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 to those in enriched enclosures and they also displayed increased intraspecific aggression behaviour. Infections caused hosts to have significantly increased Standard Metabolic Rates compared to 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.

Keywords: 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 if 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 (Erikson 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 test 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). *P. 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,* 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 tanks (closed systems- 60 cm x 40cm x 30 cm) utilising dechlorinated water from a main source at 24 $\pm$ 0.5°C under a 12-h light: 12-h dark photoperiod (lights on 07:00-19:00) 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<0.21 mg/l, Nitrate levels: <20mg/l (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*, 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).

97

98 *Experimental design*

99 All fish used for this study were size matched with callipers under mild anaesthesia
100 (0.02% MS-222- see above) . Experimental fish were assigned to one of two treatments:
101 enriched or barren tanks (16 L- 36 cm x 21 cm x 21 cm). Each enriched tank contained gravel
102 (2 cm pea gravel substrate), plastic tube, flowerpot and plastic reeds (purchased from Aquatic
103 World, Cardiff) and these enrichments were consistent between each batch. Barren tanks
104 contained no enrichment and were visually isolated from enriched tanks. Guppies were
105 removed from stock tanks and a batch of fish (5 fish per batch x 12 replicates per treatment)
106 randomly assigned to an enriched or barren treatment tank. To ensure the effect of
107 displacement and a novel environment did not confound results, fish prescribed to enriched
108 and barren treatments were maintained in their respective experimental tanks for 2 weeks
109 to allow acclimatisation prior to starting experiments; this is sufficient time for the formation
110 of shoals based on familiarity (Griffiths and Magurran, 1997).

111

112 *Behavioural observations*

113 To investigate the effect of enrichment deprivation on guppy behaviour, focal
114 observations were conducted pre-infection (days 13 and 14 of acclimatisation) as *G. turnbulli*
115 is known to influence guppy inter-specific interactions (Reynold et al., 2018). Focal
116 observations involved an observer choosing a single male, identifiable from distinct
117 colouration (out of 5 fish per tank) and recording all interactions between the focal male and
118 conspecifics. For the enriched tanks, the time spent interacting with the structural enrichment
119 was also recorded as preliminary observations revealed that guppies will interact with
120 enrichment by either pecking at structures (gravel, flowerpot, plastic tube and reeds) or
121 seeking refuge (in flowerpots, plastic tubes and reeds). To ensure that observer bias did not
122 influence recording behavioural metrics, two observers (one who was unaware of the
123 expected outcomes of this study) recorded agonistic behaviours for a subsample of tank
124 treatments (5 enriched and barren tanks). A Kendall's Tau correlation analysis (chosen
125 because several 'tied' observations were reported between observers) revealed no significant
126 difference between observer data (i.e. a significant association was detected; $z = 11.729$, $p < 0.001$).
127

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

Experimental infection

To investigate the effect of enrichment deprivation on susceptibility to disease, guppies from tank treatments (barren = 40 fish, enriched= 40 fish) were lightly anaesthetised with 0.02 % MS222 and all fish 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 hosts by placing the tail of a heavily infected donor fish close to that of a naïve host. Control fish (barren= 20 fish, enriched= 20 fish) were treated the same way infected fish were (anesthetizing 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 anaesthetising fish and counting the total number of gyrodactylids. Individual male guppies could be recognized by distinct colouration 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 Standard Metabolic Rate (SMR). All measurements were conducted in a respirometry set-up that permitted

monitoring of 3 fish and 1 blank control simultaneously and temperature for the duration of measurements 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). Glass chambers were autoclaved and rinsed with ethanol prior to commencing measurements to minimise background noise before the start of each respirometry trial and each chamber contained a false bottom with a magnetic stirrer to ensure a homogenous 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 the below formula in repeated 1s measurement cycles over ca. 1h 20 mins, with 1 h acclimation time and 20 mins for recordings:

$$\text{SMR} = \frac{\Delta O2}{fishmass} \times Vc$$

Where V_c is the volume of the respirometer chamber and $\Delta O2$ 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, which is within 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 (R Development Core Team, 2015). Here, we define three host disease categories: hosts on which parasite numbers consistently increased (susceptible); those on which parasite numbers increased

191 followed by a consistent decline indicative of an immune response (responders) or hosts
192 which cleared their parasites (resistant) (Bakke et al., 2002). Total infection trajectory over 17
193 days was calculated by Area Under Curve (AUC), using the trapezoid rule. A generalized linear
194 mixed model (GLMM) with a negative binomial error family in the MASS R package was used
195 to analyse both AUC and mean parasite intensity. Host standard length and treatment were
196 treated as fixed factors. Parasite count was recorded on each fish at multiple time points over
197 a 17-day infection trajectory so 'Fish ID' was included as a random effect in the GLMM to
198 avoid pseudoreplication by incorporating repeated-measures. Fish length was included in the
199 initial model but was removed because the size range did not explain significant variation. We
200 used a Generalised Linear Model (GLM) to analyse how peak parasite day, maximum parasite
201 count and mortality varied with treatment. For analysing maximum parasite count we used a
202 negative binomial error family with a log link function; a quasiPoisson error family with a log
203 link function for peak parasite day and a Poisson error family with log link function for
204 mortality count. A Fisher's exact test was used to investigate the difference between fish
205 disease categories.
206 For analysing behaviour data, we used a GLMM with a negative binomial error
207 structure to analyse agonistic behaviour between treatments, to prevent pseudoreplication
208 as each experimental tank was observed at two time points and over two days. Agonistic
209 behaviours (number of nips and chases) were combined into a single aggression index for
210 analysis. We hypothesized that any aggression observed in enriched tanks would be
211 associated with the time spent interacting with enrichment. Thus, we also used a GLMM with
212 a Restricted Maximum Likelihood (REML) function to analyse the association between the
213 time spent interacting with enrichment and the number of agonistic interactions within
214 enriched tanks. Data in the REML model had to be rescaled due to very large eigenvalues and
215 overdispersion (Thomas et al., 2013). Rescaling maintained data structure and minimized
216 dispersion, generating a robust model structure.
217 For analyzing the effect of tank treatments (barren versus enriched) and infection on
218 SMR, we used a GLM with an inverse gaussian error family and log link function. Additionally,
219 we used a linear regression analysis to assess the relationship between parasite count and
220 SMR. All models used for analyses were chosen and refined based on the lowest Akaike
221 Information Criterion (Bates et al., 2014).
222

