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Title

Declining amphibians might be evolving increased reproductive effort in the face of devastating disease

Running title

Disease endemism changes reproduction

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Abstract

The devastating infectious disease chytridiomycosis has caused declines of amphibians across the globe, yet some populations are persisting and even recovering. One understudied effect of wildlife disease is changes in reproductive effort. Here we aimed to understand if disease has plastic effects on reproduction and if reproductive effort could evolve with disease endemism. We compared the effects of experimental pathogen exposure (trait plasticity) and population-level disease history (evolution in trait baseline) on reproductive effort using gametogenesis as a proxy in the declining and endangered frog *Litoria verreauxii alpina.* We found that unexposed males from disease-endemic populations had higher reproductive effort, which is consistent with an evolutionary response to chytridiomycosis. We also found evidence of trait plasticity, where males and females were affected differently by infection: pathogen exposed males had higher reproductive effort (larger testes), whereas females had reduced reproductive effort (smaller and fewer developed eggs) regardless of the population of origin. Infectious disease can cause plastic changes in reproductive effort at an individual level, and population-level disease exposure can result in changes to baseline reproductive effort; therefore, individual- and population-level effects of disease should be considered when designing management and conservation programs for threatened and declining species.

Key words.

Reproductive evolution; terminal investment; chytridiomycosis; reproduction; plasticity; compensatory recruitment; sex-specific stress

Introduction

Emerging infectious diseases are a major cause of catastrophic wildlife declines around the globe (Tompkins et al. 2015). Yet despite declines and extinctions in many species, some populations persist and recover following disease introductions. Changes in recruitment and reproductive effort in response to disease are an understudied but potentially important consequence of wildlife diseases that could contribute to population recovery. For example, disease-exposed populations of Tasmanian devils sexually mature earlier and have shifted from multiple breeding seasons of small litters over their lifetime, to a single large litter, when compared with disease-naïve populations (Jones et al. 2008; Lachish et al. 2009; Farquharson et al. 2018). Reproductive success in Tasmanian devils is associated with specific genes, some of which have high variability that might allow for population-level evolution of reproductive traits, although the frequency of these reproductive genes have not yet been analysed across populations (Russell et al. 2018; Brandies et al. 2020). Changes in reproduction and recruitment in Tasmanian devils demonstrates the possibility that evolution of reproductive effort can occur in response to disease endemism and might be an important effect of disease in other wildlife systems.

One of the most impactful diseases causing declines and extinctions in wildlife is chytridiomycosis (Skerratt et al. 2007; Scheele et al. 2019b). Chytridiomycosis is caused by the introduced fungal pathogen *Batrachochytrium dendrobatidis* and impacts amphibian species across the globe (Skerratt et al. 2007; Scheele et al. 2019b). Chytridiomycosis emerged globally several decades ago, and has become permanent and endemic in many regions. Yet even with endemic disease, adult mortality remains high in some species (Brannelly et al. 2020b). Endemic wildlife diseases can cause sublethal effects in individuals and at a population level; one unexpected effect is change in reproductive effort. Increased reproductive effort in response to a pathogen is termed "terminal investment". Terminal investment is a plastic response to disease, where an individual invests more in reproduction than in self-maintenance, such as immunity to fight the pathogen. In amphibians, there are several examples of terminal investment, where *B. dendrobatidis* infected

animals display increased reproductive effort. For example, pathogen-exposed males from three species invested more in sperm production, and pathogen-exposed females from one species increased their egg production (Chatfield et al. 2013; Brannelly et al. 2016b). Infection with *B. dendrobatidis* can also alter mating behavioural displays, with infected males increasing their calling effort (Roznik et al. 2015) or calling more energetically (An and Waldman 2016).

Wildlife diseases can also lead to population-level changes. Disease-endemic populations of the endangered alpine tree frog, *Litoria verreauxii alpina*, have a higher proportion of younger males actively breeding compared with disease-naïve populations (Scheele et al. 2017c). Shifts in population demographics and life-history are an important host response to multiple wildlife diseases (Jones et al. 2008); however, the mechanisms of earlier maturation in disease-endemic populations are poorly understood. Amphibians could be experiencing earlier maturation as 1) a heritable change in those populations, 2) a plastic response where pathogen exposure leads to changes in rate of maturation (Telfer et al. 2005; Tersago et al. 2012), or 3) a response to lower adult abundance where the breeding niche has opened and there is reduced intraspecific competition on younger individuals (Sato et al. 2014).

Several studies have demonstrated an effect of *B. dendrobatidis* infection on reproductive effort, recruitment, and demography; however, no studies have investigated whether changes in reproductive effort are an evolved response (Muths et al. 2011; Chatfield et al. 2013; Roznik et al. 2015; Brannelly et al. 2016b; Lampo et al. 2017; Scheele et al. 2017c; Campbell et al. 2019). It is well understood that for many amphibian species, recruitment can mitigate the effects of devastating disease (Muths et al. 2011; Newell et al. 2013; Scheele et al. 2015; West et al. 2020). There is also evidence that disease epidemics can impact genetic diversity and structure (Lachish et al. 2011; Robinson et al. 2012), which can lead to an evolutionary response. Disease-associated population-level changes or recovery are often attributed to evolved resistance or tolerance (Savage and Zamudio 2011; Robinson et al. 2012; Langwig et al. 2017). However, in cases where there is little

93 evidence of increased resistance or tolerance, reproductive changes associated with disease
94 endemism might be occurring. Research into reproductive evolution in response to disease is
95 important for informing management actions, especially for populations that continue to decline
96 (Brannelly et al. 2020a). Furthermore, understanding whether shifts in reproductive effort
97 correspond to trait plasticity and/or changes in trait baseline is important for predicting long-term
98 population dynamics associated with endemic disease and for informing management actions.

