninfected, captive-bred, juvenile frogs were released into 16 outdoor mesocosms in which salinity was controlled (high- or low-salinity treatment). The experiment was run for 25 weeks from the mid-austral winter to the mid-austral summer of 2013 in a temperate coastal environment, Australia.
3. Increasing salinity from *c.* 0.5 ppt to 3.5–4.5 ppt reduced pathogen transmission between infected and uninfected animals, resulting in significantly reduced mortality in elevated salt mesocosms (0.13, high-salt vs. 0.23, low-salt survival at 23 weeks). Increasing water temperature associated with season (from mean 13 to 25°C) eventually cleared all surviving animals of the pathogen.
4. *Synthesis and applications*. We identified a mechanism by which environmental salinity can protect amphibian hosts from chytridomycosis by reducing disease transmission rates. We conclude that manipulating environmental salinity in landscapes where chytrid-affected amphibians are currently translocated could improve the probability of population persistence for hundreds of species. More broadly, we provide support for the paradigm that environmental manipulation can be used to mitigate the impact of emerging infectious diseases.

KEYWORDS
amphibian, *Batrachochytrium dendrobatidis*, chytrid, chytridiomycosis, disease, environmental mitigation, refugia, reintroduction, translocation, transmission

1 | INTRODUCTION

Mounting evidence indicates that novel infectious diseases can alter the demographic trajectories of naïve populations, leading them to become unstable and at risk of extinction (Smith, Sax, & Lafferty, 2006). Chytridiomycosis in amphibians (Berger et al., 1998; Olson et al., 2013) is perhaps the most extreme example of this phenomenon known to science, given the massive decline and extinction of hundreds of species due to this single pathogen (Alroy, 2015; Bower, Lips, Schwarzkopf, Georges, & Clulow, 2017; Lips et al., 2006; Skerratt et al., 2007; Stuart et al., 2004; Vredenburg, Knapp, Tunstall, & Briggs, 2010). Sometimes, the impacts of diseases such as chytridiomycosis are not uniform across the ranges of species, and species persist in remnants of former distributions, which effectively become refugia (Bower et al., 2017; Briggs, Knapp, & Vredenburg, 2010; Briggs, Vredenburg, Knapp, & Rachowicz, 2005; Puschendorf et al., 2009, 2011; Rödder, Veith, & Lötters, 2008; Rowley & Alford, 2007; Scheele, Guarino, et al., 2014; Tobler & Schmidt, 2010). Understanding the drivers of disease dynamics in remnant populations destabilized through the coexistence of pathogen and host is thus an important area of investigation in conservation biology and disease ecology because it offers the potential for improved outcomes for species impacted by emerging diseases.

The ongoing impact of pathogens on host post-emergence population dynamics is determined by the transmission rate (Begon et al., 2002; Woolhouse, Haydon, & Antia, 2005) and levels of pathogen load, morbidity and mortality expressed in infected individuals (reflected in survivorship). In theory, it may be possible to mitigate the impacts of a pathogen on a host population by altering components of the environment of the host if they reduce the rates of transmission, morbidity and mortality. There is evidence that environmental factors mitigate the biological activity of *Batrachochytrium dendrobatidis* (*Bd*), the aetiological agent in chytridiomycosis, and as a result the disease dynamics in host populations. The viability and disease impact of the aquatically transmitted *Bd* may be inhibited and limited by elevated temperature (Forrest & Schlaepfer, 2011; Johnson, Berger, Phillips, & Speare, 2003; Piotrowski, Annis, & Longcore, 2004; Savage, Sredl, & Zamudio, 2011; Stevenson et al., 2013) and salinity (Heard, Scroggie, Clemann, & Ramsey, 2014; Stockwell, Clulow, & Mahony, 2012, 2015; Stockwell, Storrie, Pollard, Clulow, & Mahony, 2015). As well, there is evidence that post-metamorphic stages of some susceptible host species may be cleared of the disease in wild populations (Brannelly et al., 2015; Briggs et al., 2005, 2010; Kriger & Hero, 2006; Murray, Skerratt, Speare, & McCallum, 2009). This suggests that manipulation of environmental temperature and salinity may alter vital rates under some circumstances in a manner that improves the demographic status of some impacted amphibian populations and species.

A popular conservation strategy globally for declining wildlife is to reintroduce or translocate individuals of affected species to new or formerly occupied habitats (Germano et al., 2015). Often, as is especially the case with mitigation-driven translocations, such habitats are purpose-built or modified specifically for translocation purposes (Germano et al., 2015), providing a unique opportunity to incorporate design features that could mitigate threats such as disease. Considering that translocations are a popular conservation strategy for endangered amphibians (Germano & Bishop, 2009), and that many such programmes fail due to the presence of disease (Stockwell, Clulow, Clulow, & Mahony, 2008), understanding whether, and by what mechanisms, environmental manipulations could mitigate such impacts in real-world scenarios is an important conservation goal.

