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Embryo mortality in a captive-bred, Critically Endangered amphibian

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ABSTRACT: The Critically Endangered southern corroboree frog *Pseudophryne corroboree* is dependent upon captive assurance colonies for its continued survival. Although the captive breeding programme for this species has largely been successful, embryonic mortality remains high (40–90% per year). This study aimed to investigate the causes of mortality in *P. corroboree* embryos in the captive collection at Melbourne Zoo. During the 2021 breeding season, we investigated 108 abnormal embryos to determine the impact of infections and anatomical deformities on survival and used culture and molecular methods to identify microbes. Overall, 100% of abnormal embryos had fungal infections, and of these, 41.6% also had anatomical deformities. The mortality rate in abnormal embryos was 89.8%; however, we detected no difference in survival in any of the 3 observed fungal growth patterns or between deformed and non-deformed embryos. Sanger sequencing of the ITS region identified fungal isolates belonging to the genus *Ilyonectria*, the first record in a vertebrate host, and another as a *Plectosphaerella* sp., which is the first record of infection in an embryo. Dominant bacteria identified were of the genera *Herbaspirillum* and *Flavobacterium*; however, their role in the mortality is unknown. Fungal infection and deformities have a significant impact on embryo survival in captive-bred *P. corroboree*. In a species which relies on captive breeding, identifying and reducing the impacts of embryonic mortality can inform conservation efforts and improve reintroduction outcomes.

KEY WORDS: Southern Corroboree frog · *Pseudophryne corroboree* · Fungi · Infection · Deformities · Assurance colony

1. INTRODUCTION

Embryonic mortality is a major constraint to reproductive performance in amphibians, with disease, predation and unfavourable abiotic conditions being key causes. In response to such threats, the breeding behaviours and reproductive strategies in amphibians are incredibly diverse, although some strategies have remained conserved across taxa. In oviparous amphibians, the use of an external jelly coating to surround their embryos functions as a protective barrier to reduce disease, desiccation and to a limited extent, predation. Despite this, a range of pathogens have been documented in both captive and wild amphibian embryos (Green & Converse 2005) including viruses (Tweedell & Granoff 1968, McKinnell 1973), bacteria (Bishop et al. 2021, Khalifa et al. 2021), ciliates (Gilbert 1942), protists (Schuetz et al. 1978, Holz et al. 2015), algae (Gilbert 1942, Bachmann et al. 1986, Kim et al. 2014) and fungi (Forester 1979, Villa 1979, Blaustein et al. 1994, Gomez-Mestre et al. 2006, Banning et al. 2008). Although representative across pathogen taxa, this list is likely an underrepresentation of the

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true number of pathogenic microorganisms that impact amphibian embryos.

Whilst investigations of disease events in tadpoles and post-metamorphic amphibians have been undertaken, an understanding of the causes of morbidity and mortality in embryos of these species is lacking. Knowledge in this regard is likely to become of increasing relevance as the impacts of climate change and the increased risks of infectious disease necessitate the maintenance of captive assurance colonies. Captive assurance colonies are an essential management strategy for species threatened with extinction (Brannelly 2014). One species where such action has been taken is the Australian endemic southern corroboree frog *Pseudophryne corroboree*, which suffered catastrophic declines due to the introduction of the amphibian chytrid fungus (Hunter et al. 2010). Now functionally extinct in the wild, the remaining breeding animals are held within bio-secure facilities managed by Zoos Victoria and Taronga Conservation Society. Their goal is to ensure the survival of the species by preventing mortality from chytridiomycosis, while also generating animals for conservation research and reintroduction.

P. corroboree are seasonal, terrestrial breeders with breeding occurring from mid- to late summer. Males create small nest chambers on the edges of vernal pools to attract females (Hunter et al. 2009), with clutches containing on average 16–38 eggs (Fig. 1A) (McFadden et al. 2013). Embryos develop in the nest chamber until autumn rains initiate hatching (Hunter et al. 2009). The free-swimming tadpoles then migrate to the main pool where they overwinter until metamorphosing in late spring (McFadden et al. 2013). On average, the developmental time (i.e. egg-laying to metamorphosis) in *P. corroboree* is 10–12 mo.

Establishing and maintaining captive breeding programmes is an ongoing process involving trial and error, with continued refinement. Captive breeding for *P. corroboree* has largely been successful (McFadden et al. 2013). However, there were several challenges initially which resulted in the captive colonies experiencing up to 100% embryonic mortality in the first few years (McFadden et al. 2013). Since then, efforts have focussed on understanding the life-cycle requirements of the species, leading to improved husbandry practices with greater recruitment success. Despite these changes, the captive colonies have continued to experience high embryonic mortality (40–90%, depending on the year and captive facility) (Zoos Victoria and Taronga Conservation Society unpublished records).

