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RESEARCH ARTICLE

Cost of a deprived environment – increased intraspecific aggression and susceptibility to pathogen infections

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ABSTRACT

A lack of environmental enrichment can be severely detrimental to animal welfare. For terrestrial species, including humans, barren environments are associated with reduced cognitive function and increased stress responses and pathology. Despite a clear link between increased stress and reduced immune function, uncertainty remains on how enrichment might influence susceptibility to disease. For aquatic vertebrates, we are only now beginning to assess enrichment needs. Enrichment deprivation in fish has been linked to increased stress responses, agonistic behaviour, physiological changes and reduced survival. Limited data exist, however, on the impact of enrichment on disease resistance in fish, despite infectious diseases being a major challenge for global aquaculture. Here, using a model vertebrate host–parasite system, we investigated the impact of enrichment deprivation on susceptibility to disease, behaviour and physiology. Fish in barren tanks showed significantly higher infection burdens compared with those in enriched enclosures and they also displayed increased intraspecific aggression behaviour. Infections caused hosts to have significantly increased standard metabolic rates compared with uninfected conspecifics, but this did not differ between enriched and barren tanks. This study highlights the universal physiological cost of parasite infection and the biological cost (increased susceptibility to infection and increased aggression) of depriving captive animals of environmental enrichment.

KEY WORDS: Environmental enrichment, Transmissible disease, Host–pathogen interactions, Fish welfare, Respirometer

INTRODUCTION

Lack of environmental enrichment for captive terrestrial species is an established global welfare concern (Erwin et al., 1976; Appleby and Wood-Gush, 1988; Carughi et al., 1989). Even for humans, environments lacking enrichment such as colour and structural variation cause reduced cognitive stimulation and are implicated in early onset neurodegenerative diseases (reviewed by Kramer et al., 2004; Milgram et al., 2006). For non-human vertebrates, commercial farming, in particular, represents a major welfare challenge with its focus on maximizing outputs often at the cost of depriving species of enrichment (Ashley, 2007; Wells, 2009; Stevens et al., 2017). But addition of structural enrichment, in the poultry industry, for example, can reduce intra-specific aggression, mortality levels and stress responses to human contact (Jones and Waddington, 1992; Gvaryahu et al., 1994). Reducing stress is particularly important in captive animals as it has knock-on positive effects for immunity. Much of our understanding of this connection between stress and immunity is based on research conducted in fish (see Tort, 2011), where enrichment has been shown to reduce stress that is linked to decreased cortisol production (e.g. Pounder et al., 2016; Giacomini et al., 2016). However, it remains to be seen whether using structural enrichment will translate to improved disease resistance.

Managing disease burden in fish is a global priority; fish are the most consumed source of animal protein and aquaculture is the fastest growing food industry globally (Shinn et al., 2015; FAO, 2018). Parasitic diseases pose the most significant biosecurity and economic risk for aquaculture (Shinn et al., 2015) and stock management strategies are now emphasizing husbandry practices that minimize stressors to prevent stress-related immunosuppression (Conte, 2004; Ashley, 2007). The monogenean gyrodactylids are a group of hyperviviparous ectoparasites that historically have been a challenge to manage in aquaculture and the ornamental trade, with no effective cures that can be applied to fish stocks *en masse* (Schelkle et al., 2009). Norwegian salmon were decimated by *Gyrodactylus salaris* in the 1970s (Johnsen, 1978; Appleby and Mo, 1997) and despite the use of rotenone in rivers to kill all potential fish hosts, the parasite persisted in adjacent water bodies (Eriksen et al., 2009). Even for parasite species that may not cause mortality, the metabolic cost of infection will have life history consequences, such as reduced growth and fecundity, for hosts (Sheldon and Verhulst, 1996; Bonneaud et al., 2016).

Here, we tested the hypothesis that inclusion of environmental enrichment for captive animals can increase disease resistance using a model host–parasite system (guppy–*Gyrodactylus turnbulli*). The guppy host, *Poecilia reticulata*, is an established ecological and parasitological model (Magurran, 2005). *Poecilia reticulata* has been introduced as a pet and biological agent to every major continent, except Antarctica (Deacon et al., 2011), and is a key economic species in the ornamental trade (Maceda-Veiga et al., 2016). The hyperviviparous ectoparasite *G. turnbulli* is a primary monogenean parasite of the guppy and a major concern in the ornamental trade (reviewed by Cable, 2011). This is the first study of its kind to investigate the impact of enrichment deprivation simultaneously on fish disease resistance, behaviour and physiology (standard metabolic rate, SMR).