The green and golden bell frog (*Litoria aurea*) is one species in which there has been a major spatial decline in its range due to chytridiomycosis, with coexistence of pathogen and host in all but one of its persisting populations (Mahony, 1999; Mahony et al., 2013; Stockwell et al., 2008). In the case of *L. aurea*, chytridiomycosis appears to be the proximate cause of this decline, and ongoing population instability (Mahony et al., 2013). This species is no longer encountered over more than 90% of its former range, but persists in what appears to be demographically unstable remnant populations close to the coast in New South Wales and eastern Victoria, Australia (Mahony et al., 2013). Over time, some of those persisting populations are going extinct, while few, if any remaining ones can be regarded as secure (Mahony et al., 2013; White & Pyke, 2008). Coexistence of pathogen and host in remnant populations arguably leads to ongoing demographic instability in those populations (Bower et al., 2013), and understanding this is complicated by a temporal (seasonal) component to disease expression and impact (Stockwell et al., 2008), as well as spatial heterogeneity of environmental variables such as salinity that may affect survival and demographic parameters (Stockwell, Clulow, et al., 2015). Considering that this species has been subject to numerous translocation and reintroduction attempts (Germano et al., 2015; James, Stockwell, Clulow, Clulow, & Mahony, 2015; McFadden et al., 2008), many of which have failed due to the ongoing presence of *Bd* in the wild (Stockwell et al., 2008), it is an ideal species to explore the concept of environmental manipulation to mitigate the threat of disease.

We carried out an experiment using open mesocosms located within 5 km of a persisting, but unstable, population of *L. aurea* (from which captive-bred animals were derived to perform the current study) to test whether altering environmental salinity could influence disease dynamics (pathogen transmission and host survivorship) and improve translocation success. The source population is close to the mouth of the Hunter River, NSW Australia (32°51′49S, 151°44′29E) and thus subject to maritime influences on water body salinities and

climate, both of which may be factors in the persistence of the species (Hamer, Lane, & Mahony, 2008; Lane, Hamer, & Mahony, 2007; Stockwell, Clulow, et al., 2015). Specifically, we aimed to determine whether salinity reduced *Bd* transmission or provided a curative effect (or both) in real-world environments and thus increased survival, which might explain persistence of *L. aurea* in coastal environments. The experiment was conducted commencing in the austral winter, and concluding in the austral summer to expose the frogs to the maximal seasonal thermal challenge that this species faces in the geographical location of the source population. All of our environmental manipulations were carried out in such a way that might realistically be incorporated into translocation programmes for threatened amphibians in the future.

2 | MATERIALS AND METHODS

2.1 | Source of animals and captive husbandry

Juvenile green and golden bell frogs *L. aurea* were grown from tadpoles bred at the University of Newcastle (animal ethics approval A-2013-302; NSW NPWS licence SL100190). Tadpoles were raised in outdoor tubs (200 cm × 1,100 cm × 60 cm) and fed a mixture of ground and whole trout pellets (Ridley Aqua-Feed, Ridley AgriProducts Pty Ltd, Narangba, Australia) ad libitum. Metamorphosing individuals (Gosner stage 42) were moved into the laboratory where they were housed in small plastic aquaria (30 cm × 20 cm × 15 cm) with gravel and aged tap water to a depth of 5 cm. Once each metamorph had undergone full tail regression and reached a minimum SVL of 25 mm, a small passive integrated transponder (Hongteng HT-157 PIT tag, Hongteng, Gangzhou) was positioned under the skin of the right lateral surface for identification. Juvenile frogs were fed live crickets (*Acheta domesticus*, Pisces Enterprises, Brisbane) ad libitum and maintained at room temperature on a 12 hr day (5% UV lamp)/night cycle.

2.2 | Cultivation of Chytrid fungus (*Bd*)

Batrachochytrium dendrobatidis (strain: Gibbo River-Llesueuri-00-LB-1) was derived from pre-existing stock held at the University of Newcastle, NSW. In vitro cultivation was carried out by seeding TGhL agar plates (16 g tryptone, 4 g gelatine hydrolysate, 2 g lactose, 10 g bacteriological agar and 200 mg penicillin-G in 1,000 ml distilled water) with 2 ml of a 1-week-old actively growing liquid chytrid broth. Plates were incubated at 17–19°C and periodically checked for growth using an inverted light microscope until colonies of zoosporangia and free-swimming zoospores could be detected. A zoospore suspension was then prepared by flooding inculcated TGhL agar plates with 1.5 ml of liquid media (TGhL). These plates were left to stand for 5 min, before the supernatant was collected.

2.3 | Chytrid infection

Two hundred and fifty juvenile frogs were moved from the laboratory aquaria in which they were held after metamorphosis to new containers filled with gravel and tap water for infection with *Bd*. Approximately 200 μl of *Bd* zoospore suspension was pipetted into the water of each container. An additional 290 frogs that were to remain uninfected prior to introduction to outdoor mesocosms were placed in identical containers to which an equivalent volume of a sterile agar broth solution without *Bd* zoospores was added. Water changes ceased for a period of 5 days to allow animals contact with the inoculated water to acquire infection. A subset of frogs from each of the "infected" and "uninfected" containers were swabbed to test for the presence of *Bd* 7 days after exposure to determine if frogs had become infected. This time interval is sufficient to allow the *Bd* to undergo a complete life cycle of reproduction in infected animals (Longcore, Pessier, & Nichols, 1999; Piotrowski et al., 2004). Upon analysis of the swabbing data (see below for details), it was found that no individual tested positive for infection. Animals were, therefore, exposed to an additional 200 μl of a zoospore suspension 2 weeks later and reswabbed. After this exposure period, it was found that frogs from two treatment containers tested positive for *Bd*. Individuals from these containers were distributed evenly amongst the other "infected" containers to allow for the natural spread of the pathogen, which was shown to occur for all animals in "infected" tanks via swab testing *c.* 2 weeks later. "Uninfected" containers remained free of *Bd*. The experiment commenced shortly after confirming the infection status of all animals (within 1 week).