As amphibians are among the most threatened groups of vertebrates, breeding programmes are key to the conservation of many species worldwide (Stuart et al. 2004). Captive management is currently the only survival strategy for *P. corroboree*; therefore, embryo production is a key outcome for the conservation breeding programme and has significant flow-on

Fig. 1. (A) Adult male *Pseudophryne corroboree* on a nest of captive-bred embryos at Melbourne Zoo (photo: D. Goodall), and (B) healthy captive bred *P. corroboree* embryo

effects to the recovery programme for this species. While embryo mortality in this species has remained high, the causes of mortality have yet to be investigated. The aim of this study was to identify factors contributing to mortality in the captive-bred *P. corroboree* embryos produced at Melbourne Zoo. We hypothesised that microbial infections would be a leading cause of mortality in the captive-bred embryos.

2. MATERIALS AND METHODS

During the 2021 breeding season (February to April), 718 naturally laid *Pseudophryne corroboree* embryos from the captive colony at Melbourne Zoo were culled from the breeding programme due to abnormal appearance. Abnormal embryos were characterised by any deviation from a healthy embryo, such as a change in the embryo colour as a likely result of microbial growths, and/or the presence of an anatomical deformity, and/or a change in the capsule composition (e.g. softening or decomposition). We examined these embryos with a dissecting microscope (Nikon SMZ745T) to look for anatomical deformities and the presence of obvious microbial growth. Embryos were then classified according to the presence or absence of deformities and the pattern of microbial growth (based on the 4 morphological appearances identified in Section 3.1) and randomly assigned to 2 groups. The first group was used to study infection progression and quantify embryonic survival, and the second was destructively sampled for histopathology, culturing, and/or molecular identification of the microbes present. Due to the Critically Endangered status of *P. corroboree,* access to healthy embryos was limited to 4 individuals (Fig. 1B).

2.1. Gross observations

Embryos were maintained individually between 16 and 18°C in Petri dishes on a moist paper towel. Every 24–48 h from Day 0 (collection day) until hatching they were examined using a dissecting microscope (Nikon SMZ745T). The morphology of each embryo was recorded noting any microbial growth. The severity of colonising microbes was scored as the percentage of growth occupying the perivitelline space. Mild infection was characterised as occupying approximately 10% of the perivitelline space, moderate infection 10–50%, and severe infection >50%. Appreciable deformities were also recorded.

As embryos became unviable, characterised by halted development or excessive microbial growth, they were fixed for histological examination. The jelly coating surrounding the vitelline envelope was removed prior to histopathology and microbial culture to reduce microbial contamination. We removed jelly coatings manually under sterile conditions and washed with 70% ethanol to remove any contaminants introduced during the removal procedure.

2.2. Histopathology

For histopathology, embryos were fixed in either 10% buffered neutral formalin or Bouin's solution. Samples were then processed using routine histologic tissue processing. Briefly, samples were processed on a standard 12 h cycle and paraffin-embedded, and 5 µm sections were taken and stained with a combination of haematoxylin and eosin, Gram stain or Grocott's methenamine silver stain.

2.3. Microbiology

Culturing was performed using homogenised embryos which were plated onto non-selective (tryptic soy agar with 5% sheep blood) and fungal-selective (Sabouraud's dextrose agar) media, and then incubated at 25°C for 2–7 d. Plates were examined for the presence of bacteria and fungi, with dominant microorganisms isolated and purified for identification.

2.3.1. Bacterial identification

Bacterial cultures were presumptively identified by conventional methods using Gram stain, colony morphology, atmospheric requirements and primary identification tests (catalase, oxidase). Taxonomic identification was performed by 16S rRNA sequence analysis. Genomic DNA was extracted from a subset of bacterial cultures using a QIAmp DNA Mini Kit (Qiagen) according to the manufacturer's instructions and quantified using a NanoDrop spectrophotometer (Thermo Scientific) and a Quantus fluorimeter (Promega).

The 16S rRNA gene was amplified by PCR using MyTaq Mix (Bioline), with 20 µM of the primers 27F and 1492R (Lane et al. 1985) and approximately 50 ng of genomic DNA. The reaction was performed on a

T100 Thermal Cycler (Bio-Rad) with the following cycle parameters: 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s; with a final extension of 72°C for 5 min. Sanger sequencing of the 16S rDNA was performed on purified amplicons by the Australian Genome Research Facility (Melbourne, Australia). Amplicon sequences were aligned against the NCBI GenBank nucleotide database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and species identification was confirmed using the Ribosomal Database Project (RDP) taxonomy classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp).