MATERIALS AND METHODS

Study system

For this study, we used size matched male guppies measured using callipers under 0.02% MS-222 induced mild anaesthesia (*Poecilia reticulata* W. Peters 1859; size range: 14–19 mm) bred from a stock caught in the Lower Aripo River in Trinidad in 2012 and initially housed at Exeter University before being transferred to Cardiff University in 2014. All guppies were maintained in 70 l breeding

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tanks (closed systems: 60 cm×40 cm×30 cm) utilizing dechlorinated water from a main source at 24±0.5°C under a 12 h light:12 h dark photoperiod (lights on 07:00–19:00 h) and fed dry food flakes (Aquarian®) *ad libitum* and freshly hatched *Artemia* nauplii every alternate day. Water quality levels are tested on a weekly basis and prior to removing fish for experimental investigations the water quality level was: ammonia, non detectable, pH 7.8; nitrite levels, >0 to <0.21 mg l^{-1}; nitrate levels, <20 mg l^{-1} (API® Freshwater Master Test Kit). All fish stock tanks are consistently aerated with air stones connected to a main air supply. Each stock tank was provided with the same environmental enrichment consisting of 2 cm pea gravel substrate, plastic flowerpots, plastic reeds and tubing. Sufficient refugia were available to ensure all individual fish were able to use them when required.

For investigating susceptibility to disease, experimental infections used the Gt3 strain of *Gyrodactylus turnbulli* Harris 1986, isolated from a Nottingham aquarium shop in October 1997 and subsequently maintained at Cardiff University on inbred guppies prior to this study (see King and Cable, 2007).

Experimental design

All fish used for this study were size matched with callipers under mild anaesthesia (0.02% MS-222; see above). Experimental fish were assigned to one of two treatments: enriched or barren tanks (16 l, 36 cm×21 cm×21 cm). Each enriched tank contained gravel (2 cm pea gravel substrate), a plastic tube, a flowerpot and plastic reeds (purchased from Aquatic World, Cardiff) and these enrichments were consistent between each batch. Barren tanks contained no enrichment and were visually isolated from enriched tanks. Guppies were removed from stock tanks and a batch of fish (5 fish per batch×12 replicates per treatment) was randomly assigned to an enriched or barren treatment tank. To ensure the effect of displacement and a novel environment did not confound results, fish prescribed to enriched and barren treatments were maintained in their respective experimental tanks for 2 weeks to allow acclimatization prior to starting experiments; this is sufficient time for the formation of shoals based on familiarity (Griffiths and Magurran, 1997).

Behavioural observations

To investigate the effect of enrichment deprivation on guppy behaviour, focal observations were conducted pre-infection (days 13 and 14 of acclimatization) as *G. turnbulli* is known to influence guppy inter-specific interactions (Reynolds et al., 2018). Focal observations involved an observer choosing a single male, identifiable from distinct coloration (out of 5 fish per tank) and recording all interactions between the focal male and conspecifics. For the enriched tanks, the time spent interacting with the structural enrichment was also recorded as preliminary observations revealed that guppies will interact with the enrichment by either pecking at structures (gravel, flowerpot, plastic tube and reeds) or seeking refuge (in the flowerpot, plastic tube and reeds). To ensure that observer bias did not influence recording behavioural metrics, two observers (one who was unaware of the expected outcomes of this study) recorded agonistic behaviours for a subsample of tank treatments (5 enriched and barren tanks). A Kendall's Tau correlation analysis (chosen because several 'tied' observations were reported between observers) revealed no significant difference between observer data (i.e. a significant association was detected; Z=11.729, P<0.001).

All observations were conducted between 10:00 h and 14:00 h, and prior to each behavioural recording, the experimenter allowed 10 min for the fish to acclimatize to their presence. Aggression between male guppies is characterized by chasing and nipping behaviour (Houde, 1997). We report on two behavioural metrics for this study: (1) aggression index, i.e. the number of nips plus the number of chases; and (2) time spent associating with the enrichment, i.e. time spent nibbling the enrichment plus swimming into the plastic pot or tubing and swimming between the plastic reeds.