2.4 | Outdoor mesocosm configuration

Sixteen cylindrical polyethylene mesocosms (Duraplus Aquapoly Aquaculture tubs: 3.5 m diameter, 1 m height, 10,000 L volume) were filled over half their bottom surface area with gravel to a depth of 30 cm; with the remaining half of the bottom surface filled with aged rain water to a maximum depth of 30 cm (Figure 1). Habitat

FIGURE 1 An experimental mesocosm showing the configuration of refuge habitat [Colour figure can be viewed at wileyonlinelibrary.com]

was provided in the form of brick piles and plastic plants which were placed in three rows—on the dry gravel, along the shoreline and within the water body. Bricks were placed individually (flat on the ground and perpendicular to the water) and in groups consisting of five bricks stacked in a lattice formation (three bricks laid 1 cm apart, parallel and perpendicular to the water, with two bricks laid on top at right angles to the bricks below; Figure 1). These brick piles have been shown to be a preferred refuge of juvenile *L. aurea* in many outdoor field trials that we have conducted (data not presented). A small drainage hole was drilled through the wall of the tub to enable excess water to drain out of the mesocosm during periods of heavy rainfall, and to allow maintenance of a constant water level in each mesocom. Each tub was covered with heavy netting to form an enclosed system, with the connection between the netting and rim of the tub bordered with an additional 10-cm-wide strip of fine mosquito netting to prevent frog escapes.

2.5 | Experimental design

Each salinity treatment (high or low) was replicated across eight mesocosms, with treatments assigned randomly using a random numbers generator in Excel. Natural sea salt (Cheetham Salt Limited, Sunray Swimming Pool Salt) was added to the "high salinity" tubs to achieve a concentration of *c.* 4 ppt (target range: 3.5–4.5 ppt). Non-salted water bodies ("low salinity" treatments) were found to fluctuate between 0 and 1 ppt. These ranges of salt levels in water bodies occupied by *L. aurea* are observed regularly in the field in natural *L. aurea* ponds and are within the known physiological limits of the species (Mahony et al., 2013; Stockwell, Clulow, et al., 2015). Salinity values were recorded periodically across the study period using a water quality meter (YSI professional plus water meter, Xylem, USA) to measure salt levels, and additional water or salt was added where necessary to maintain the targeted salt concentrations. Water temperatures were monitored every 10 min during the study period using temperature data loggers (iButton®) wrapped in a single layer of paraffin film and positioned along the central line of tub, 10 cm away from the edge (deepest point).

Originally, a two-way factorial experiment was planned between water temperature (high/low) and salinity (high/low) as the main effects. However, attempts to heat the water in mesocosms passively using solar powered pumps to circulate water through black polypipe exposed to the sun (considered a potential option in the field for created habitats for reintroduction programmes) and by using aquarium heaters powered by a diesel generator failed to heat the water bodies in "high temperature" treatments by more than 0.5°C mean daily difference compared to "low temperature" treatments during the study period, despite a difference of 3–4°C being achieved during preliminary trials. Thus, the experiment effectively became a salinity only experiment.

2.6 | Animal release into mesocosms

Prior to release, the PIT number, initial weight (g), snout-vent length, head width and right tibia length (recorded to the nearest mm using dial callipers) of infected and non-infected frogs were recorded. Each individual was swabbed to test for *Bd* infection immediately prior to release by swabbing the hands, feet, thighs and lateral sides with a sterile swab (Tubed Sterile Dryswab™ Tip MW100) in a standardized manner, involving four strokes posterior to anterior and back (eight strokes total) of each of the lateral sides and inner and outer thighs, and four strokes (applied in a twirling motion) of the hands and feet. Eighteen non-infected frogs and 10 infected frogs were released into each of the experimental mesocosms on the 12 July 2013 (the middle of the austral winter). Individuals were distributed randomly among the 16 mesocosms to avoid bias in allocating to treatments arising from variations in size, sex and age at the time of release. All animals were released separately into the water so that they were not forced to come into contact with other frogs at the time of release. The experiment was terminated on the 24 December (mid-summer), 2013. The start and end dates of the experiment were chosen due to observations in the wild that *Bd* infection prevalence and loads (and resulting effects of chytridiomycosis) are most intense in the winter months and largely benign in the summer months (believed to be linked to temperature; Mahony et al., 2013; Stockwell et al., 2008). Thus, disease intervention is most likely to be optimal during the winter months.

2.7 | Mesocosm surveys

A census of the experimental mesocosms was carried out at 1, 2, 5, 8, 13, 18 and 23 weeks post-release. During each census, each frog was caught by hand in an individual plastic bag to prevent cross-contamination of frogs during handling before being identified by PIT number, weighed, measured and swabbed as per the standardized method above. Frogs were then released back onto the gravel surface of their respective tubs, making sure that no individual made contact with another to prevent cross-infection during the release. Mesocosm tubs were checked daily for dead or moribund frogs. These were collected, identified by PIT tag number, swabbed and, if alive but moribund, euthanized. The diets of animals in mesocosms were supplemented with live crickets ad libitum throughout the experiment.