2.3.2. Fungal identification

DNA was extracted from each morphologically distinct fungal isolate with a QIAmp DNA Mini Kit (Qiagen) using a modified protocol for plate cultures. To facilitate cell wall digestion, 30 mg of silicon microbeads (BioSpec) were added to the lysate mix, which was then homogenised using the TissueLyser II (Qiagen) at 25 Hz repeated for 4 cycles of 2 min each. The homogenate was incubated at 56°C for 5 h. DNA concentration and purity were then checked with a NanoDrop (Thermo Scientific), and samples were stored at −20°C until use.

For species identification, the internal transcribed spacer (ITS) region was amplified by PCR using previously published protocols and primers (ITS1 and ITS4) (White et al. 1990). All PCR reactions were performed in a final volume of 50 µl containing 25 µl MyTaq mix (Bioline), 0.4 µM of both forward and reverse primers and 2 µl of genomic DNA template. The reaction consisted of 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final step at 72°C for 10 min. PCR products were visualised by gel electrophoresis on a 1% agarose gel, using the ChemiDoc XRS+ (Bio-Rad) imaging system and sequenced by Sanger sequencing at the Australian Genome Research Facility (Melbourne, Australia). We analysed DNA sequence data with Geneious Prime (v. 2021.0.3), and trimmed reads were blasted against the NCBI GenBank nucleotide database using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

To rule out culture contamination and confirm fungal identity, 2 formalin-fixed, paraffin-embedded tissue samples were sent to the Clinical Mycology Reference Laboratory (Institute of Clinical Pathology and Medical Research, NSW Health Pathology, Westmead Hospital, New South Wales, Australia) for panfungal PCR (Lau et al. 2007).

2.4. Statistical analyses

All statistical analyses were conducted in R v.4.1.2 using the RStudio interface (RStudio Team 2020, R Core Team 2021). Survival differences between growth patterns and deformities were analysed by Cox regression analysis using the 'Survival' package (Therneau & Lumley 2015). Censoring events were included to show samples which were removed from the survival analysis. Embryos classified as having Growth-X morphology (see Section 3.1) were excluded from the survival analysis, due to low sample size (N = 1), thus reducing the overall sample size to 80 embryos.

3. RESULTS

Over the course of the 2021 breeding season, the captive population of *Pseudophryne corroboree* at Melbourne Zoo laid 2546 eggs, of which 42.9% were unviable (includes unfertilised and fertilised-deceased embryos).

3.1. Gross observations

Gross examination of 718 abnormal embryos performed on the day of collection suggested that there were prolific fungal infections in all embryos. Fungal presence was later confirmed by histology and microbiology. Examination indicated that 582 of the 718 embryos were unviable upon collection. Unviability was characterised by a lack of movement, fungal overgrowth and/or decomposition. Of the 136 viable embryos, 108 were between Gosner stage 15 and 20 (Gosner 1960), thus it was possible to reliably confirm their viability via embryonic movement, and so this subset was used for this study. The remaining 28 embryos were in early embryonic development and were excluded.

We identified 4 morphologically distinct gross patterns of fungal growth in the *P. corroboree* embryos, which hereafter are referred to as Growths-W, -X, -Y and -Z (Fig. 2). Anatomical deformities were also identified in 45 embryos. We classified these into 3 categories based on the location of the deformity, which encompassed deformities of the head (Fig. 3A), tail (Fig. 3B) and yolk sac (Fig. 3C).

Examination of the 108 embryos that were viable on the day of collection revealed that 60 (55.6%) were colonised by a fungus with Growth-W, 20 (18.5%) with Growth-Y, 17 (15.7%) with Growth-Z