99 In this study we experimentally tested whether increased reproductive effort is a plastic
100 and/or an evolved response in a species that has dramatically declined due to chytridiomycosis. We
101 investigated reproductive investment in the endangered alpine tree frog, *Litoria verreauxii alpina*, in
102 males and females from four populations: three with endemic *B. dendrobatidis* infection (>20 years),
103 and population that was naïve to *B. dendrobatidis* (i.e., it had never experienced an epidemic). We
104 explored the plasticity of reproductive effort by comparing individuals that were exposed or not
105 exposed to the pathogen in a common garden setting. In addition, we looked for an evolutionary
106 response of changes in baseline reproductive effort by comparing individuals originating from
107 populations that either experienced endemic disease or were naïve to the pathogen. We note that
108 plasticity can be an evolved and heritable trait; however, testing for the evolution of plasticity of
109 reproductive response is outside the scope of this study. The samples used here were part of a larger
110 clinical infection experiment, exploring multiple facets of disease plasticity and evolution (Bataille et
111 al. 2015; Grogan et al. 2018a,b,c).

112 To quantify reproductive effort, we explored gonad characteristics and stages of
113 gametogenesis in males and females. We also compared gonad development across populations of
114 origin and experimental pathogen exposure to determine if exposure or population-level disease
115 history caused precocial maturity. We predicted that if reproductive effort and development are
116 plastic, we would see an effect of experimental exposure on reproductive traits. If reproductive
117 effort and development vary with population-level disease exposure, we predicted that the animals

from the disease-endemic populations would have higher baseline reproductive effort compared with the disease-naïve population. Changes in reproductive effort can occur at the individual- and at the population-level, such that these two hypotheses are not mutually exclusive.

Methods

Study species

The alpine tree frog (*Litoria verreauxii alpina*) is a species of conservation concern because this once widespread species experienced extreme decline due to disease in the Australian Alps beginning in the 1980s. It is currently listed as an endangered species in both New South Wales and Victoria, and high mortality due to *B. dendrobatidis* infection still occurs (Brannelly et al. 2015; Scheele et al. 2015). There is empirical evidence that recruitment, rather than environmental or immune factors, is most critical for population survival where the disease is endemic (Brannelly et al. 2020b).

Prior to the introduction of *B. dendrobatidis*, *L. v. alpina* males sexually matured by two years of age, and females by three years of age (Scheele et al. 2015, 2016). Demographic analysis after the arrival of *B. dendrobatidis* indicates that both males and females have the ability to sexually mature by their first year of age (Scheele et al. 2015, 2016). At the time of sample collection and fixation, animals in this experiment were between 9 and 11 months post-metamorphosis, which we assumed is similar to young-of-year in their first possible breeding season (see Text S1). These frogs were held in a stable captive environment, but because *L. v. alpina* is a temperate species that relies on seasonality to stimulate breeding, we expect that the results of this experiment reflect baseline breeding capacity, not breeding season optimum. Therefore, our study represents a conservative measure of reproductive effort and investment.

142 ***Populations of origin***

143 Experimental animals came from four populations in Kosciuszko National Park, New South
144 Wales, Australia. The populations are geographically distinct and have similar ecology, consisting of
145 open bog habitat, with an alpine stream running through the site and pools for breeding habitat. The
146 range in elevation for these four sites is 1307–1525m. Three of these populations – Eucumbene,
147 Kiandra, and Ogilvies – were disease-endemic, having become infected with *B. dendrobatidis* about
148 20 years prior to this study. The fourth population – Grey Mare – was *B. dendrobatidis* naïve during
149 this experiment; one of only two known disease-naïve populations of *L. v. alpina* at the time. Since
150 then, both naïve populations have experienced *B. dendrobatidis* introductions and near complete
151 population crashes (Howard and Clemann 2014; Grogan et al. 2018b; Banks et al. 2020). While the
152 use of one disease-naïve population is a small sample size, it was the only disease-naïve population
153 ecologically similar to the infected populations nearby. These four populations have been used in
154 population-level comparison studies for adaptive and evolved traits in *L. v. alpina* (Bataille et al.
155 2015; Brannelly et al. 2016a; Grogan et al. 2018a,b,c; Banks et al. 2020). A map and site description
156 are available in Brannelly et al. (2016*a*).

157

158 ***Experimental design***

159 We conducted a common garden experiment in which individuals from four populations
160 (one disease-naïve: Grey Mare; and three disease-endemic: Eucumbene, Kiandra and Ogilvies) were
161 collected as eggs from the wild, brought into captivity, and raised in a bio-secure, pathogen-free
162 environment (Bataille et al. 2015; Grogan et al. 2018a,b,c). At approximately eight months after
163 metamorphosis, we experimentally exposed the frogs to 750,000 *B. dendrobatidis* zoospores in
164 25mL of dilute salt solution for 18hrs, or mock exposed (exposed n=215, unexposed-controls n=39,
165 see Table S1). Sample size between exposed and unexposed-control groups are not even because

166 these animals were part of a larger clinical infection experiment (Bataille et al. 2015; Grogan et al.
167 2018a,b,c). We tested the animals for *B. dendrobatidis* infection every 14 days using quantitative
168 PCR until the end of the experiment (first signs of morbidity or day 86 after inoculation) (see Text S1
169 husbandry details).

170

171 ***Sample fixation and processing***

172 On the day that animals first showed clinical signs of infection (from day 19 onwards after
173 inoculation) or at the end of the experiment if they remained healthy (day 86 after inoculation), we
174 swabbed the animals for *B. dendrobatidis* infection, weighed them to the nearest 0.01g, humanely
175 euthanized them with buffered Tricaine Methanesulfonate (MS-222), and fixed them in neutral
176 buffered formalin. We used mass as a proxy for animal size (Text S1), and measured mass on the day
177 of experimental pathogen exposure and on the day of euthanasia for all individuals.

178 We dissected the formalin fixed animals and removed the left gonad. We conducted visual
179 gross examination blind to experimental pathogen exposure and population of origin. All males
180 dissected had well developed testes, which indicated sexual maturity. We assigned females to one of
181 three developmental categories based on gross examination of the ovaries: 1) small,
182 underdeveloped ovaries, 2) mid-sized almost developed ovaries with some developed eggs, and 3)
183 ovaries with developed eggs.

