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The effects of inbreeding on disease susceptibility: *Gyrodactylus turnbulli* infection of guppies, *Poecilia reticulata*

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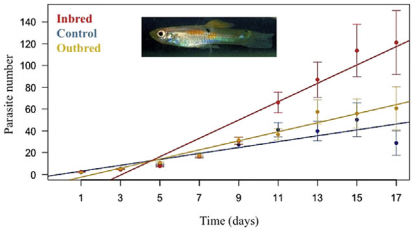
HIGHLIGHTS

- Inbreeding effects on resistance were tested using the *Gyrodactylus*-guppy model.
- Inbred individuals had higher parasite intensity compared to outbred fish.
- The most inbred individuals were significantly less able to clear the infection.
- Parasite infections may raise the extinction risk of inbred populations.

GRAPHICAL ABSTRACT



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ABSTRACT

Inbreeding can threaten population persistence by reducing disease resistance through the accelerated loss of gene diversity (i.e. heterozygosity). Such inbreeding depression can affect many different fitness-related traits, including survival, reproductive success, and parasite susceptibility. Empirically quantifying the effects of inbreeding on parasite resistance is therefore important for *ex-situ* conservation of vertebrates. The present study evaluates the disease susceptibility of individuals bred under three different breeding regimes (inbred, crossed with full siblings; control, randomly crossed mating; and fully outbred). Specifically, we examined the relationship between inbreeding coefficient (F-coefficient) and susceptibility to *Gyrodactylus turnbulli* infection in a live bearing vertebrate, the guppy *Poecilia reticulata*. Host-breeding regime significantly affected the trajectories of parasite population growth on individual fish. Inbred fish showed significantly higher mean parasite intensity than fish from the control and outbred breeding regimes, and in addition, inbred fish were slower in purging their gyrodactylid infections. We discuss the role of inbreeding on the various arms of the immune system, and argue that the increased disease susceptibility of inbred individuals could contribute to the extinction vortex. This is one of the first studies to quantify the effects of inbreeding and breeding regime on disease susceptibility in a captive bred vertebrate of wild origin, and it highlights the risks faced by small (captive-bred) populations when exposed to their native parasites.

1. Introduction

Small and isolated populations are particularly vulnerable to environmental and demographic stochasticity, which can result in the loss of genetic variation due to random genetic drift (Keller and Waller, 2002). Both drift and inbreeding tend to accelerate the loss

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of gene diversity (i.e. heterozygosity), and this can result in individuals being less resistant to environmental change and increase mortality (e.g. Fox and Reed, 2010; Bijlsma and Loeschcke, 2011). Inbreeding depression can affect many different fitness-related traits (Saccheri et al., 1998; van Oosterhout et al., 2000a; Keller and Waller, 2002), including survival (Coltman et al., 1998), reproductive success (Spielman et al., 2004), sexual ornamentation and courtship behaviour (van Oosterhout et al., 2003), and parasite susceptibility (MacDougall-Shackleton et al., 2005; Rijks et al., 2008). Inbred individuals tend to have higher pathogen susceptibility compared to outbred counterparts (e.g. Coltman et al., 1999; Hedrick et al., 2001). Genetic diversity has been negatively associated with susceptibility to parasitism in many animals, including insects (e.g. Whitehorn et al., 2011), birds (e.g. MacDougall-Shackleton et al., 2005; Ortego et al., 2007), mammals (e.g. Roelke et al., 1993; Rijks et al., 2008) and fish (e.g. Arkush et al., 2002; Consuegra and de Leaniz, 2008; Ellison et al., 2011; Eszterbauer et al., 2015).

Understanding the effects of inbreeding on fitness traits in fish is important because of their economic value and the constraints imposed on aquaculture by limited brood stock, high stocking densities and infectious disease. Reduced sexual activity has been reported in several inbred fish species (Farr and Peters, 1984; van Oosterhout et al., 2003; Mariette et al., 2006; Frommen et al., 2008). Even one generation of full sibling inbreeding can lead to a reduction in male sexual motivation and mating success (Mariette et al., 2006; Frommen et al., 2008), but the effects of inbreeding are significantly more pronounced after multiple generations possibly due to epistatic interactions (van Oosterhout et al., 2003). Several inbreeding avoidance mechanisms have evolved in vertebrates in nature, including disassortative mating based on MHC dissimilarity, which is thought to increase offspring resistance to parasitism (Penn et al., 2002; Rauch et al., 2006; Forsberg et al., 2007; Consuegra and de Leaniz, 2008; Evans and Neff, 2009).