2.8 | Detection of chytrid infection by real-time qPCR analysis of swabs

Nucleic acids were extracted from swabs and *Bd* DNA quantified using a qPCR Taqman assay (Boyle, Boyle, Olsen, Morgan, & Hyatt, 2004). Each swab sample was analysed in triplicate using a Rotor Gene 6000 real-time DNA amplification system (Corbett Life Science, Sydney, Australia) following a 1/10 dilution of the original DNA extract. After amplification, the number of *B. dendrobatidis* genomic equivalents (GE) detected at a standardized cycle threshold was calculated across all three replicates as a geometric mean in samples considered positive. Samples were considered positive for *B. dendrobatidis* when amplification occurred in at least two of the three replicates. Non-positive (zero) values were included in the calculation of the geometric mean as these were assumed to be the

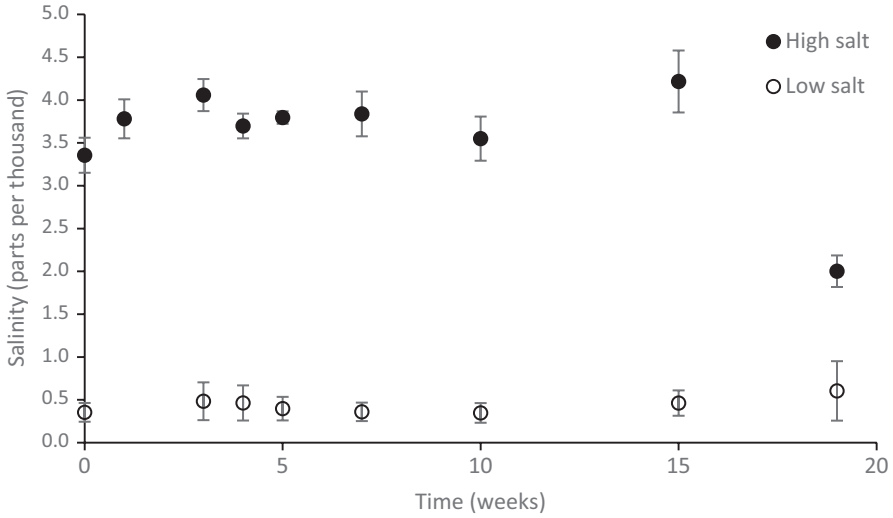

FIGURE 2 Mean salinity levels in high- (N = 8) and low (N = 8)-salt treatment mesocosms across the study period of 23 weeks. The study began on 12 July 2013 (mid-winter) and ended 24 December 2013 (mid-summer). Error bars = ±1*SE*

result of a low quantity of DNA present within the sample. For all samples, the mean GE value was multiplied by 10 to account for the dilution step carried out during the extraction process.

Samples were considered negative for the presence of *Bd* if the process of amplification did not occur in two or more of the three replicates from each sample, providing that the PCR reaction was not inhibited. In order to detect such inhibition, an internal positive control was tested in a single replicate of each sample, along with one replicate of the negative *B. dendrobatidis* template control. Following qPCR, the data for the cycle number in which the sample crossed a threshold set halfway up the amplification curve was noted. Samples were considered inhibited if the amplification curves for positive controls crossed the threshold point more than five cycles after that of the negative control.

2.9 | Data analyses

Univariate survival analysis was initially carried out using Kaplan–Meier plots in JMP (version 11), testing separately for the effects of salt for all frogs released, as well as frogs released initially as infected and uninfected alone. Log-rank and Wilcoxon tests were used to determine the significance of differences between treatments in the survival curves over time.

The proportion of individuals infected with chytrid over time was analysed using a mixed effects logistic regression model in SAS version 9.4 (glimmix procedure), with time and salt treatment as fixed effects and a random effect for mesocosm. The repeated measures per individual were modelled using a residual covariance matrix with autoregressive order one covariance structure. The Kenwood–Roger adjustment was applied to correct for downward bias in the variance–covariance matrix.

Log of infection load was used to correct for skewness in infection load data. Infection load within the infected proportion of the population was analysed over time using linear mixed effects models (LMM) in SAS (PROC MIXED procedure), with salt and time and all two-way interactions as fixed effects. Mesocosms were added as a random effect and repeated measures over time were modelled with a residual covariance matrix with compound symmetry covariance structure.

Effects of the salt treatments on *L. aurea* growth rates were examined using LMM in SAS, with a full factorial model run for the effects of salt and time on tibia length, head width and snout-vent length.

Differences in salt levels between "high" and "low" treatments were investigated using *t*-tests in Excel.

3 | RESULTS

3.1 | Fluctuations in salt concentrations and seasonal water temperature

Mean salt concentrations in "high salt" treatment mesocosms were consistently higher than those in "low salt" mesocosms ($p < .001$; Figure 2). Mean high-salt treatment concentrations generally fluctuated between 3.5 and 4.5 ppt, except for a drop to around 2 ppt in week 19 following a sustained heavy rainfall event (Figure 2). Low-salt treatment means generally fluctuated around 0.5 ppt and rarely exceeded 1 ppt (Figure 2). Mean weekly water temperature increased from approximately 13°C to *c.* 25°C across the study period, which ran from mid-winter to mid-summer (Figure 3).

3.2 | Effect of salt and water body temperature on morphometrics

Neither salt nor water temperature effects resulted in any significant differences between treatments for any of the morphometrics (tibia length, head width or snout-vent length) over time.