Experimental infection

To investigate the effect of enrichment deprivation on susceptibility to disease, guppies from tank treatments (barren n=40 fish, enriched n=40 fish) were lightly anaesthetised with 0.02% MS-222 and infected with two gyrodactylids each. Parasite transfer was conducted using a dissection microscope with fibre optic illumination (following standard methods of King and Cable, 2007). Briefly, two parasites from donor fish were transferred to the caudal fin of each recipient host by placing the tail of a heavily infected donor fish close to that of a naive host. Control fish (barren n=20 fish, enriched n=20 fish) were treated (anaesthetized) the same way as infected fish (but without pathogen inoculation), to ensure that handling was not a confounding variable.

After experimental infections, fish were returned to their respective experimental tanks where they were housed for a further 17 days. As gyrodactylids naturally transfer between fish upon contact, every 48 h guppies were removed from their tanks and mean parasite intensity was calculated for each fish. Parasite infections were monitored by anaesthetizing fish and counting the total number of gyrodactylids. Individual male guppies could be recognized by distinct coloration based on photographs taken on an iPhone (Apple Inc.).

Respirometry

For investigating how environmental enrichment and infection impacted SMR, individual infected (n=29) or uninfected (n=28) guppies from both barren (n=14) and enriched (n=15) tanks were placed in respirometer chambers on days 3 and 13 of the 17 day infection trajectory to determine the impact of low and high parasite burden on SMR. All measurements were conducted in a respirometry set-up that permitted monitoring of n=3 fish and n=1 blank control simultaneously, and temperature for the duration of measurements was maintained at 24±0.5°C. All water used for experimental purposes was autoclaved. The static respirometry set-up consisted of individual glass chambers (130 ml, sealed Duran™ square glass bottle with polypropylene screw cap, Fisher), which were autoclaved and rinsed with ethanol prior to commencing measurements to minimize background noise before the start of each respirometry trial; each chamber contained a false bottom with a magnetic stirrer to ensure a homogeneous distribution of oxygen within it. Chambers were fitted with individual contactless oxygen sensor spots attached to probes that were connected to a FireSting O_2 meter (PyroScience, Aachen, Germany). Food was withdrawn for 24 h before each fish was tested to ensure they were in a post-absorptive state so SMR measurements were not influenced by thermal effects of food in the digestive tract. The decline in O_2 concentration within respirometry chambers was measured using Eqn 1 in repeated 1 s measurement cycles over ca. 1 h 20 min, with 1 h acclimation time and 20 min for recordings:

$$\mathrm{SMR} = \frac{\Delta \mathrm{O}_2}{M} \times V_\mathrm{c}, \quad (1)$$

where M is fish mass, V_c is the volume of the respirometer chamber and ΔO_2 is the rate of oxygen decline (Bonneaud et al., 2016) calculated as the slope of a linear regression. During measurements, dissolved oxygen levels never fell below 7 mg l^{-1}, which is within

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recommended levels for freshwater tropical fish (OATA, 2008). The mean background oxygen consumption (typically ca. 20% of fish SMR) was subtracted from fish SMR for analysis.

Ethics statement

All animal work was approved by the Cardiff University Animal Ethics Committee and conducted under UK Home Office licence PPL 303424.

Statistical analysis

All statistical analyses were conducted using RStudio version 1.0.143 (http://www.R-project.org/). We defined three host disease categories: susceptible – hosts on which parasite numbers consistently increased; responders – those on which parasite numbers increased followed by a consistent decline indicative of an immune response; and resistant – hosts that cleared their parasites (Bakke et al., 2002). Total infection trajectory over 17 days was calculated from the area under the curve (AUC), using the trapezoid rule. A generalized linear mixed model (GLMM) with a negative binomial error family in the MASS R package was used to analyse both AUC and mean parasite intensity. Host standard length and treatment were treated as fixed factors. Parasite count was recorded on each fish at multiple time points over a 17 day infection trajectory so 'fish ID' was included as a random effect in the GLMM to avoid pseudoreplication by incorporating repeated measures. Fish length was included in the initial model but was removed because the size range did not explain significant variation. We used a generalized linear model (GLM) to analyse how peak parasite day, maximum parasite count and mortality varied with treatment. For analysing maximum parasite count, we used a negative binomial error family with a log link function, a quasiPoisson error family with a log link function for peak parasite day and a Poisson error family with log link function for mortality count. A Fisher's exact test was used to investigate the difference between fish disease categories.