184 We processed a subset of those dissected individuals (16 unexposed females, 16 unexposed
185 males, 21 exposed females and 23 exposed males, see Table S1) for histological examination
186 following standard methods (Woods and Ellis 1994). We analysed the histological samples blinded to
187 experimental exposure and population of origin.

188

189 ***Gonad and gamete characteristics***

190 We assessed six histosections per male individual to determine testis area, number of
191 seminiferous tubules, density of seminiferous tubules, and germinal epithelium depth. We measured
192 the largest tubule size and identified spermatogenesis cell clusters to stage (spermatogonia,
193 spermatocytes, spermatids, spermatozoa) following Brannelly et al. (2016*c*), (see Text S1). For most
194 of the characteristics measured, larger depth, area, or higher count indicates higher reproductive
195 output. However, a lower density of seminiferous tubules and a lower proportion of spermatogonia
196 indicates higher reproductive output: lower tubule density per unit of testis area allows for more
197 spermatogenesis to occur due to increased intra-tubule space (i.e., more space inside the testis
198 devoted to spermatogenesis) and is therefore associated with higher reproductive effort when
199 spermatogenesis is active (Marion 1982). Because spermatogonia are an early stage of
200 spermatogenesis, a lower proportion of spermatogonia cell clusters (i.e., more cells in later stages of
201 development) are indicative of higher spermatogenesis effort (Delgado et al. 1992; Rastogi et al.
202 2005).

203 We assessed six histosections per female individual to determine ovary size, number of eggs,
204 size of the largest eggs, oviduct width, density of eggs within the ovary, and oogenesis stages (Stage I
205 – pre-vitellogenic; Stage II-V – vitellogenic; Stage VI – post-vitellogenic) (Text S1). Similar to the
206 interpretation of the male gonad/gamete characteristic analyses, for females, a lower density of
207 eggs per ovary correlates with larger average egg size, which indicates higher reproductive effort
208 because larger egg cells are associated with later oogenesis. A smaller proportion of pre-vitellogenic
209 eggs indicates an overall higher proportion of combined vitellogenic and post-vitellogenic eggs (i.e.,
210 eggs that have yolk development), which we interpreted as higher reproductive effort. In both males
211 and females, we analysed gonad size relative to body size, rather than unscaled gonad size.

212

213 ***Statistical analysis***

214 We modelled the proportional change in body mass from the beginning of the experiment to
215 the end of the experiment, and the mass of the animals at the end of the experiment using a linear
216 model (LM) where experimental pathogen exposure (unexposed-controls or exposed), sex of the
217 animal, population of origin (Kiandra, Eucumbene, Ogilvies [disease-endemic populations], and Grey
218 Mare [disease-naïve population]), and the interaction of experimental exposure and sex were fixed
219 effects. All analyses were conducted in R in the RStudio interface (RStudio Team 2016; R Core Team
220 2017), see Text S1 for package and citation details, and Text S1 and Tables S1-S3 for full model
221 details.

222 The effect of population of origin and experimental exposure on female reproductive status
223 was analyzed using a multinomial logistic regression (MR), where reproductive category (1:
224 underdeveloped eggs, 2: developing eggs, and 3: developed eggs) was the dependent variable, and
225 the fixed effects were experimental exposure, population of origin, and an interaction between
226 experimental exposure and population of origin.

227 For each gonad/gametogenesis measure we conducted a linear mixed effects model (LME)
228 for continuous dependent variables, generalized linear mixed effects model (GLME) with a beta
229 distribution for dependent variables that were proportions, and GLME with a Poisson distribution for
230 dependent variables that were count data. The fixed effects were experimental exposure,
231 population of origin, and their interaction. The random effect was individual. To assess the oogenesis
232 stages of the cells, we compared the proportion of total eggs counted within the three stages (pre-
233 vitellogenic; vitellogenic; post-vitellogenic) per individual using a general linear model (GLM) with a
234 beta distribution, where the fixed effects were experimental exposure, population of origin, and
235 their interaction. To understand the effect of population-level disease history without the direct
236 influence of infection we assessed the effect of population of origin with a separate model on a
237 subset of the data (only unexposed-control animals) in instances where there was a statistical trend

238 (p-value between 0.05 and 0.08) or statistical significance ($p<0.05$) for the effect of population of
239 origin on a trait (e.g., proportion of spermatogonia cell clusters) or an interaction between
240 population of origin and experimental exposure (e.g., tubule density) in the full model. In these
241 analyseses, population-level disease history (disease-naïve or disease-endemic) was the fixed effect
242 and individual was a random effect.

243

244 **Results**

245 ***Experimental pathogen exposure***

246 All *B. dendrobatidis* exposed animals (n=215) developed severe chytridiomycosis. Pathogen-
247 exposed animals survived on average 29.4 days (range 19-63 days) after inoculation, and infection
248 load at morbidity was 5.58±0.89 $\log_{10}$ zoospore equivalents (mean ± standard deviation). There was
249 an effect of population of origin on survival and infection intensity, which has been explored in detail
250 in other published work, where animals from the disease-endemic population, Kiandra, had higher
251 survival and lower infection loads compared to the other populations of origin (Bataille et al. 2015).
252 All unexposed-control animals (n=39) for this study remained *B. dendrobatidis* negative and healthy
253 to the end of the experiment (day 86).

254 Pathogen-exposed animals lost mass over the experimental timeframe, while the
255 unexposed-control animals gained mass (Fig 1a). There was a significant effect of experimental
256 exposure on proportional change in mass where pathogen-exposed animals lost 7.9±13.9% body
257 mass over the course of the experiment (average of 29.4 days after experimental exposure), while
258 unexposed-control animals gained 16.3±18.5% in body mass (84 days after experimental exposure)
259 (LM: experimental exposure, $F_{1, 243}=93.802$, $p<0.001$; Table S3).

260 At the end of the experiment, pathogen-exposed animals were smaller than unexposed-
261 control animals, and females were heavier than males (Fig 1b). Exposed females were 17.7% smaller

than unexposed-control females (exposed: 3.81±1.11g, unexposed: 4.63±1.26g; *d*=0.488); and exposed males were 16.5% smaller than unexposed-control males (exposed: 2.84±0.79g, unexposed: 3.40±0.73g; *d*=0.521). Overall, individuals from the disease-naïve Grey Mare population were larger than individuals from the other populations (Fig S1) (LM: sex, $F_{1, 243}$=80.231, p<0.001; experimental exposure, $F_{1, 243}$=18.402, p<0.001; population of origin, $F_{3, 243}$=11.651, p<0.001; Table S3).