3.3 | Effect of salt concentration on survival

Litoria aurea survival was higher in mesocosms containing high salt concentrations after 23 weeks than in those containing low salt concentrations (Figure 4a). Survival was 0.23 in the high salt treatment at 23 weeks compared to 0.13 in the low-salt treatment. The Wilcoxin test for determining the significance of differences in the survival curves suggested that these survival curves were not

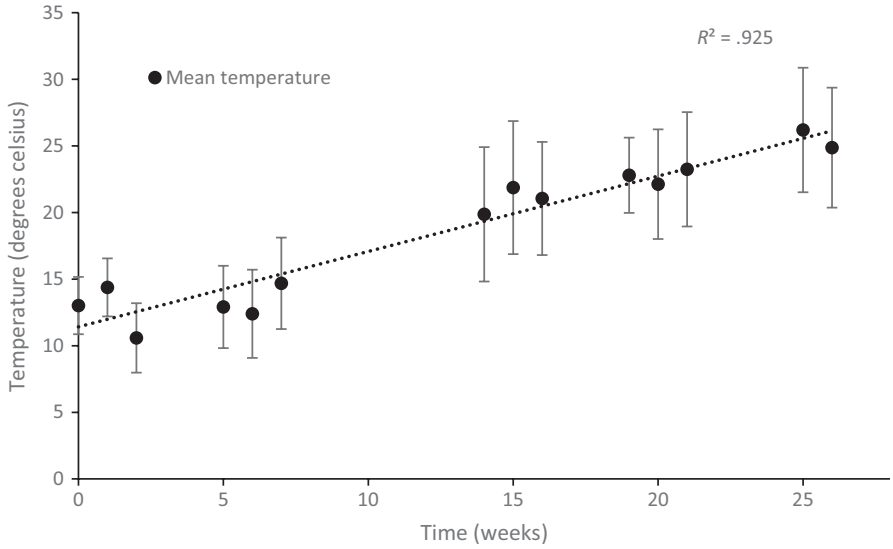

FIGURE 3 Mean weekly water temperatures in mesocosms across the study period. The study began on 12 July (mid-winter) and ended 24 December (mid-summer), 2013. Error bars = ±1 *SD*

significantly different between salt treatments (χ^2 = 1.04, *df* = 1, *p* = .31), while the log-rank test suggested that they were significantly different (χ^2 = 4.66, *df* = 1, *p* = .03). The different outcomes of the two tests are likely due to the breaking of the proportional hazard assumption implicit in these tests (i.e. through the crossing over of the survival curves early on in the experimental period) which are handled differently by each test. The Wilcoxin test places more emphasis on the early part of the survival curves while the log-rank test places emphasis more evenly across the experimental period. Taking both tests together, it can be suggested that there was no significant difference in the early period of the survival curves, but that the curves deviated significantly in the latter period of the experiment.

Almost all of the increased survival in the high-salt treatments was attributable to the higher survival rates of animals that were initially released into the mesocosms without *Bd* infection (Figure 4b). These animals experienced survival of 0.34 in high-salt treatments at 23 weeks compared to 0.20 in low-salt treatments. For the animals released initially without *Bd*, this difference was found to be significant with both the log-rank test (χ^2 = 6.36, *df* = 1, *p* = .01) and Wilcoxin test (χ^2 = 4.37, *df* = 1, *p* = .04). Animals initially released with *Bd* infection had similarly low survival irrespective of salt treatment, with 0.04 survival in high-salt compared to 0.01 in low-salt treatments by 23 weeks (Figure 4c). These survival curves were not significantly different by either test (log-rank χ^2 = 0.33, *df* = 1, *p* = .56; Wilcoxin χ^2 = 0.003, *df* = 1, *p* = .96). Collectively, survival was much higher overall in animals initially released without *Bd* infection compared to those released with *Bd* infection (Figure 4b,c).

The rate at which survival decreased also changed markedly between the two groups of animals. Survival in the initially uninfected animals decreased very slowly in the first 5 weeks, with survival decreasing to 0.77 and 0.72 in low and high salt, respectively, before decreasing rapidly in the low-salt treatment to 0.34 by week 8 (Figure 4b). In contrast, the survival of uninfected animals released into the high-salt treatment only declined to 0.59 by week 8. The survival for the *Bd*-infected animals on the other hand decreased very rapidly, down to 0.09 and 0.14 in low and high salt, respectively, in the first 5 weeks alone (Figure 4c), and very few initially *Bd*-infected animals survived to week 23 in either treatment.

3.4 | Clearance of infection from surviving animals over time

There was a strong effect of time on the proportion of individuals in the mesocosm population that were infected with *Bd* ($F_{5, 1,249}$ = 53.42, *p* < .001; Figure 5a). The population experienced a rapid increase in the prevalence (i.e. proportion of individuals infected) of *Bd* infection by week 8 (early September) before a decrease shortly after week 13 (mid-October) when infected individuals either died or clear themselves of the infection, with no animals infected by week 23 (late December; Figure 5a). The clearance of infection from the mesocosm population was associated with the seasonal increase in mean water temperature across the experimental period (Figure 3).

The overall proportion of infected individuals in the mesocosm population over time was consistently higher in the low-salt treatment than the high-salt treatment (Figure 5a), although this was not found to be statistically significant with α = 0.05 ($F_{1, 44.13}$ = 2.92, *p* = .09). The size of this effect as characterized by the odds ratio was 0.78 (95% CI 0.55–1.11), indicating that the chance of *Bd* infection in the high-salt treatment was 0.78 times, or 22% lower than that of the low-salt treatment.

There was also a strong effect of time on *Bd* infection load within the infected mesocosm population ($F_{5, 764}$ = 92.68, *p* < .001; Figure 5b). This effect closely matched the pattern of infection prevalence, with infection loads increasing rapidly in infected animals with peaks between 8 and 13 weeks post-release (Figure 5b). Declines in infection load occurred after week 8 and were close to zero by week 13, which is the point at which the population began to clear itself of infection (Figure 5a). Salt did not have an effect on *Bd* load over time ($F_{1, 27.9}$ = 0.03, *p* = .86; Figure 5c).