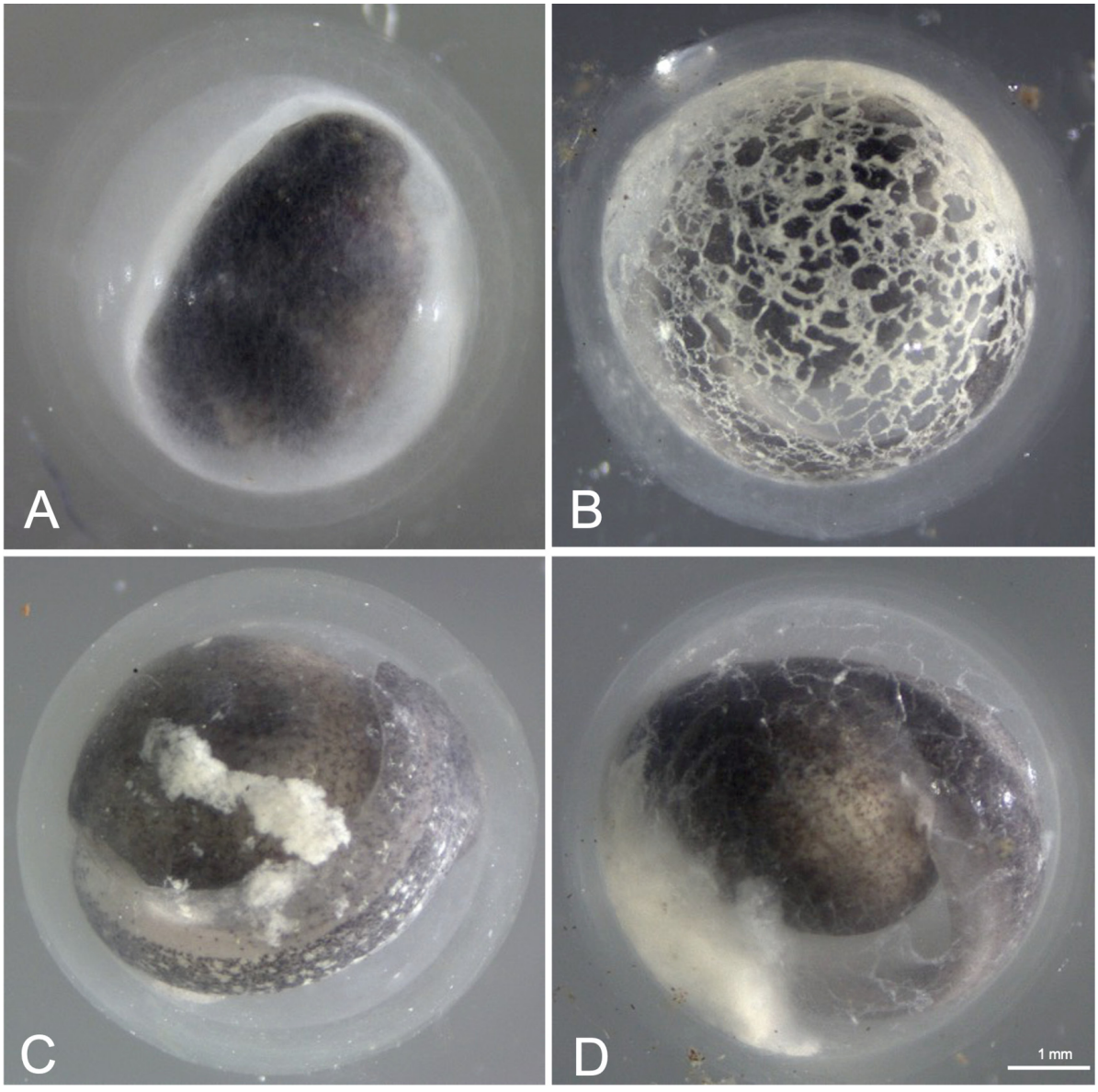

Fig. 2. Gross fungal growth patterns in captive *Pseudophryne corroboree* embryos. (A) Growth-W: categorised by fine, white filaments, with a diffuse pattern of growth. (B) Growth-X: cream to tan-coloured growth, forming a honeycomb pattern progressing to coalescing dense plaques. (C) Growth-Y: irregular, dense floccular aggregates of white to cream-coloured material. (D) Growth-Z: fine filamentous to reticular growth pattern, coalescing to form thick aggregates and plaques

and 7 (6.5%) by Growth-X. No fungal growths were observed in the 4 controls. Of the 81 embryos left to develop, 11 successfully hatched into free-swimming tadpoles despite evidence of fungal infection. These embryos hatched between Gosner stages 20 and 22 (Gosner 1960). Of the hatchlings, 7 were colonised by fungi with Growth-W, 2 with Growth-Z, and one each of Growth-X and -Y. The other 71 embryos became unviable between 2 and 46 d post collection. There was no significant difference in survival between the embryos infected with Growths-W, -Y and -Z (log-rank: $p = 0.72$) (Fig. 4). However, the survival of embryos infected with Growths-W, -Y and -Z was significantly greater than that of embryos which were also deformed (Cox regression: $p = 0.025$, $p = 0.29$, $p = 0.027$, respectively) (Fig. 5).

The severity of infections on day 0 ranged from mild (59%) to moderate (26%) and severe (15%). Most infections increased in severity until death (N = 64), with the remainder showing no change (N = 17).