It is important to understand the impact of *ex-situ* breeding of wild populations on an individual's ability to fight off pathogenic infection, which may affect their reintroduction success and is important for conservation (see van Oosterhout et al., 2007a; IUCN/SSC, 2013). Inbreeding is a key consideration in conservation genetics; reducing the effective population size will accelerate the rate of inbreeding and disease susceptibility. Most natural vertebrate populations have had long-term exposure to host-pathogen interactions (May, 1988). Resistance traits in the wild are costly and captive bred animals are likely to lose resistance in the absence of pathogen infection and parasitism, due to a lack of acquired immunity from previous pathogen exposure and stress-induced immunosuppression (Altizer et al., 2003; Mathews et al., 2006). Reintroduction programmes may lead to increased pathological effects (Viggers et al., 1993), particularly when susceptible captive bred individuals are released in genetic supplementation programmes (Faria et al., 2010). For example, parasitic infection in reintroduced naïve fish (*Poecilia reticulata*) reached similarly high levels to those reported on experimentally infected naïve laboratory individuals when released into mesocosms alongside native fish (van Oosterhout et al., 2007a). Reintroduced inbred individuals had a lower survival rate from parasitic infection than their outbred counterparts (van Oosterhout et al., 2007a).

This study examines the relationship between the level of inbreeding and susceptibility to *Gyrodactylus turnbulli* infection in the live bearing guppy under controlled laboratory conditions. Guppies are introduced widely for mosquito control, the success of which depends on their ability to survive under natural conditions, including exposure to parasites (Elias et al., 1995; Cavalcanti et al., 2007). Examining the susceptibility of inbred individuals to parasitism in a controlled environment has been scarcely studied (but see Hedrick et al., 2001; Spielman et al., 2004). Infection with gyrodactylids can have significant fitness consequences in both wild and captive fish populations, including marked effects on host behaviour, including courtship and feeding (Kennedy et al., 1987; van Oosterhout et al., 2003; Bakke et al., 2007; van Oosterhout et al., 2007b) and survival of fish stocks (Cable et al., 2000). This study aims to determine whether inbreeding reduces the immunocompetence of guppies and increases their susceptibility to gyrodactylid infection. We analyse the parasite trajectories on individual fish from three different breeding regimes (i.e. the inbred, the control and the outbred regime) and quantify (i) parasite intensity; (ii) parasite persistence; and (iii) maximum parasite numbers.

2. Material and methods

2.1. Study system

Gyrodactylus turnbulli is a highly contagious, monogenean ectoparasite with a direct transmission pathway (Cable, 2011) and "Russian doll" reproduction (Bakke et al., 2007). Guppies are small, live-bearing tropical fish, native to the streams of Trinidad, Tobago and South America (Houde, 1997) that are an important ecological and evolutionary model (Magurran, 2005) with a short generation time. The study system allows for parasite trajectories to be monitored regularly over a period of time without the need for destructive sampling.

2.2. Host populations

Guppies (standard length: 13.5–29.0 mm) were collected from the Upper Aripo (UA, grid reference PS 931817) and Lower Aripo (LA, PS 938786) River in the northern mountain range of Trinidad and transported to the UK in October 2001.

2.3. Breeding regimes

The present study analyses guppies that have been bred for four generations in one of three breeding regimes: control, inbred, and outbred. The control group was derived from random mating within a small population ($n = 16$) of breeding individuals. Pairings were assigned randomly using computer simulations, which occasionally resulted in full-sibling crosses. Individual inbreeding coefficients (F-coefficients) were calculated using the co-ancestries in pedigrees using a Minitab 12.1 macro (van Oosterhout et al., 2000b).