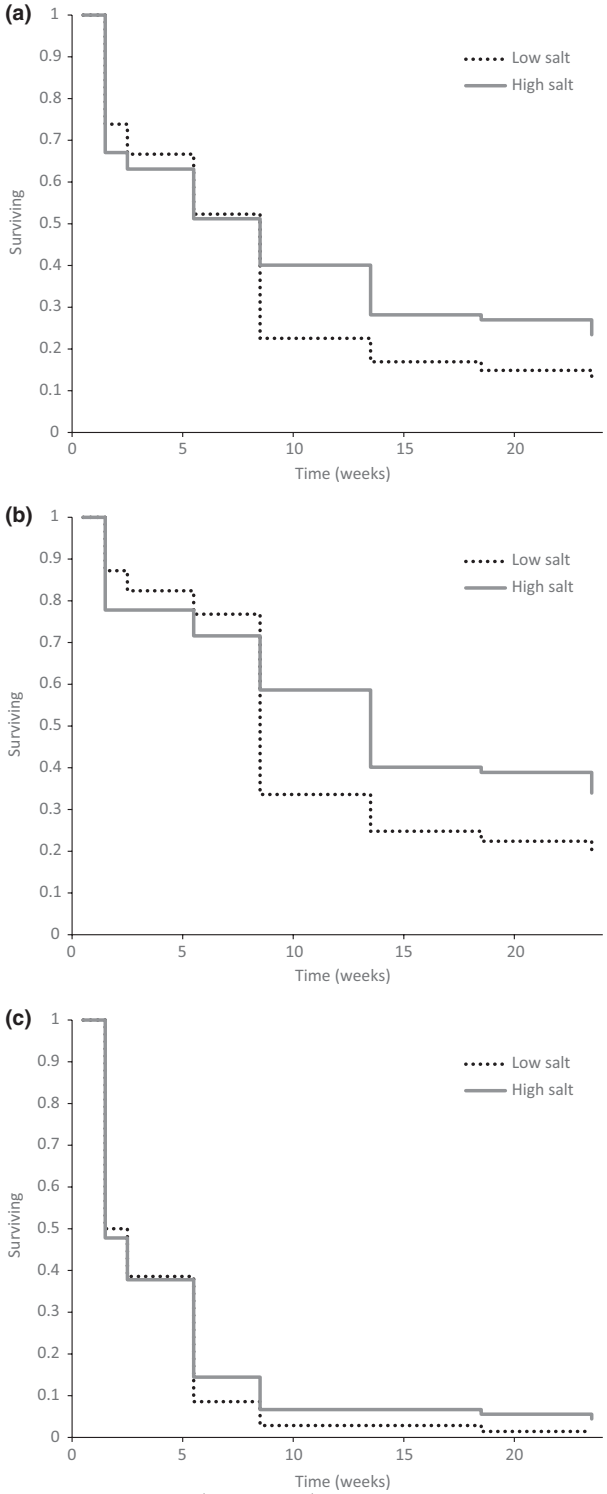

FIGURE 4 Survival (proportion) of frogs released into experimental mesocosms containing low- and high salt concentrations over the course of the study period. (a) All frogs released at time zero (n = 8 mesocosms and 224 animals per treatment level at time zero); (b) Frogs released initially without *Bd* infection only (n = 8 mesocosms and 144 animals per treatment at time zero); (c) Frogs released initially with *Bd* infection only (n = 8 mesocosms and 80 animals per treatment at time zero)

3.5 | Differences in rates of infection state transitions between high- and low-salt treatments

The patterns of change in infection state within individuals in the population (i.e. from uninfected to infected or vice versa) differed over time (Table 1). A much higher proportion of the mesocosm population in both low- and high-salt treatments changed from uninfected to infected at each survey period in the first 8 weeks of the experiment (when the population was nearing its infection prevalence peak), before switching to a state in which a much greater proportion of the population cleared itself of the infection (changing from infected to uninfected) after week 8 (Table 1). All of the surviving animals in both salt treatments changed infection state from infected to uninfected between weeks 13 and 18 (Table 1). In all, bar one, time periods (weeks 5–8), the proportion of animals changing from uninfected to infected was lower in the high-salt treatment (Table 1).

4 | DISCUSSION

This study has shown that it is possible to manipulate salinity levels in a realistic environmental setting in a way that can lead to significant improvements in host survival for a threatened frog that coexists with *Bd* and is impacted by chytridiomycosis. Importantly, for the first time, we have demonstrated that the probable primary mechanism of a previously demonstrated beneficial effect of salinity on survival of *L. aurea*, is reduced disease transmission rather than increased survival of infected frogs (i.e. there appears to be no curative effect for already infected individuals in a field setting). This is an important finding for the management of species in which there has been a shift in demographics with a reduced proportion of adults in reproductively mature age classes due to chytridiomycosis. Based on the results of this study, there may be a measurable gain in the population viability of host species if salinity in water bodies can be increased to a level where the viability of the pathogen is sufficiently reduced to reduce transmission rates. This could become an important management strategy for not only managing the threat of affected species that coexist with the disease in existing populations and habitats, but in particular could be incorporated into created or modified habitats that form the basis of a swathe of translocation programmes around the globe (Germano et al., 2015), provided that the salinity levels are within the physiological limits of the host species.