Male gonad characteristics

There was an effect of both experimental pathogen exposure, and its interaction with population of origin on seminiferous tubule density. We found that unexposed-control males from the disease-naïve population had significantly higher tubule density than other experimental groups: 40.8% higher density than the unexposed-control animals from disease-endemic populations, and 65.9% higher density than all pathogen exposed animals (LME: experimental exposure, χ^2_1=14.236, p<0.001; experimental exposure*population of origin, χ^2_3=9.828, p=0.020; Table S3; Fig 2a&3).

Because we observed a significant interaction between experimental exposure and population of origin in seminiferous tubule density, we explored the effect of population-level disease history (disease-endemic or disease naïve) on the tubule density of male gonads in the unexposed-control animals only. We found that indeed, unexposed males from disease-endemic populations had significantly lower tubule density than the unexposed males from the disease-naïve population (disease-naïve: 346.0±134.7 tubules/mm^2; disease-endemic: 204.8±89.7 tubules/mm^2; *d*=0.87), indicating higher baseline reproductive effort in unexposed-control males from disease-endemic populations (LME: χ^2_1=4.820, p=0.028; Table S3, Fig 2a). Pathogen-exposed animals did not vary in seminiferous tubule density across populations of origin (Fig 2a; see Text S1).

We found that population of origin had a marginal effect on the proportion of spermatogonia (GLME: Population of origin, χ^2_3=7.515, p=0.057; Table S3, Fig S2). Therefore, we explored the effect of population-level disease history the proportion of spermatogonia in the unexposed-control animals. Unexposed-control males from disease-endemic populations had a 45.2% lower proportion of spermatogonia (disease-endemic populations: 23.0±8.1% spermatogonia; disease-naïve population: 33.4±8.5% spermatogonia; *d*=0.89; Fig 2b), indicating higher baseline reproductive effort (GLME: χ^2_1=8.850, p=0.003; Table S3).

The number of seminiferous tubules within the testis was associated with population of origin (Fig 2c), where individuals from one disease-endemic population had fewer tubules within the testis in total than those from the disease-naïve population. Individuals from Eucumbene, a disease-endemic population, had the fewest number of tubules within the testis (GLME: population of origin, χ^2_3=19.600, p<0.001; Tukey's post hoc: Eucumbene–Kiandra, p=0.001; Eucumbene–Grey Mare, p=0.002; Kiandra–Ogilvies, p=0.067; all other comparisons p>0.103; Table S3). In unexposed animals alone, population-level disease history was not associated with the total number of seminiferous tubules counted (GLME: χ^2_1=1.427, p=0.232; Table S3).

Experimental pathogen exposure affected testis size relative to body size. Exposed animals had 119.4% larger testis per body mass than unexposed-control animals (experimentally exposed: 0.32±0.14 testis size mm^2/animal mass g; unexposed: 0.14±0.09 mm^2/g; *d*=1.04, Fig 4). There was no effect of population of origin nor an interactive effect with experimental exposure on testis size relative to body mass (LME: experimental exposure, χ^2_1=20.531, p<0.001; Table S2, S3).

The gonad tissues in all samples looked healthy and normal; there were no obvious changes consistent with oedema (Fig 3). We found that regardless of population of origin or experimental pathogen exposure, individuals were producing an equivalent number of sperm per unit area of seminiferous tubules (see Text S1). There was no effect of experimental exposure or population of

origin on germinal epithelium depth, the size of the largest seminiferous tubule, or the proportion of spermatozoa, spermatids, or spermatocytes (see Table S2 and S3 for model results and summary).

Female gonad characteristics

While there was no effect of population of origin on female gonad/gamete characteristics, experimental pathogen exposure affected reproductive effort such that exposed females had reduced gametogenesis (Figs 5&6). Experimental exposure affected the proportion of eggs per oogenesis stage (Fig. 5b), where exposed animals had a smaller proportion of eggs in vitellogenesis (oogenesis stages II-V) than unexposed-control animals (exposed: 22.0%, unexposed-control: 33.0%; odds ratio, 0.57; GLM: experimental exposure, χ^2_1=6.809, p=0.009; Table S3). Exposed females had 97.0% more eggs per ovary area, indicating smaller average egg cells (exposed, eggs per mm^2: 19.7±17.2; unexposed-control, eggs per mm^2: 10.0±10.4; *d*=0.48; LME: experimental exposure, χ^2_1=4.691, p=0.030; Table S3; Fig 6a). Among the largest egg cells within the ovaries, exposed animals had 24.7% smaller eggs compared with unexposed-control animals (exposed egg width: 0.65±0.35mm; unexposed egg width: 0.86±0.309mm; *d*=0.45; LME: experimental exposure, χ^2_1=4.444, p=0.035; Table S3; Fig 6b).

There was no effect of experimental exposure, population of origin, or their interaction on number of eggs present within the ovary histosections, area of the ovary relative to body size, or on oviduct size (see Table S2 and S3 for model results and summary).

Maturity

Male frogs (n=125) from all populations of origin were morphologically sexually mature. Female morphological sexual maturity (n=139) was variable, where pathogen-exposed animals were

less likely to be sexually mature than unexposed-control animals (Fig 5a). Exposed females were half as likely (odds ratio, 0.53) to have fully developed eggs in the ovaries (31.8% of 110 experimentally exposed females; 60.0% of 20 unexposed-control females), 2.54 times more likely to have developing eggs in the ovaries (25.5% of experimentally exposed females; 10.0% of unexposed-control females), and 1.71 times more likely to have underdeveloped ovaries (42.7% experimentally exposed females; 25.0% of unexposed-control females) (MR: experimental exposure, χ^2_2=7.157, p=0.028; Table S3; Fig 5a).