For analysing behaviour data, we used a GLMM with a negative binomial error structure to analyse agonistic behaviour between treatments, to prevent pseudoreplication as each experimental tank was observed at two time points and over 2 days. Agonistic behaviours (number of nips and chases) were combined into a single aggression index for analysis. We hypothesized that any aggression observed in enriched tanks would be associated with the time spent interacting with enrichment. Thus, we also used a GLMM with a restricted maximum likelihood (REML) function to analyse the association between the time spent interacting with the enrichment and the number of agonistic interactions within enriched tanks. Data in the REML model had to be rescaled because of very large eigenvalues and over-dispersion (Thomas et al., 2013). Rescaling maintained data structure and minimized dispersion, generating a robust model structure.

For analysing the effect of tank treatments (barren versus enriched) and infection on SMR, we used a GLM with an inverse gaussian error family and log link function. Additionally, we used linear regression analysis to assess the relationship between parasite count and SMR. All models used for analyses were chosen and refined based on the lowest Akaike information criterion (http://CRAN.R-project.org/package=lme4).

RESULTS

Mortality did not significantly differ between fish in enriched tanks and barren ones (GLM: $Z=-0.11$, s.e.=0.21, $P=0.91$) but fish from barren tanks were significantly more susceptible to infection (barren: 26/42, 62%; enriched: 12/40, 30%) and showed significantly higher mean parasite intensity compared with fish housed in enriched tanks (Fig. 1A; GLMM: $Z=-8.16$, s.e.=0.08, $P<0.001$). Fish from barren tanks also had a significantly higher peak pathogen burden (Fig. 2A; GLM: $Z=-16.03$, s.e.=0.07, $P<0.001$) and this peak was achieved significantly later in fish from barren tanks compared with enriched ones (Fig. 2B; GLM: $t=-7.893$, s.e.=0.02, $P<0.001$). In addition, significantly more fish (Fisher's exact text: 95% confidence interval, CI=3.29, $P<0.001$) cleared infections (resistant) in enriched tanks (13/40, 33%) compared with barren tanks (1/42, 2%). Enrichment did not significantly affect SMR (Fig. 3A; GLM: $t=-1.66$, s.e.=0.11, $P=0.09$) but fish with a high parasite burden (parasite range: 30–330; parasite mean: 120) had significantly greater SMR compared with uninfected ones regardless of enrichment (Fig. 3B; GLM:

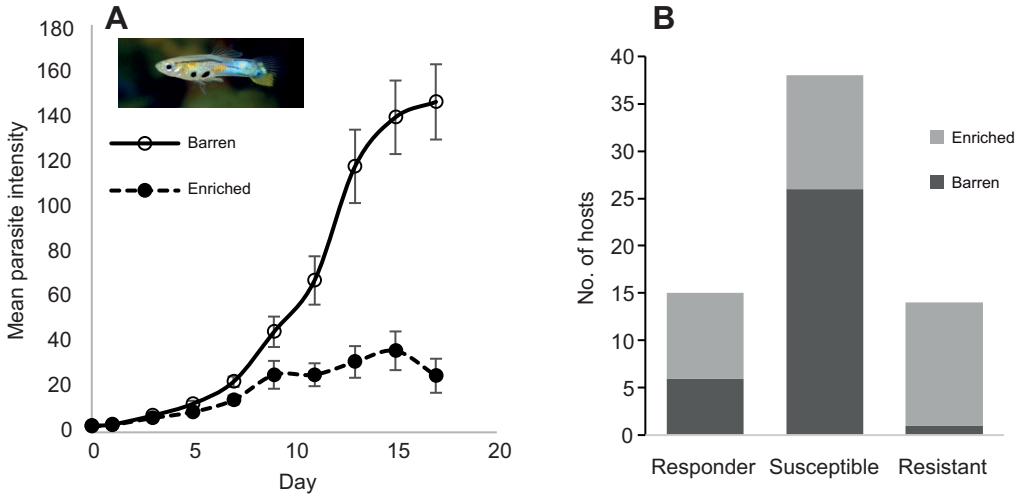

Fig. 1. Parasite (*Gyrodactylus turnbulli*) infection in guppies (*Poecilia reticulata*) from barren and enriched tanks. (A) Mean (±1 s.e.m.) parasite intensity in guppies exposed to *G. turnbulli* was significantly higher in fish in barren tanks ($n=40$) than in enriched ones ($n=40$). (B) The number of hosts raised in either enriched or barren tanks classed as susceptible (hosts on which parasite numbers consistently increased), responders (hosts on which parasite numbers increased followed by a consistent decline indicative of an immune response), or resistant (hosts that cleared their parasites). Hosts from barren tanks were significantly more susceptible to disease ($n=26$) compared with those from enriched tanks ($n=12$).

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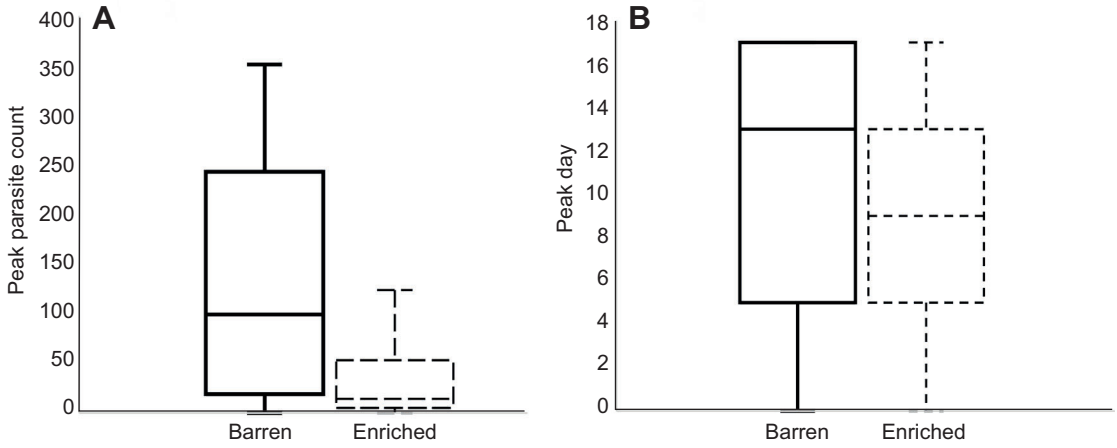

Fig. 2. Parasite load. (A) Hosts from barren tanks (n=40) had a significantly higher peak parasite count than their enriched counterparts (n=40). (B) Peak parasite burden occurred significantly later (peak day) for hosts in barren tanks compared with those in enriched tanks. Box plots show median (line), interquartile range (box) and 1.5x interquartile range (whiskers).

t=3.38, s.e.=0.25, P<0.001). Moreover, a linear regression analysis revealed that a significant proportion of the SMR of infected fish could be explained by parasite count (Fig. 3C: R^2=0.31, t=5.16, P<0.001).

Fish in barren tanks displayed significantly more aggressive behaviour (nipping and chasing) towards conspecifics compared with those in enriched tanks (GLMM: Z=−11.21, s.e.=0.15, P<0.001). In addition, aggression observed in enriched tanks was