The increased survival in the high-salt treatment in this study was not trivial; an increase in survival from 13% to 23% among juvenile *L. aurea* through the winter months represents a relative increase in survival of 77% over that time period compared to the low-salt treatment. Such an increase in survival could translate into large differences in the number of juvenile frogs that survive the winter period post-metamorphosis, and adults in the population capable of contributing to recruitment through reproduction if applied

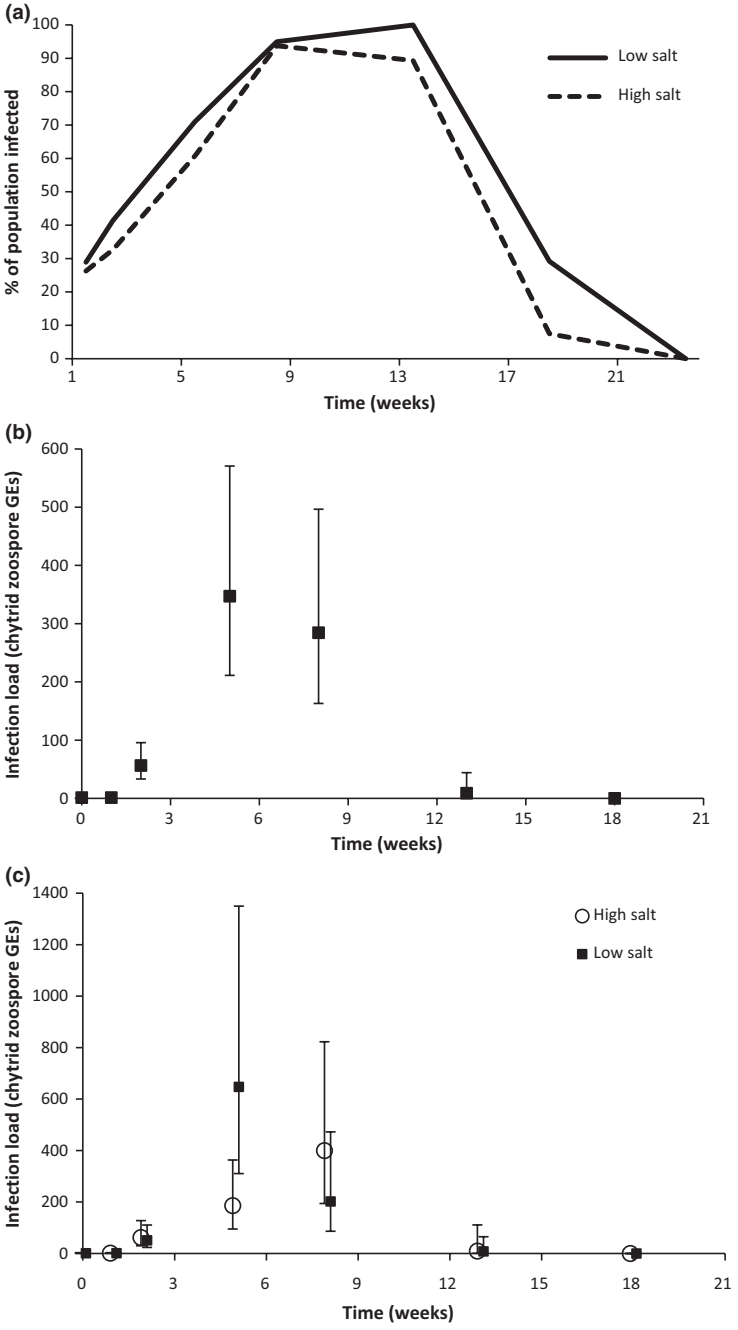

FIGURE 5 *Bd* prevalence and infection load over time. (a) The proportion of surviving frogs in low- (N = 8) and high (N = 8)-salt mesocosms that were infected (tested *Bd* positive) across the experimental period; (b) Mean *Bd* infection loads for all surviving frogs at various time intervals across the experimental period; (c) Mean *Bd* infection loads for all surviving frogs at various time intervals across the experimental period, separated by low- and high-salt treatments (N = 8 mesocosms per treatment). Error bars = 95% confidence intervals. GEs, genomic equivalents. The study period lasted 23 weeks, from 12 July (mid-winter) to 24 December (mid-summer), 2013

to the source population. Modelling and field studies by Pickett et al. (2014) on another coastal population of *L. aurea* located *c.* 200 km south of the population/outdoor mesocosms in this study support this view. That modelling indicated that an increase in female survivorship at 2 years (the age at which females reach sexual maturity) and beyond into older adult age classes could lead to a significant improvement in the population viability and a reduced probability of extinction in *L. aurea* populations.

The salinity range in this study (0–4 ppt) was similar to a previous study in ponds of a nearby population which showed lower *Bd* loads and increased survival of *L. aurea* at 2–4 ppt compared to ponds with salinities at or close to 0 ppt (Stockwell, Storrie, et al., 2015), although the mechanism by which this occurred (curative effect or reduced disease transmission) was not determined. However, the timing of the release and establishment of *L. aurea* progeny into the ponds of the earlier study was different to the current study. In the earlier study, *L. aurea* were released as tadpoles in the middle of summer (also in different years to the current study), and growth, infection status and survival was monitored over 1 year from summer to summer. An advantage of the current study was that juvenile frogs were released in mid-winter, at a time when the impact of chytridiomycosis is known to be at its seasonal peak in *L. aurea* (Mahony et al., 2013; Stockwell et al., 2008), as in many other susceptible species. In addition, in the current study, a combination of both *Bd*-infected and *Bd*-free animals were released together to specifically study the effects of saline influences on infection transmission. The demonstration in this study that

TABLE 1 Changes in infection state in surviving frogs within intervals from 0 to 23 weeks in low- and high-salt treatments. N indicates uninfected frogs (negative for *Bd*). I indicates infected (*Bd* swab positive) frogs. # = number of individuals that changed state in the indicated interval (numerator = number changing state; denominator = number in starting state at beginning of respective interval); % = the percentage of individuals in one state (N or I) that changed to another state (N or I) within the indicated interval. Blank cells indicate no change in state was possible within particular intervals due to the absence of frogs in the starting state in the respective treatment in that interval. Mean water temperature is across all mesocosms in all treatments for the second week in each interval