Discussion

Understanding how infection can affect individuals and populations is important for identifying the impacts of wildlife disease and developing procedures to protect endangered and declining species (Brannelly et al. 2020a). However, the direct impacts of infection on reproduction at both the individual- and population-level in declining amphibian species are still understudied. In this study we tested whether gamete production, gonad characteristics, and maturation rate were directly influenced by *B. dendrobatidis* infection (i.e., trait plasticity in response to experimental pathogen exposure), and if these traits varied among populations with different pathogen exposure histories (i.e., possible evolutionary changes in baseline effort with disease endemism). To date, there are very few examples of trait evolution after chytridiomycosis enters a population (but see Voyles et al. 2018), and even fewer that are not related to immune function. Testing for population-level shifts in specific traits is challenging because there are few *B. dendrobatidis* naïve populations left to compare to disease-exposed populations across the globe (Scheele et al. 2019a; Brannelly et al. 2020a). Using the *L. v. alpina* system, we had the unique opportunity to assess gametogenesis in an endangered frog where recruitment is important for population persistence following disease introduction. Through this research we found multiple lines of evidence that support changes in reproductive effort in response to disease. We found support for both trait plasticity in individual

males and females, as well as population-level changes in baseline male reproductive effort that are consistent with trait evolution after disease endemism.

Population-level adaptation

Previous research has explored the plastic effects of disease on reproductive traits (Chatfield et al. 2013; Roznik et al. 2015; Brannelly et al. 2016b), but has not explicitly tested whether disease endemism could result in evolution of reproductive effort. We found that in disease-endemic populations, unexposed-control males had lower seminiferous tubule density and a lower proportion of spermatogonia in the testes, which is indicative of increased baseline reproductive effort. This higher reproductive effort in disease-endemic populations is consistent with the evolution of reproductive effort following disease endemism, but we cannot exclude other causes such as parental effects.

Interestingly, the population-level variation in gamete production disappeared when the animals were experimentally exposed to the pathogen. Population-level change in gamete production might have a relatively lower total impact on recruitment compared with the direct impact of infection, which has been repeatedly tested and the results were similar (Chatfield et al. 2013; Brannelly et al. 2016b). Our study design on wild-caught animals limits our ability to conclude that the changes in reproductive effort observed across populations are due to an evolved response, or other causes such as parental effects and epigenetics. Regardless, this study presents valuable data on threatened natural populations of amphibians, and the observed reproductive changes in the disease-endemic populations highlight that reproductive effort is important for this species and warrants future research.

Development of *B. dendrobatidis* endemism has had a short evolutionary timescale since pathogen emergence at these sites approximately 20 years prior to the time of the experiment

(Scheele et al. 2017b), yet we still see an observable difference in male reproductive effort among populations. We expect that if recruitment remains an important factor in population persistence, over time there will be more observable differences among populations with varying disease-exposure histories. Our experimental results indicate that impacts of disease on reproduction might be more important than previously thought. Future work on this species should focus on verifying whether disease-endemism can cause evolution of increased reproduction. Possible methods to test for evolution of reproduction include genetic techniques (such as exploring whether genes associated with gametogenesis are under selection across populations), establishing that increased fitness is associated with reproductive changes in a disease context, and minimizing the impacts of parental effects and epigenetics following several generations of captive rearing animals for additional common garden experiments. When working with endangered species, small samples sizes are a reality. While we only tested one *B. dendrobatidis* naïve population, similar studies demonstrate that variation among populations is consistent with evolution of a phenotype (Grogan et al. 2018a).

Disease-related reproductive plasticity

We found that *B. dendrobatidis* infection causes a plastic response of reproductive effort in both male and female frogs. We found that exposed males had over two-fold larger testes relative to body size than unexposed-control males across populations, which is a similar result to previous studies in *L. v. alpina* and other species of frogs (Chatfield et al. 2013; Brannelly et al. 2016b). These findings support the idea of terminal investment in males. In this study, we found that pathogen-exposed males lost mass during the experiment and were smaller than the unexposed-controls, yet still increased their reproductive effort in gametogenesis; therefore, sperm production might not be associated with size or body condition in *L. v. alpina*. Increased sperm production can greatly increase reproductive potential (Gage et al. 1995; Vellnow et al. 2018), where total testis size and

testis size relative to body size is positively correlated with number of offspring (Trivers 1972; Gage et al. 1995; Byrne et al. 2002; Macias-Garcia and Saborio 2009). In our experiment we found that both testis size and testis size relative to body mass were higher in the exposed males, indicating increased sperm production in experimentally exposed animals.

Female frogs in this study did not show terminal investment. We found that experimentally exposed females exhibited traits that were consistent with a negative effect on reproduction, including smaller egg cell size of the largest eggs in the ovaries, fewer vitellogenic cells within the ovaries, and a higher density of eggs (more eggs per unit of ovary area). Sexual development and egg production is often correlated with female body size as they are associated with energy availability (Trivers 1972; Berven 1990; Prado and Haddad 2005; Cabrera-Guzmán et al. 2013). While our experimentally exposed females had smaller, less developed eggs in their ovaries, we found that experimentally exposed and unexposed animals had equivalent total numbers of eggs in the ovaries and equivalent ovary size relative to body size. Ovary size appeared to scale with body size, because experimentally exposed females were smaller than unexposed-control females and lost mass over the course of the experiment. The effect of *B. dendrobatidis* infection on female size is a likely explanation for their reduced reproductive activity (e.g., fewer developed eggs), especially because the females in this experiment were young, and it is uncommon for them to breed in their first year (Scheele et al. 2016).

Our results for female oogenesis differ from a previous experiment with *L. v. alpina* (Brannelly et al. 2016b). In the previous study, *B. dendrobatidis* exposed females had much larger ovaries, oviducts, and produced more developed eggs than unexposed-control females (Brannelly et al. 2016b). One explanation for these opposing results is differences in female age. In the present study, females were under one year of age, whereas in the previous study, females were at least three years old when the clinical infection experiment began. Females often take longer to sexually mature than males, and a wide range of female maturity is expected in young animals (Rastogi et al.