2.4. Experimental infections

Guppies were kept individually in 1.1 L containers where they remained isolated for the duration of the infection trial. The population origin of individual guppies remained concealed until completion of the blind experiment. Fish were fed live newly hatched *Artemia* every day and water was changed every second day. Fish from each population were assigned randomly to the experimental *Gyrodactylus turnbulli* infection group or the negative control group, which was kept uninfected. A total of 72 experimental fish (size range: 12.5–29 mm; 18 UA males, 14 UA females, 32 L A males and 8 L A females) and 30 control fish (12 UA males, 6 UA females, 5 L A males and 7 L A females) were parasite screened to account for variation in mortality. Experimental infections utilised the Gt3 strain of *Gyrodactylus turnbulli*, which was isolated from a Nottingham aquarium shop in October 1997 and subsequently maintained in laboratory culture for ca. 4 years on inbred guppies prior to this study.

At the start of the experiment on Day 0, fish were lightly anaesthetised with 0.2% MS222 and each experimental fish was infected with two individual gyrodactylids. Extreme care was taken in transferring parasites using a dissection microscope with fibre optic illumination (following standard methods of King and Cable, 2007). Worms from donor fish were transferred to the caudal fin of recipient hosts after they had naturally attached to insect pins. To avoid the possibility that the initial two parasites transferred were too old to reproduce, all fish were re-examined the day after infection and those fish which had lost both parasites were immediately re-infected with a further two specimens of *G. turnbulli,* and for these fish the time was re-set to Day 0. Parasite infections were then monitored every 48 h when fish were anaesthetised (both controls and infected fish) and the total number of gyrodactylids counted. This study reports on the infection dynamics during the first 17 days after inoculation. Control fish were anaesthetised and sham-infected, and were monitored at the same time as experimental fish.

2.5. Statistical analysis

All statistical analysis was conducted using R version 2.15.1 (R Development Core Team, 2008). A survival curve of parasitised fish with different breeding regimes (inbred, control and outbred) was created using the survival library and the survfit function; this determines the percentage of fish survival over time. A survival curve was also plotted using the survival library and survfit function to determine the rate of extinction of parasites (parasite persistence) depending on the breeding regime of their host. A Chi-Square (χ^2) test was performed to determine any significant difference between fish survival/parasite persistence and breeding regime.

G. turnbulli intensity, defined as the number of worms on an infected host (Bush et al., 1997) were analysed using a generalised linear mixed model (GLMM) with negative binomial distribution using the glmmadmb package and the glmmadmb function with breeding regime, host standard length, sex and time (days from initial infection) as fixed factors. As parasite intensity was recorded for each individual fish at different time points, 'Fish ID' was included as a random effect in the GLMM to avoid pseudo-replication by incorporating repeated-measures. Breeding regime and time were also included as interactive terms to determine the effect on intensity of parasites over time. Fish length was included in the initial model but was removed because they did not explain significant variation, which makes the model more efficient (Thomas et al., 2013). A negative binomial general linear model (GLM) was used to analyse the effect of breeding regime, host standard length and sex on the maximum parasite intensity, i.e. the highest parasite intensity reached during the experimental period, using the function glm.nb in the library MASS. Host standard length was not significant and was removed from the model. The models were refined using analysis of variance and stepwise deletions of the least significant terms until only the significant terms remained (Crawley, 2007). An analysis of variance ("Anova" function in R) was employed as a measure of goodness of fit using the maximum likelihood-ratio test. Only significant terms are reported.

2.6. Ethical note

Approved by Cardiff Ethical Committee and conducted under UK Home Office License (PPL 30/2876).

3. Results

The mean(±SEM) individual inbreeding coefficient (F-coefficient) of fish within the control regime was F = 0.042(±0.010), which is representative of many zoo populations of larger vertebrates. Inbred lines were full-siblings inbred over four generations, resulting in an inbreeding coefficient of F = 0.5(±0.0). The outbred regime prevented inbreeding by using pedigree information and equalising the family size by allowing only two offspring per family to reproduce into the next generation. The F4 individuals of this regime were fully outbred, and there had been no consanguineous reproduction for (at least) 4 generations (F = 0.0(±0.0)).

There was no significant difference in host survival rates between the three breeding regimes over the 17 day experiment ($\chi^2 = 0.179$; d.f. = 2; p = 0.915). The persistence of the infection did, however, differ significantly between the breeding regimes (Fig. 1; $\chi^2 = 15.970$; d.f. = 2; $p < 0.001$). The infection persisted for longest (on 89% of hosts) on inbred guppies, which differed significantly from that on the control (46%; $\chi^2 = 14.081$; d.f. = 1; $p \leq 0.001$) and outbred regime fish (57%; $\chi^2 = 7.082$; d.f. = 1; p = 0.008).