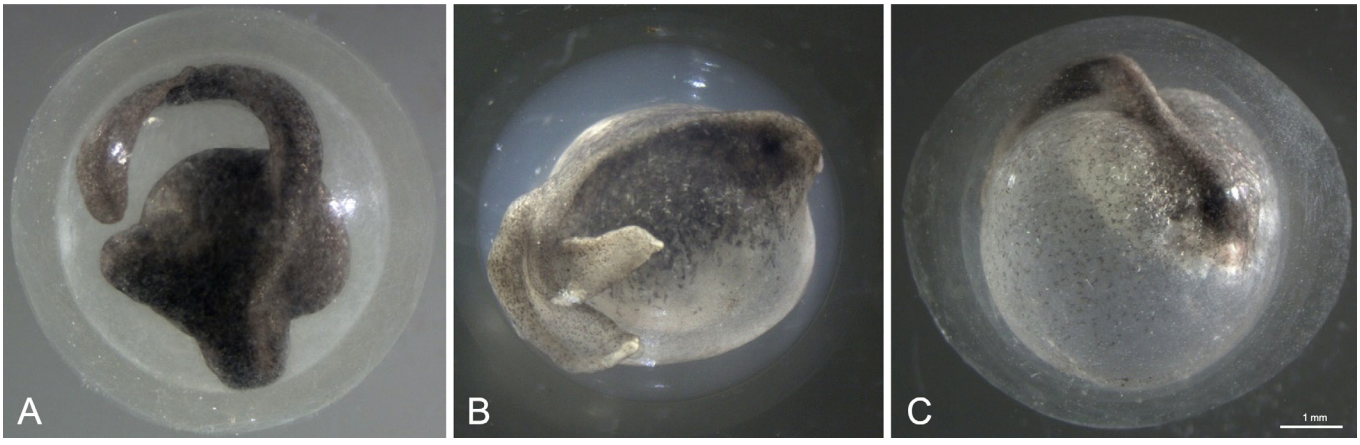

Fig. 3. Anatomical deformities in captive bred *Pseudophryne corroboree* embryos. (A) Bicephaly, (B) twin tails and (C) malformed yolk sacs

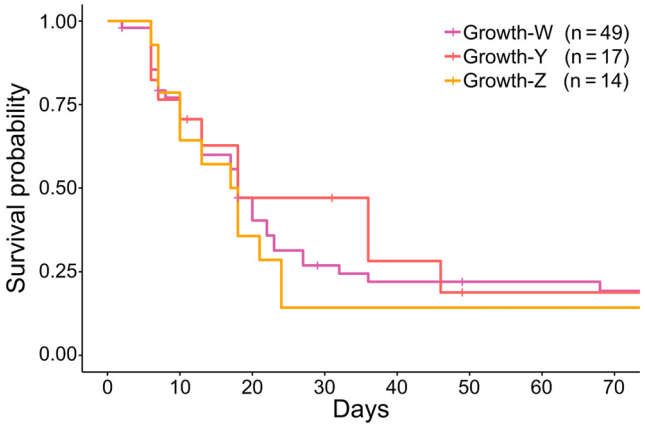

Fig. 4. Kaplan-Meier survivorship curves of infected *Pseudophryne corroboree* embryos (N = 80) from the 2021 breeding season. Day 0 indicates the first day infection was noticed. All embryos that survived beyond Day 70 hatched (N = 11). Notches on lines indicate censoring events. Growth-X was excluded due to small sample size

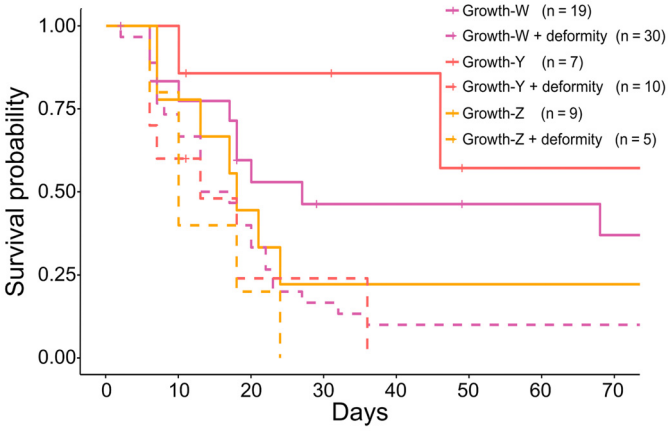

Fig. 5. Kaplan-Meier survivorship curve of *Pseudophryne corroboree* embryos (N = 80) from the 2021 breeding season. Day 0 indicates the first day infection was noticed. All embryos that survived beyond day 70 hatched (N = 11). Infected embryos are represented with solid lines and infected embryos which also had a deformity by dashed lines. Notches on lines indicate censoring events. Growth-X was excluded due to small sample size

Of the 52 embryos that developed severe infections, 51 were colonised by Growth-W. Only 3 of these embryos classified with severe colonisation of Growth-W survived to hatching. These 3 embryos were the only embryos, out of 45, which also had a deformity and survived to hatching (93.3% mortality). The 3 survivors were characterised by deformities of the yolk sac (N = 2) or head (N = 1).