1983; Morrison and Hero 2003; Brannelly et al. 2019), which is consistent with what we found through our assessment of maturity. As predicted by the terminal investment hypothesis, young animals might prioritize self-maintenance rather than reproductive effort or sexual development (Velando et al. 2006; Duffield et al. 2018). Young animals prioritizing self-maintenance following pathogen exposure has been suggested before, where young infected *Litoria aurea* males (<1 year old) had smaller testes when recovering from infection, but equivalent body size compared to non-recovering individuals (Campbell et al. 2019).

It is important to note that this species experiences very high mortality due to chytridiomycosis in the wild, with the adult breeding cohort experiencing near complete population turnover every year (Brannelly et al. 2015; Scheele et al. 2015). Terminal investment leading to increased reproductive output might be a viable strategy for male animals, but harmful for young females that do not increase their reproductive effort. The sex differences in the plastic effects of disease on reproduction are likely due to differences between male and female reproductive developmental patterns. Females often take longer to sexually mature, and sexual maturity is often a function of body size in frogs (Rastogi et al. 1983; Berven 1990). It is possible that these young females are not prioritizing self-maintenance, but rather are energetically constrained and physically cannot increase their reproductive effort when infected. However, in the wild, female *L. v. alpina* typically enter the breeding season at 2-4 years of age (Scheele et al. 2017c), which was the age range tested in Brannelly *et al.* (2016*c*). Most female *L. v. alpina* do not breed in their first year; therefore, the impacts of pathogen exposure on female gamete development and production might not be relevant to breeding in wild populations. However, these results highlight the sublethal effects of *B. dendrobatidis* infection and the differences between male and female responses to infection. It would be prudent to investigate the effect of disease and population of origin on females of varying age to ensure that the effects seen here and in Brannelly *et al.* (2016*c*) are consistent with age-dependent responses to pathogen exposure. Such an approach could be

456 adopted in field studies using skeletochronology to determine age (Scheele et al. 2017c; Brannelly et
457 al. 2018), and ultrasound to estimate if females are gravid (Graham et al. 2018; Calatayud et al.
458 2019).

459

460 ***Male maturation rate***

461 Rate of maturation in males was consistent, and all males in this study were sexually mature,
462 confirmed by active spermatogenesis during histological examination. Our results indicate that
463 regardless of population of origin or experimental pathogen exposure, males were sexually mature
464 for their first breeding season when raised in captivity. While rates of maturity in the wild might
465 differ, with either increasing or decreasing maturation possible (O'Regan and Kitchener 2005; Antor
466 et al. 2007; Ritz et al. 2010; Zupa et al. 2017), we assume that this timeframe for sexual maturity
467 after metamorphosis would be similar to these individuals breeding as young-of-year in their first
468 possible breeding season.

469 Our results have implications for interpreting previous field observations of maturation
470 rates. In wild populations, a higher proportion of young male and female *L. v. alpina* are commonly
471 found among breeding cohorts in disease-endemic populations (Scheele et al. 2017c), which is a
472 clear shift in population age structure after disease endemism. However, this shift in demographics
473 might not be due to early maturation. Instead, a younger breeding cohort might be due to high adult
474 mortality, resulting in a lower proportion of animals in their second or third breeding season. The
475 results of our study demonstrate that both males and females are physically capable of sexual
476 maturation at less than one year of age, and maturation rate is unrelated to population-level disease
477 history of the source population. We suggest that the shift in age structure and higher proportion of
478 younger males (1 yr) and females (2 yrs) breeding at disease-endemic populations is a response to
479 lower abundance of older individuals: where the breeding niche has opened and reduced the

intraspecific competition on younger individuals (Sato et al. 2014). A younger cohort of breeding adults is an important shift in demographics, but in the case of *L. v. alpina*, we suggest that it is not likely due to an evolutionary change at the population-level, unlike what we observed in sperm production in this study.

Management implications

Populations that rely on increased recruitment in response to disease are still susceptible to other threats such as climate change and habitat destruction. In *L. v. alpina*, distribution and abundance declined dramatically from short hydroperiod ephemeral water bodies with the introduction of *B. dendrobatidis*, where recruitment was likely to fail due to drought (Scheele et al. 2017a). The remaining populations are surviving only at the few permanent to semi-permanent water bodies in the alpine region. However, with a heavy reliance on recruitment for population persistence, a whole population could be lost with one or two successive years of failed recruitment. Tadpole death, and thus failed recruitment, can be caused by natural disasters such as the recent devastating fires and extreme droughts in Australia (Ward et al. 2020; Wintle et al. 2020). These natural disasters are anticipated to become more frequent with climate change (Williams et al. 2001). Protection of breeding at permanent water bodies is key to the survival of *L. v. alpina* (Scheele et al. 2015, 2017c; Brannelly et al. 2016b), and has become especially important now because most of the remnant populations' habitat was burned in the 2019/2020 bush fires.

Confirming an evolutionary response to disease is important for identifying management strategies. For example, interventions that involve captive breeding or translocations should include consideration of possible changes in reproduction when choosing individuals. If populations are evolving with disease endemism it would be important to include wild type genotypes that have undergone selection rather than maintaining the genetic diversity of the founder animals, which is

the current standard practice in captive management. However, there are also likely fitness trade-offs with the evolution of reproductive effort and immunity; high reproductive effort might lead to higher susceptibility to disease (Nordling et al. 1998; Knowles et al. 2009). If conservation interventions are needed, then understanding and mitigating these tradeoffs are important and require thoughtful consideration. Our study highlights the importance of understanding the impact of disease from an ecological and evolutionary perspective. We can use this knowledge to develop conservation strategies and techniques that will align with successful evolutionary trajectories of species and therefore be more sustainable.

Author contributions

LAB, LFS, LB and LFG designed the experiment. LAB, RJW, ZJ and LFG collected the data and analysed the samples. LAB analysed the results and wrote the manuscript. All authors contributed to editing the manuscript and approve the submission.

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Data accessibility

All data is accessible though Dryad. Brannelly et al. (2021), Male reproductive effort might be evolving in the face of devastating disease in a threatened amphibian, Dryad, Dataset, https://doi.org/10.5061/dryad.f1vhhmgxf

Animal ethics statement

This study was conducted with approval by the James Cook University Animal Ethics Committee (A1589) and Scientific License number: S12848.