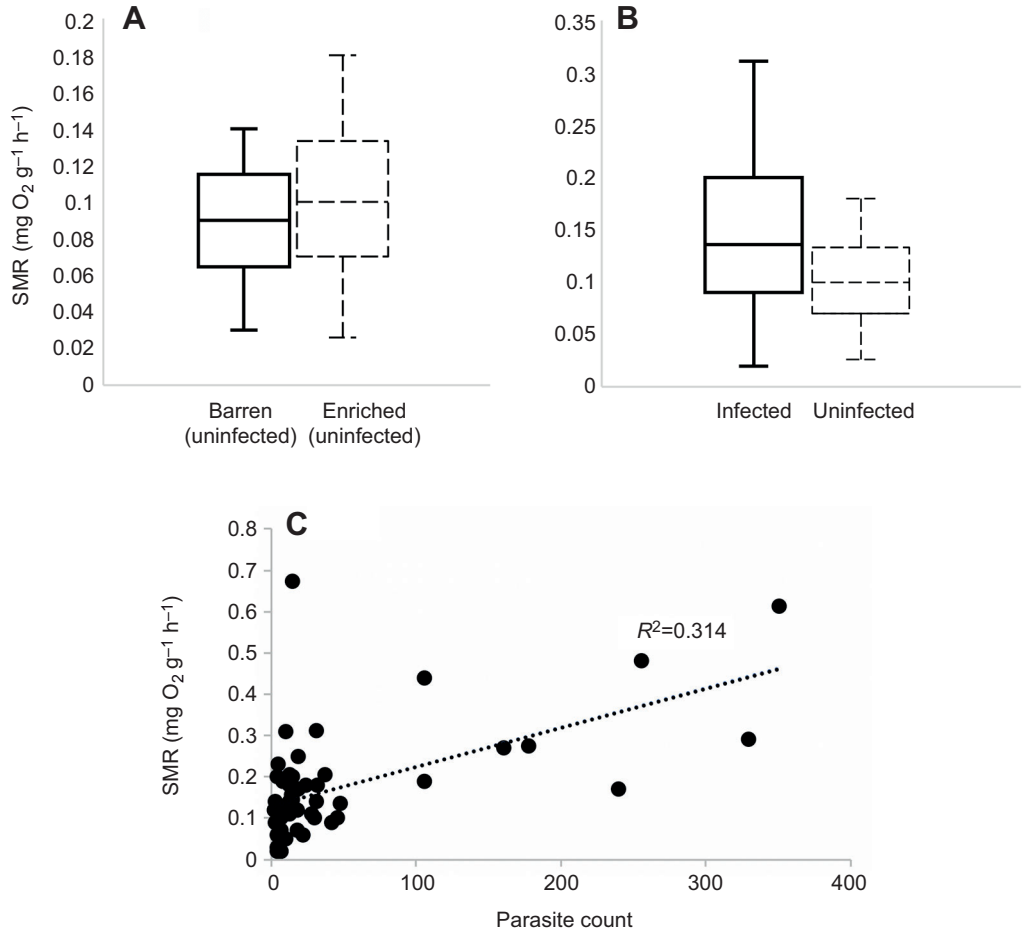

Fig. 3. Relationship between fish standard metabolic rate (SMR), tank treatment (barren versus enriched) and infectious status. (A) No significant association was found between SMR and tank treatment (n=29 barren and n=28 enrichment – no infections). (B) However, fish that were infected (n=29) had significantly higher SMR compared with uninfected conspecifics (n=28). (C) Moreover, a significant proportion of SMR of infected hosts could be explained by parasite count.

significantly associated with time spent interacting with the structural enrichment and fish that spent more time using the enrichment showed significantly less agonistic behaviour compared with those that used the enrichment less (GLMM: $t=-5.34$, s.e.=0.0008, $P<0.001$).

DISCUSSION

Transmissible disease is one of the most significant factors limiting the expansion of aquaculture globally (Stentiford et al., 2017) and there is now a renewed emphasis on developing sustainable methods for disease management. Here, we show that inclusion of environmental enrichment significantly reduces disease burden of ornamental fish. We also reveal behavioural modification (i.e. increased aggression) caused by depriving hosts of enrichment that could facilitate disease transmission and we show how increased disease burden significantly increases SMR of hosts. Taken together, these results show how relatively simple measures could sustainably improve the welfare of captive animals by reducing disease burden and maladaptive behaviours.

Previous studies on the impact of environmental enrichment on host–pathogen dynamics are so limited, and use different methodologies, that this precludes direct comparisons. Our findings, however, do directly support the observation that farmed piglets reared with environmental enrichment and subsequently inoculated with both Porcine Reproductive and Respiratory Virus (PRRSV) and *Actinobacillus pleuropneumoniae* showed greater disease resistance compared with piglets treated the same way but reared in barren enclosures (van Dixhoorn et al., 2016). In our study, it was clear that fish from barren enclosures were less resistant to pathogen infections compared with hosts from enriched tanks, and peak pathogen burden was also significantly higher in fish from barren enclosures (Fig. 1B). Moreover, hosts from enriched tanks cleared pathogen infections more effectively, suggesting that application of environmental enrichment can improve immune responses to infectious disease. This finding is particularly compelling as pathogen exposure is likely to occur in most captive environments because sterile enclosures are not sustainable, especially in large-scale facilities. Therefore, ensuring maintenance conditions maximize host immune responses should be a priority.