3.2. Histopathology

In total, 46 embryos were processed for histological examination. Poor fixative penetration through the jelly capsule resulted in poor section quality, which prevented detailed microscopic assessments. All samples examined had heavy fungal colonisation that was localised to the perivitelline space (Fig. 6A). We found no evidence of fungal penetration through the outer epidermal layer of the developing embryo, and there were no significant morphological changes in the outer epithelial cells. However, fungal hyphae clearly penetrated through the capsule layers (Fig. 6B). Dense accumulations of fine bacilli bacteria were observed in some sections. There was no evidence of an inflammatory response in any sample infected with bacteria or fungi.

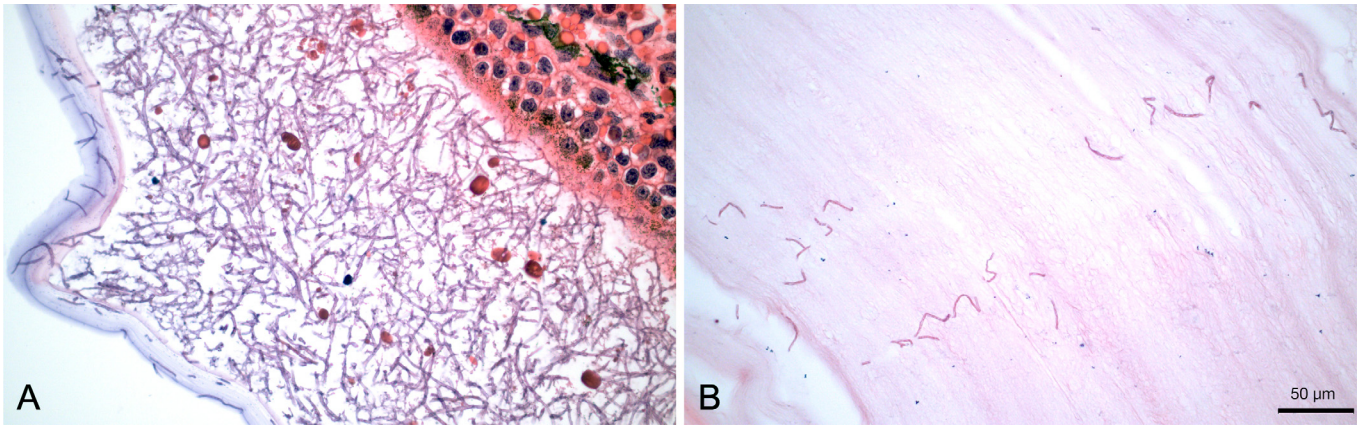

Fig. 6. Histological section of a *Pseudophryne corroboree* embryo infected with Growth-W stained with haematoxylin and eosin. (A) Fungi localised to the perivitelline space, and (B) fungal hyphae penetrating through the multiple capsule layers of the embryo

3.3. Microbiology

Bacterial and fungal cultures were established from 20 embryos; 13 on Day 0 and 7 throughout the experimental period. All embryos cultured yielded either bacteria (N = 13), fungi (N = 5) or both bacteria and fungi (N = 2). None of the control embryos had any visible bacterial or fungal growth, but both samples produced bacterial growth when cultured. Hence, all embryos cultured yielded microbial growths.

3.3.1. Bacterial identification

All samples cultured had mixed bacterial growth that were dominated by the genera *Herbaspirillum* (N = 7), *Flavobacterium* (N = 4) or both (N = 4). DNA sequence analysis of the 16S rRNA identified the first isolate as *H. frisingense* with 99% identity (1091/1097 bp) (MH910131.1). This isolate was further confirmed as belonging to the genus *Herbaspirillum* using the RDP taxonomy classifier. DNA sequence analysis of the second isolate could not discriminate between *F. tructae* (1089/1092 bp) (NR_133749.1) and *F. johnsoniae* (1087/1092 bp) (EU730945.1); this result was also supported by RDP classifier results. The phenotypes largely matched previous descriptions for these organisms (Baldani et al. 2015, Bernardet & Bowman 2015) as outlined in Table S1 in the Supplement at www.int-res.com/articles/suppl/d152p073_supp.pdf.

3.3.2. Fungal identification

We established fungal cultures from 7 embryos, from which 4 isolates were morphologically distinct. ITS sequence analysis using the BLASTn search identified 2 of the 4 isolates as *Ilynectria radicicola* (GQ169022.1) (505 bp and 503/505 bp). The third sample showed 99% identity (492/494 bp) to *I. cyclaminicola* (MF440369.1), and the fourth isolate showed 100% identity (500 bp) to *Plectosphaerella cucumerina* (MT032517.1). Differences between sequences are outlined in Fig. S1.