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Figure legends

Figure 1. Proportional change in mass (a) from inoculation (the start of the experiment) to the end of the experiment, calculated as [(mass at experiment end – mass at inoculation)/mass at inoculation]. Each point represents an individual animal (males and females combined). The grey line represents the mean proportional change for each group (experimentally exposed to *B. dendrobatidis* or unexposed-control animals). (b) Individual mass at the end of the experiment. The middle lines in the box plots represent the median, and box hinges represent the first and third quartile, the whiskers represent 1.5x the interquartile range, and dots represent outlying data points.

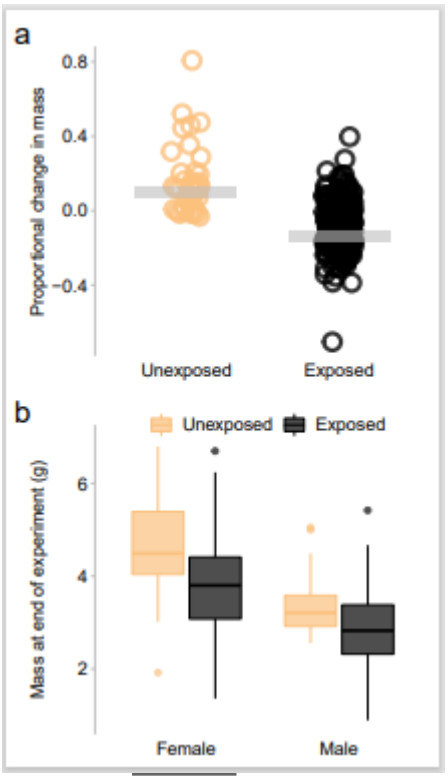
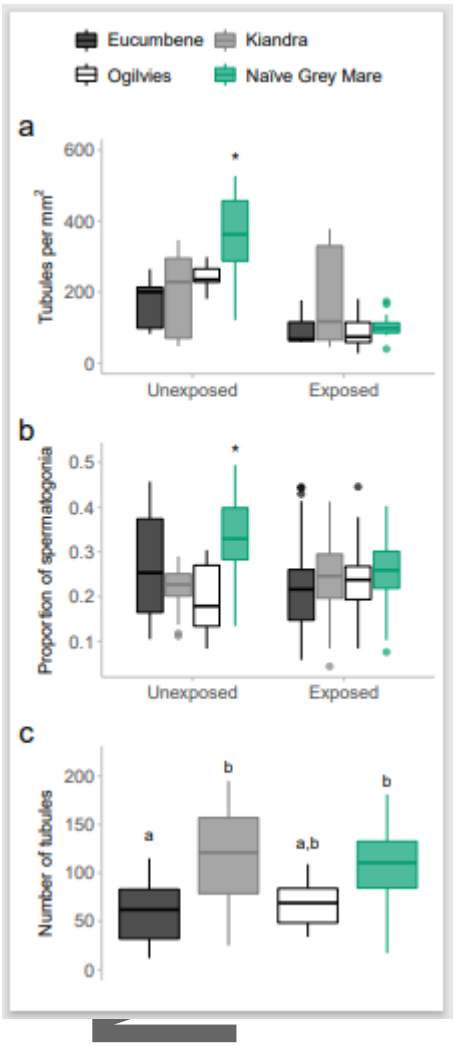


Figure 2. Male testis characteristics affected by population of origin. a) The density of seminiferous tubules per testis area across population and experimental exposure to the pathogen *B. dendrobatidis*. b) The proportion of spermatogonia cells across population of origin and experimental exposure to the pathogen *B. dendrobatidis*, where the proportion of spermatogonia cells is influenced by population-level disease history for unexposed-control animals only. c) The effect of population on number of seminiferous tubules per whole testis histosection, represented as experimentally exposed and unexposed-control animals combined because experimental exposure did not statistically influence the number of tubules per histosection. The middle lines in the box plots represent the median, and box hinges represent the first and third quartile, the whiskers represent 1.5x the interquartile range, and dots represent outlying data points. For panels (a) and (b) the (*) indicates statistical significance.



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770 ***Figure 3***. An example of a histosection view of an unexposed-control male testis (a) and a *B.*

771 *dendrobatidis* exposed male (b). Both photographs were taken at 40x magnification, and the scale

772 bar represents 250µm for both panels. Notice visually that the experimentally exposed male (b) has

773 a larger testis area, and many large tubules. Both histosections demonstrate active spermatogenesis,

774 with all spermatogenesis stages present.

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Figure 4. Testis area per histosection, measured in mm^2 among males that were experimentally exposed to *B. dendrobatidis* and unexposed-control males. The middle lines in the box plots represent the median, and box hinges represent the first and third quartile, the whiskers represent 1.5x the interquartile range, and dots represent outlying data points.