		Time (weeks)						
Salt	**State change**	**0–1**	**1–2**	**2–5**	**5–8**	**8–13**	**13–18**	**18–23**
Low	N to I (#)	27/101	50/72	29/31	2/4		0/17	0/24
	N to I (% of N)	27	69	94	50		0	0
	I to N (#)	9/41	9/51	4/76	7/75	19/28	7/7	
	I to N (% of I)	22	18	5	9	68	100	
	Number alive	142	123	107	79	28	24	24
High	N to I (#)	26/118	53/101	44/51	4/7	0/8	0/62	0/57
	N to I (% of N)	22	52	86	57	0	0	0
	I to N (#)	11/42	5/49	1/79	10/105	60/67	5/5	
	I to N (% of I)	26	10	1	10	90	100	
	Number alive	160	150	130	112	75	67	57
Mean water temperature (°C)		14.4	10.6	12.9	14.7	19.9	22.8	26.2

transmission is reduced under conditions of elevated salinity during the season when the transmission and mortality rates are highest was possible because of the timing and design of the study. Furthermore, commencing this study in mid-winter and following the survival and infection dynamics until mid-summer showed that infected *L. aurea* that survive winter are capable of clearing the disease at a high rate heading into summer. This reinforces the view supported by some published data on *L. aurea* (Stockwell et al., 2008) that increased mortality in colder months of the year was the driving force of demographic shifts in the species responsible for its decline across its former range.

It is worth noting that attempts to manipulate water temperature in this study did not produce a large enough thermal shift to directly confirm this as an experimental treatment, despite attempts by several methods that might have been able to be applied in a field setting. This is important because active and passive strategies have been proposed in the literature to raise air and water temperatures, such as using dark substrates in ponds and removal of aquatic and overhanging terrestrial vegetation to reduce shading and increase exposure to solar radiance (Becker, Rodriguez, Longo, Talaba, & Zamudio, 2012; Becker & Zamudio, 2011; Heard et al., 2014; Raffel, Michel, Sites, & Rohr, 2010; Scheele, Hunter, et al., 2014). Our experience suggests that directly managing temperature in external water bodies by passive or active management of temperature will be a challenging environmental management strategy to implement, even if a potentially important one (Scheele, Hunter, et al., 2014). Such an investigation awaits the development of a better system for heating water bodies in outdoor environments.

Few studies have dealt with the manipulation of environmental salinity as a management approach for mitigating *Bd* load and impact in habitats, although it has been suggested (Scheele, Hunter, et al., 2014; Stockwell, Clulow, et al., 2015; Stockwell et al., 2012; Stockwell, Storrie, et al., 2015). The susceptibility of *Bd* to increasing salinity has been demonstrated in culture through reduced growth rates and motility/viability (Johnson et al., 2003; Stockwell et al., 2012) and there is evidence in support of benefits of salinity to frogs and tadpoles in some studies (Heard et al., 2014; Stockwell, Clulow, et al., 2015; Stockwell et al., 2012; Stockwell, Storrie, et al., 2015) in addition to the current study. It is worth noting that there are probably scenarios where manipulating salinity on a scale that would be detectable in systems where salinity is naturally low could be difficult. Nevertheless, some systems such as coastal estuarine systems that are a mosaic of oligohaline and fresh water, as is the case in the environment of many persisting *L. aurea* populations (Klop-Toker et al., 2016; Valdez et al., 2015), may offer opportunities to pursue this as a passive strategy, for example, by manipulation of tidal flows and inundation at a landscape level, or actively by the addition of sea salt to water bodies.

The findings of this study suggests that where attempts are made to reintroduce or establish new populations of amphibians to secure species from the pandemic disease *Bd*, these should have the best chance of success in environments where salinities can be increased either passively or manually to reduce infection rates and transmission, and lead to higher pathogen clearance rates and adult survival. Such effects, mediated through salinity may be employed to benefit the demographics by shifting the age class structure towards older animals. On the basis of the results of this study, addition of salt to water bodies (to achieve concentrations of *c.* 2–4 ppt) should be considered for incorporation into the design of constructed supplementary or modified habitats for the management of amphibians sensitive to chytridiomycosis, where it is feasible to do so. This study demonstrated a fundamental principle in relation to management of species whose conservation status is impacted by emerging disease, but which persist, albeit at a higher risk of

extinction. Understanding mechanisms of disease transmission and dynamics as they play out in realistic environmental scenarios is a strategy worth pursuing, since such investigations may identify management strategies that increase resilience of susceptible species at the landscape level.

ACKNOWLEDGEMENTS

We thank Tim Callaghan for assistance with carrying out surveys. Port Waratah Coal Services provided funding for the study.

AUTHORS' CONTRIBUTIONS

S.C., J.C., M.S. and M.M. conceived the ideas and designed methodology; S.C., J.G., and H.J. carried out the experiments and collected the data; S.C. analysed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.r0904 (Clulow et al., 2017).

ORCID

Simon Clulow http://orcid.org/0000-0002-5700-6345

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How to cite this article: Clulow S, Gould J, James H, et al. Elevated salinity blocks pathogen transmission and improves host survival from the global amphibian chytrid pandemic: Implications for translocations. *J Appl Ecol*. 2018;55:830–840. https://doi.org/10.1111/1365-2664.13030