Variations in the amount and type of enrichment can also impact host–pathogen interactions. Certain enrichment substrates may act as a medium for pathogen growth and actually increase the chances of infection. However, enrichment substrates are unlikely to facilitate reproduction in directly transmitted microparasites such as *Gyrodactylus* spp. used in this study, which cannot survive for long off a host (reviewed in Bakke et al., 2007). Under certain enriched conditions, conversely, bacterial pathogens such as *Flavobacterium columnare* can actually increase propagation as a result of the formation of biofilms, increasing host susceptibility to disease (see Karvonen et al., 2016; Räihä et al., 2019). Moreover, the source of enrichment might not only influence biofilm growth but also present an additional hazard as a source of macrofauna contamination; for instance, intermediate hosts, such as snails, vectoring other infectious pathogens. Ultimately, the importance of managing disease burden with interventions such as environmental enrichment is linked to the trade-off between the labour costs of enrichment maintenance and risk of contamination versus the potential to reduce the economic and welfare costs imposed by pathogens.

Most infections lead to the reallocation of metabolic resources to the immune system from general physiological functions (Sheldon and Verhulst, 1996). Our study is the first to show that gyrodactylosis increases the SMR of hosts. *Gyrodactylus* spp. are of major welfare concern in both the ornamental and aquaculture trade (Bakke et al., 2007; Maceda-Veiga and Cable, 2019), particularly because there are no effective *en masse* treatments. This increased metabolic demand, even if hosts survive, will impact health, reducing physical condition and potentially, fecundity. Increased metabolic rate linked to parasitism has been demonstrated in both invertebrate and vertebrate hosts (e.g. crabs: Haye and Ojeda, 1998; brown trout: Filipsson et al., 2017), and our results further highlight the universal physiological impact of parasitism. Enrichment deprivation on its own, however, did not affect fish SMR, suggesting that the increased aggression seen in fish in barren tanks was not driven by increased basal metabolism.

Increased aggression, as seen in our study for hosts in barren tanks, may have increased disease burden. Chronic aggression can elevate stress levels (see Giacomini et al., 2016) and chronic stress does suppress immunity and increase disease susceptibility (Khansari et al., 1990; Dhabhar, 2009). Furthermore, higher aggression levels will lead to increased contact rates, which can increase the probability of direct transmission for pathogens such as *Gyrodactylus* (e.g. Reynolds et al., 2018). While we did allow 2 weeks for fish to acclimate in experimental tanks, which is sufficient for this species to form familiar shoals (Griffiths and Magurran, 1997), we acknowledge that removing fish from enriched stock tanks might have impacted stress levels. However, as fish hosts in our study demonstrated significantly higher aggression levels in only barren tanks, this does suggest that enrichment deprivation has an overriding influence on stress-related behaviour. Through aggression-associated nips and chases, contact rates would have increased, and it is plausible that this facilitated pathogen transmission.

To conclude, our study highlights the biological costs of enrichment deprivation: increased susceptibility to disease and interspecific aggression levels. We also show how elevated disease burden linked to enrichment deprivation has a significant metabolic impact. Aquaculture industries have displayed reluctance in using environmental enrichment because of the additional time spent cleaning structures and catching fish. However, if we are to prioritize animal welfare, we recommend that industries investigate which enrichment conditions are most effective at managing aggressive behaviour and disease outbreaks while minimizing cleaning and capture time. Here, we show that at least on a small scale, enrichment can be a useful tool in health management.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.M., A.E., J.C.; Methodology: N.M., E.P., J.C.; Validation: N.M., E.P., J.C.; Formal analysis: N.M.; Investigation: N.M.; Resources: E.P., J.C.; Data curation: N.M.; Writing - original draft: N.M., A.E., J.C.; Writing - review & editing: N.M., A.E., E.P., J.C.; Visualization: N.M., A.E., J.C.; Supervision: A.E., J.C.; Project administration: N.M.

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