Parasite number significantly increased through time on all fish, and breeding regime significantly affected the parasite number ($\chi^2 = 20.552$; d.f. = 2; $p < 0.001$), (Fig. 2). Parasite mean intensity was higher on inbred fish compared to the control and outbred fish over time (glmm,: $z_{2,1438} = 4.49$, $p < 0.001$; glmm: $z = -4.03$, $p < 0.001$, respectively) (Fig. 2). Females had a significantly higher maximum parasite intensity compared to males (glm: $t_{1,149} = -3.144$, p = 0.002), but the mean parasite intensity did not differ significantly between the sexes ($\chi 2 = 3.750$; d.f. = 1; p = 0.053). Larger fish, however, had a higher overall parasite intensity than smaller fish (Anova: $F_{1,1438} = 6.30$, p = 0.012).

Over a 17 day period there was significantly higher maximum parasite intensity on the inbred fish compared to those in the control (glm: $t_{2,149} = 2.914$, p = 0.004) but there was no significant difference between the maximum parasite intensity of the inbred and outbred regimes (glm: $t_{2,149} = -1.622$, p = 0.107). Fish from the control breeding regime reached their maximum parasite load earlier (Day 9) than those in the inbred regime (Day 14) (glm: $t_{2,101} = 2.134$, p = 0.035).

4. Discussion

Overall, our data suggests that the immune system of the "inbred" guppies (with inbreeding coefficient F = 0.5) was less effective in controlling the *Gyrodactylus turnbulli* parasite infection compared to the randomly mated "control" guppies (with F = 0.042) and outbred fish (F = 0), although the inbred and outbred guppies did not differ significantly in the maximum parasite intensity that was reached. The mean parasite intensity

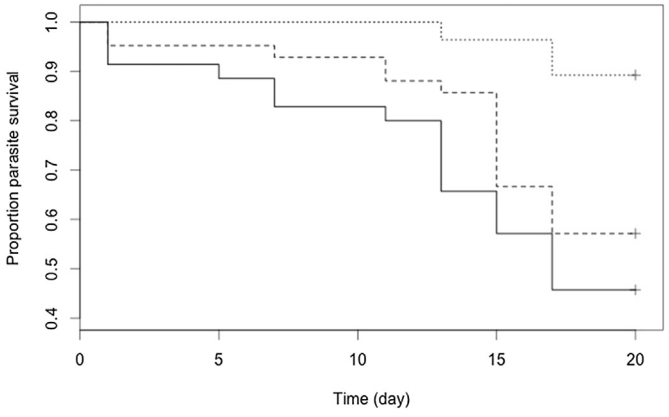

Fig. 1. Proportion of parasite (*Gyrodactylus turnbulli*) extinct over time (days) from different breeding regimes: inbred (dotted), outbred (dashed) and control (solid).

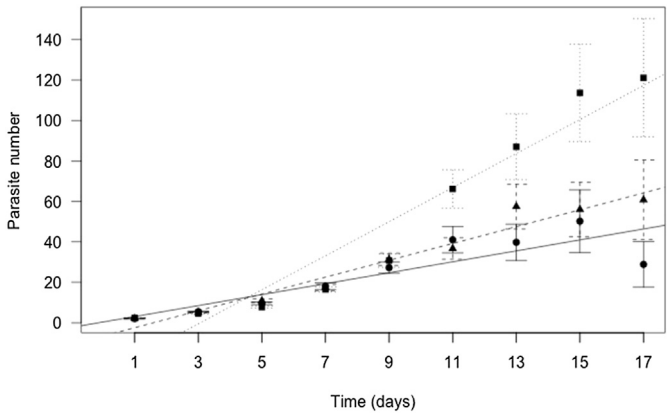

Fig. 2. The effect of breeding regime, inbred (dotted); outbred (dashed); control (solid), on the number (±SEM) of parasites (*Gyrodactylus turnbulli*) on guppies (*Poecilia reticulata*) over time (days).