Three of the fungal isolates we sequenced were from embryos that were grossly classified as Growth-Z. Of these, 2 were identified as an *Ilyonectria* spp., and the third as a *Plectosphaerella* sp. The fourth isolate was from an embryo that grossly appeared to be colonised by only Growth-W, and yielded an *Ilyonectria* sp.

The panfungal PCR detected an *Ilyonectria* sp. from an embryo colonised by Growth-W; however, the second sample, an embryo that was grossly colonised by Growth-X, failed to amplify due to inadequate DNA quantity. We were unable to establish cultures from embryos which grossly appeared to be colonised by Growth-X or -Y, and thus were unable to identify any fungi of these growth patterns.

4. DISCUSSION

Our investigation into embryonic mortality in the captive-bred *Pseudophryne corroboree* during the 2021 breeding season at Melbourne Zoo revealed a high prevalence of fungal infections and a range of anatomical deformities, with abnormal embryos experiencing 89.8% mortality. This suggests that the cause of the continuously high embryonic mortality experienced in the captive-bred *P. corroboree* is likely multifaceted.

A range of fungi have been associated with embryo mortalities in amphibians; however, many are saprophytes, and it is often unclear if infection occurred before or after embryonic mortality (Forester 1979, Villa 1979, Green 1999, Warkentin et al. 2001, Gomez-Mestre et al. 2006, Banning et al. 2008, Delia et al. 2020). Infertile or unviable embryos tend to be rapidly colonised by saprophytic fungi and can appear similar to embryos infected by pathogenic fungi (Green & Converse 2005). It is therefore essential that viable embryos are used to determine if fungi present are pathogenic or saprophytic.

We are the first to identify fungi of the genera *Ilyonectria* (of which some species are now classified as *Dactylonectria*) and *Plectosphaerella* in viable amphibian embryos. *Ilyonectria* is a soil-borne pathogen, which causes root rot in plants (Cabral et al. 2012, Farh et al. 2018) and has never been isolated from a vertebrate host before. *Plectosphaerella* is also a primary plant pathogen that affects agricultural crops across the globe (Palm et al. 1995, Carlucci et al. 2012). However, *Plectosphaerella* has been isolated from invertebrates (crayfish: Alderman & Polglase 1985; shrimp: Duc et al. 2009) and vertebrates (humans: Kamada et al. 2012; canines: Troy et al. 2013). There is one report of *Plectosphaerella* in amphibians, from a mycobiome assay of amphibian skin conducted in Ecuador, which revealed a 2% relative abundance of the fungus (Jervis et al. 2021). However, it is not possible to determine whether this detection was from infection or contamination (Jervis et al. 2021).

Vertebrate embryos were once thought to develop in a sterile, or near-sterile, environment (Escherich 1989), but with the development of culture-free, molecular-based approaches, there has been an increase in the understanding of the embryo-associated microbiome (Nyholm 2020, Kerney 2021). In addition to fungal infection, we also isolated bacteria of the genus *Herbaspirillum* and *Flavobacterium* from 15 embryos, including 2 healthy controls. Although bacteria were observed on histopathology they were not associated with pathology and their identity was not confirmed. Thus, we suspect these organisms are not significant pathogens. In a recent study, Bishop et al. (2021) isolated *Herbaspirillum* from the intracapsular fluid in wild embryos of spotted salamanders *Ambystoma maculatum*. *Herbaspirillum* spp. were also the most abundant bacteria in their samples, but effects on embryo health were not determined (Bishop et al. 2021).

In contrast to *Herbaspirillum*, *Flavobacterium* spp. have not been reported from amphibian embryos even though they are pathogens of adults (Densmore & Green 2007), and also cause severe disease and mortality in fish and their eggs (Bernardet & Bowman 2006). *Flavobacterium* spp. have been associated with antifungal activity *in vitro* (Lauer et al. 2008) so may contribute to the prevention of pathogen infections, explaining their presence in our healthy controls. Due to the limitations of our culture-based approaches (i.e. narrow range of media and conditions, focus on dominant colonies), our bacterial identification was not comprehensive. Culture-free molecular methods could improve the understanding of the microbiome in healthy and diseased embryos.

We detected a high rate of deformities, with 48 of the 108 embryos having some form of anatomical deformity, of which only 3 survived to hatching. The cause of deformities in these captive-bred *P. corroboree* embryos is unknown. Causes of deformities in wild amphibian populations include UV radiation, chemical contamination and parasitic infections (Blaustein & Johnson 2003). Other potential causes of deformities are genetic abnormalities (McFadden et al. 2011), which are particularly relevant in captive populations that are genetically closed (Williams et al. 2008), such as in *P. corroboree*. Where possible, future studies should investigate the genetic basis of malformations in embryos.