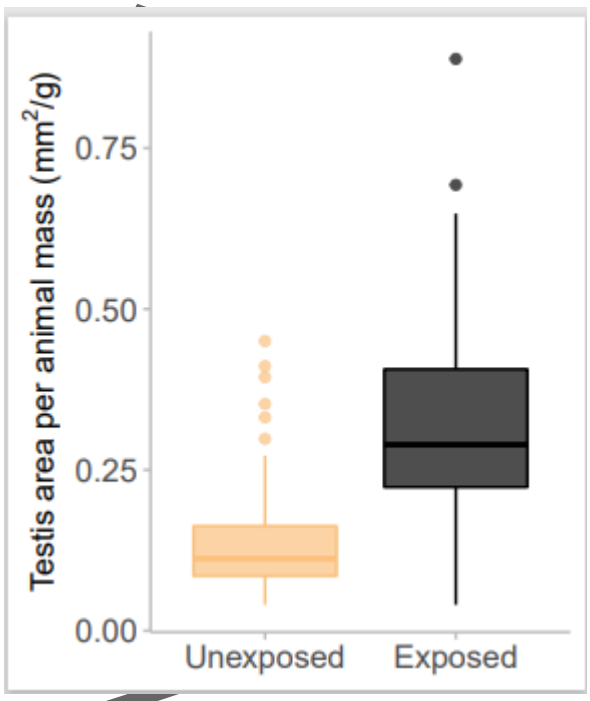
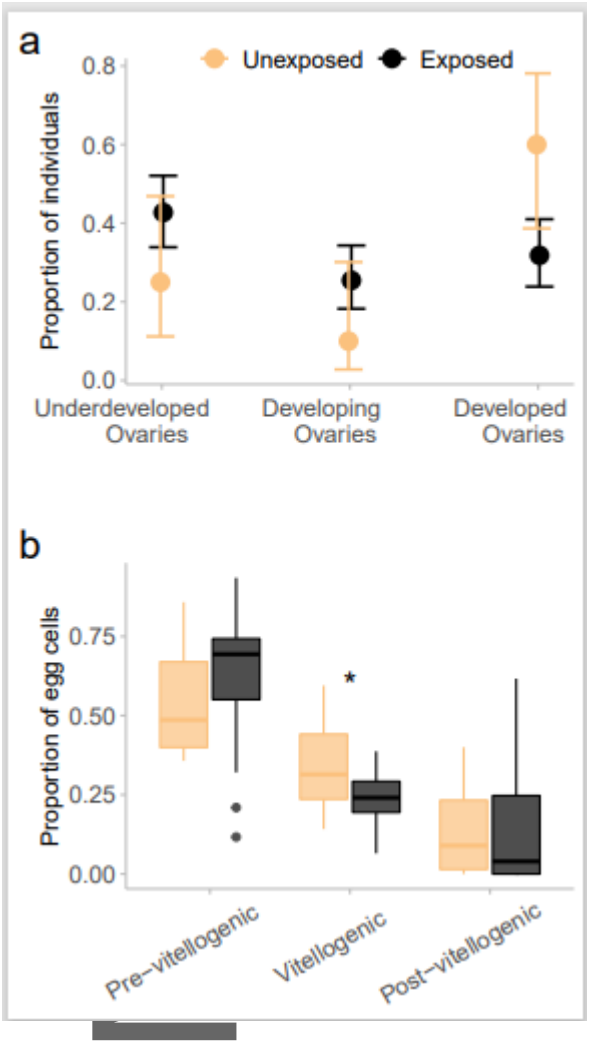


Figure 5. Female maturity and development. a) Proportion of animals based on their stage of sexual maturity. Stages of maturity as determined through gross examination of the ovaries and oviducts in all dissected females that were experimentally exposed to *B. dendrobatidis* (n=110) and unexposed-controls (n=20). Error bars are 95% confidence intervals of a proportion. b) Stages of oogenesis for unexposed-control females (n=16) and exposed females (n=21). Egg cells counted are represented as a proportion calculated per individual. The (*) represents a statistical difference. The middle lines in the box plots represent the median, and box hinges represent the first and third quartile, the whiskers represent 1.5x the interquartile range, and dots represent outlying data points.



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795 ***Figure 6.*** Ovary characteristics that were significantly different between females experimentally

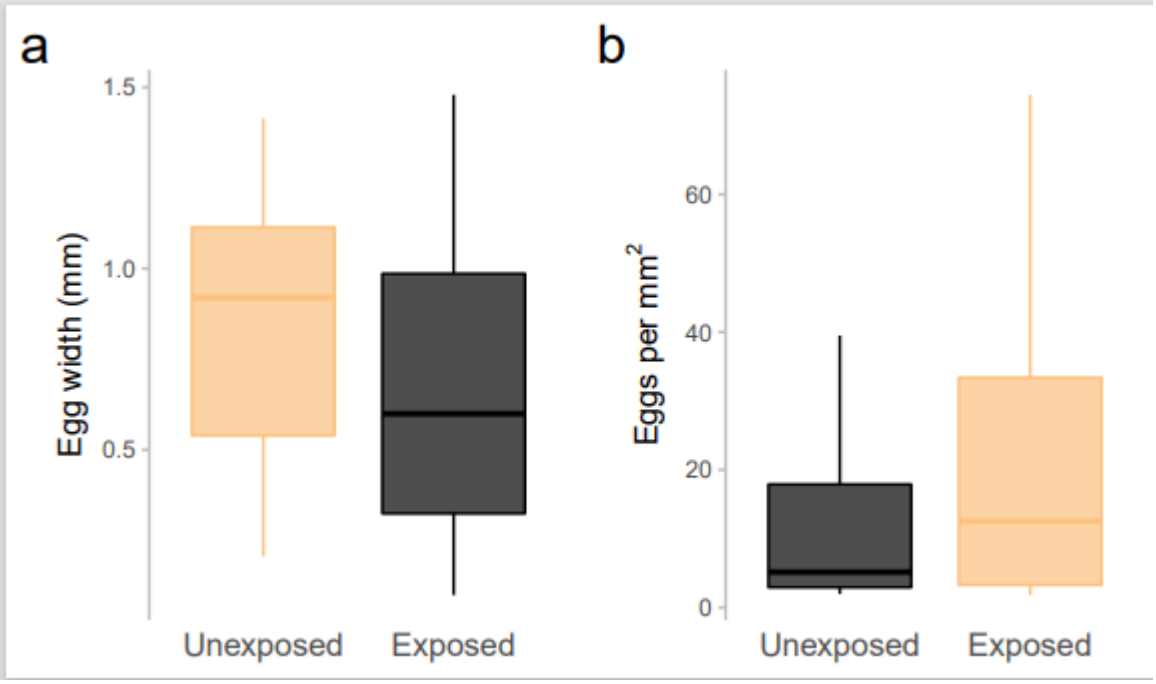
796 exposed to *B. dendrobatidis* and unexposed-controls. a) Egg width is the width (mm) of the five

797 largest egg cells per histosection. b) Egg cells per ovary area is the number of egg cells per

798 histosection divided by the ovary area (mm^2). The middle lines in the box plots represent the

799 median, and box hinges represent the first and third quartile, the whiskers represent 1.5x the

800 interquartile range, and dots represent outlying data points.



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