was higher on inbred individuals compared to their control and outbred counterparts, supporting findings from previous studies on wild animals in their natural environment (including the host-specific ectoparasitic lice on Galapagos hawk and kestrels: Whiteman et al., 2006 and Ortego et al., 2007, respectively). The parasite infection also persisted significantly longer and reached higher maximum parasite intensity on inbred hosts than on fish from the control regime (but not compared to the fish of the outbred regime), suggesting that the inbred ($F = 0.5$) were unable to clear infection. Previous studies have shown individuals with higher inbreeding coefficient have reduced parasite resistance (e.g. bacterial infections in *Drosophila* -Spielman et al., 2004). Individuals from the control regime reached their maximum parasite load earlier and at a lower level than their inbred counterparts. This suggests that either the innate immune response acts more efficiently in the control regime guppies, or that the acquired adaptive immune response was more rapidly mounted in these fish than in the inbred individuals. Maximum parasite intensity is rarely considered as a measure for parasitism; however, it is an important variable to gauge the efficiency of an individual's immune response, and assess the infectivity of an individual's infection.

In the current experiment, the inbred guppies had approximately 50% reduced gene diversity (heterozygosity; $F = 0.5$) compared to the outbred counterparts and ancestral (wild caught) fish. Low heterozygosity is likely to have impaired the ability of the inbred fish to mount a rapid immune response (Altizer et al., 2003). Genetic diversity at the Major Histocompatibility Complex (MHC) plays an important role in disease resistance to pathogens in vertebrates (Hedrick and Kim, 2000; Milinski, 2014). The MHC forms part of the adaptive immune response, and this is the second line of defence that is mounted after the innate immune response. The multigene family codes for cell surface proteins that identify foreign substances and pathogens and initiate the immune system (Zinkernagel, 1979). MHC is not the only immune defence; the vertebrate immune system is multi-layered. Nevertheless, despite the fact that there are numerous genes involved in mounting an immune defence, we argue that potentially the loss of MHC variation in the inbred regime guppies is a likely candidate that can largely explain our current results. We base this statement on four lines of evidence. Firstly, natural gyrodactylid burden has been linked to MHC variation in wild guppies (Fraser and Neff, 2010). Secondly, in the current study there was no significant difference over the first 9 days of the parasite trajectory between the fish of the three breeding regimes. This suggests that the innate immune response was not significantly different between the inbred and the control/outbred regime fish, or that there is a lot of variation in *G. turnbulli* population growth rate between each individual fish before day 9 which potentially disguises any population differences (Ramírez et al., 2012). Thirdly, the parasite population started to decline significantly later on the inbred fish (on day 11, which equates to more than 30 parasites extra on the inbred compared to the control). This suggests that the efficiency of the adaptive (MHC-mediated) immune response was impaired in the inbred fish. Finally, inbreeding is expected to have a particularly profound effect on highly polymorphic genes in multigene families (e.g. MHC) because it will simultaneously reduce the variation at multiple paralogous gene copies. The optimum MHC diversity theory suggests that an optimal level of MHC dissimilarity between mates is important in resistance to local parasitism (Eizaguirre and Lenz, 2010), this may explain why the control regime ($F = 0.042$) was less susceptible than the outbred group ($F = 0.0$). Further work is required genotyping the immune genes in inbred and non-inbred fish to examine this hypothesis explicitly.

Increased susceptibility of inbred hosts may have implications for conservation programmes in which wild populations are supplemented with *ex-situ* bred individuals (van Oosterhout et al., 2007a). Previous studies on guppies and gyrodactylids showed that parasites play a crucial role in the reintroduction success of captive-bred animals (van Oosterhout et al., 2007a), and that individuals pre-exposed to parasites before being released had lower parasite load after reintroduction (Faria et al., 2010). Guppies are used widely as a form of mosquito control (Elias et al., 1995; Cavalcanti et al., 2007). If a small founder population of wild guppies was bred in captivity for release it would be wasted effort if the introduced populations went extinct due to parasitism. This a model system and the results from this study can be scaled to other animals bred for reintroduction often originate from small gene pools without the exposure to natural enemies (both parasites and predators). Due to increased density of susceptible hosts and lack of herd immunity, such a breeding programme could exacerbate the risk of parasite outbreaks and increase mortality, which in turn could contribute to an extinction vortex (Gilpin and Soulé, 1986).

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