In diseased embryos, fungal infections are often considered secondary invasions, rather than the primary cause of mortality (Blaustein et al. 1994). It is possible that developmental errors may weaken the defence system of an embryo (e.g. jelly capsule) and allow microbial colonisation or that microbial colonisation may induce developmental errors by disrupting embryonic development. Another possibility is that underlying stressors or co-factors associated with captive conditions may increase infections and/or deformities. As we received no deformed embryos without fungal colonisation, the rate of deformities in uninfected embryos remains unknown in the captive population of *P. corroboree*.

Given that the breeding enclosures in which *P. corroboree* embryos are laid are bioactive, it is likely that fungal colonisation of the embryos occurred through contact with contaminated substrates. Hyphal penetration through the *P. corroboree* embryo capsules was observed on histopathology, and while it is unknown if the hyphae originated from the substrate or from an infected adjacent embryo, it confirms their ability to infiltrate the jelly capsule and colonise viable embryos. Hyphal penetration through amphibian jelly capsules has been observed in several other embryo-colonising fungi such as

Mucor spp. (Forester 1979), *Saprolegnia* spp. (Gomez-Mestre et al. 2006), *Mycelia sterilia* (Villa 1979) and *Mariannaea* spp. (Banning et al. 2008), and appears to be the most common route of fungal infection. However, the ability of fungal hyphae to penetrate through the jelly capsule varies with fungal and host species, with differences in pathogenicity and capsule composition likely responsible.

In some amphibian species, egg-attending behaviour decreases disease, through physical agitation (Forester 1979, Simon 1983), oophagy of abnormal eggs (Tilley 1972, Forester 1979) or by antimicrobial skin secretions (Villa 1979, Banning et al. 2008, Lauer et al. 2008). In the wild, *P. corroboree* males exhibit egg-attending behaviour, with the male remaining in the nest chamber for weeks to months post-breeding (Pengilley 1992, McFadden et al. 2013). However, in this captive colony, embryos are removed from the nest chambers and incubated separately so their development can be observed. There is no evidence that *P. corroboree* in the wild or captivity display oophagous behaviour, but it is plausible that the attending male retards fungal growth by other processes. However, a study investigating egg-attending behaviour in 3 wild *Pseudophryne* spp. (*P. bibroni*, *P. dendyi* and *P. semimarmorata*) found no evidence that the presence of the father impacted embryo mortality (Woodruff 1977). Therefore, the importance of attending behaviour in improving embryonic survival likely varies between species and their reproductive strategies.

Other captive breeding colonies of *P. corroboree* at Taronga Conservation Society and Healesville Sanctuary also experienced high levels of embryo mortality, 48 and 87%, respectively, over the 2021 breeding season (Taronga Conservation Society and Healesville Sanctuary unpublished reports). Preliminary observations from these colonies indicate that similar deformities and fungal infections are occurring, supporting the notion of multifaceted causes of mortality across these 3 captive collections.

As there is no longer a self-sustaining population of *P. corroboree* in the wild, we do not have current data on embryo survival and infections to compare with the captive breeding success. An investigation of embryo mortality in wild *P. corroboree* populations estimated 15% clutch mortality in non-drought years (Pengilley 1992), but the cause of mortality was not investigated.

Diseases of amphibian embryos are understudied, with even less work on the causes of their mortality. However, as more amphibians globally are at risk of extinction due to chytridiomycosis, climate change and habitat destruction, understanding the risks to embryo survival in wild and captive populations is imperative. For species dependent on captive breeding, understanding their reproductive behaviour and the factors that influence embryo health and survival is critical to optimizing conservation efforts.

This initial outbreak investigation on embryonic mortality in the Critically Endangered, captive-bred, southern corroboree frog shows that fungal infections and deformities are contributing to embryo mortality, but the cause of disease is likely multifaceted. Future work to understand the determinants of this mortality should include (1) determining the pathogenicity and source of the fungi isolated; (2) investigating the impacts of parental egg attendance on fungal infection and embryo survival; (3) characterising the anatomical deformities and their causes (e.g. genetic, environmental or pathogen initiated); (4) quantifying levels of infertility; and (5) investigating causes 1–4 listed above across all captive breeding colonies of this species. The results could inform experimental interventions to optimize husbandry, such as choice of substrate or genetic management. Understanding the impacts and mechanisms of fungal infection and deformities in this captive-bred Critically Endangered amphibian may improve embryonic survival in *P. corroboree* and other captive-bred amphibians.

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