Results

Mortality did not significantly differ between fish in enriched tanks and barren ones (GLM: Z=-0.11, SE=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 to fish housed in enriched tanks (Fig. 1A; GLMM: Z=-8.16, SE=0.08, p<0.001). Fish from barren tanks also had significantly higher peak pathogen burdens (Fig. 2A; GLM: Z=-16.03, SE=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, SE=0.02, p<0.001). In addition, significantly more fish (Fisher's exact text: 95% C.I. = 3.29, p<0.001) cleared infections (resistant) in enriched tanks (13/40; 33%) compared to barren tanks (1/42; 2%). Enrichment did not significantly affect SMR (Fig. 3A; GLM: t=-1.66, SE=0.11, p=0.09) but fish with high parasite burdens (parasite range: 30-330; parasite mean: 120) had significantly greater SMR compared to uninfected ones regardless of enrichment (Fig. 3B; GLM: t=3.38, SE=0.25, p<0.001). Moreover, a linear regression analysis revealed that a significant proportion of the SMR of infected fish could be explain by parasite count (Fig. 3C; LM: 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 to those in enriched tanks (GLMM: Z=-11.21, SE= 0.15, P<0.001). In addition, aggression observed in enriched tanks was significantly associated with time spent interacting with enrichment and fish that spent more time using enrichment showed significantly less agonistic behaviour compared to those that used less enrichment (GLMM: t= -5.34, SE= 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 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 standard metabolic rate of hosts. Taken together, these results

show how relatively simple measures could sustainably improve 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 to piglets 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 to hosts from enriched tanks and peak pathogen burdens were also significantly higher in barren enclosures (Fig. 1B). Moreover, hosts from enriched tanks cleared pathogen infections more effectively, suggesting 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 maximise hosts' 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 due to 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 rates 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 provide two 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 due to additional time spent cleaning structures and catching fish. However, if we are to prioritise animal welfare, we recommend industries to investigate which enrichment conditions are most effective at managing aggressive behaviour and disease outbreaks while minimising cleaning and capture time. Here we show that at least on a small-scale enrichment can be a useful tool in health management.

318 **Author contributions**

319

320 JC and NM conceived and designed the experiment. NM executed the experiment and
321 conducted all statistical analysis. ECP helped with the respirometry set-up and analysis of
322 respirometry data. Primary writing was conducted by NM and JC with all authors contributing
323 towards revisions and final manuscript.

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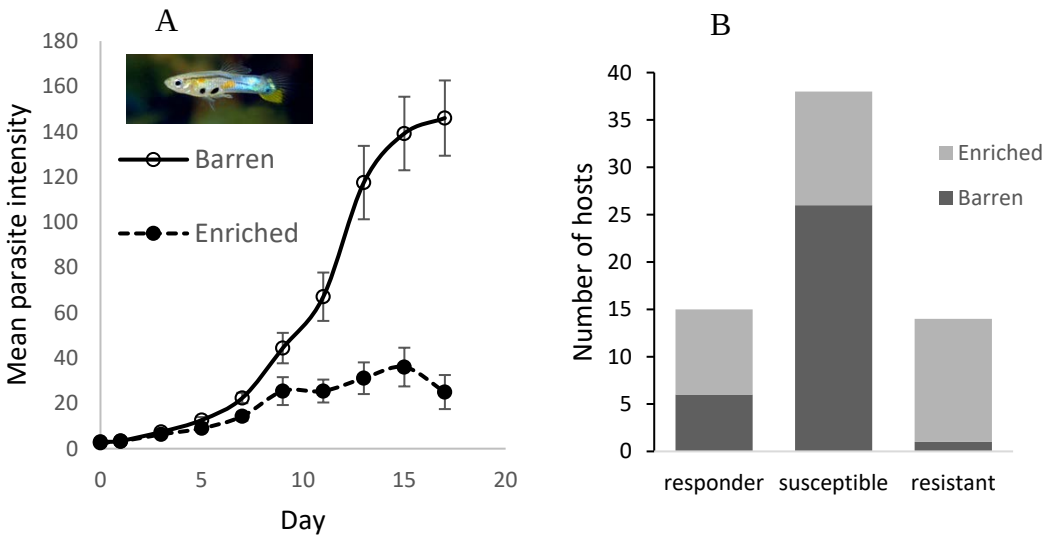
Figure 1. (A) Mean (±1 SEM) parasite intensity in guppies (*Poecilia reticulata*) exposed to *Gyrodactylus turnbulli* infection was significantly higher in fish in barren tanks (n=40) than enriched ones (n=40). (B) The number of hosts raised in either enriched or barren tanks classed as susceptible (hosts 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 which cleared their parasites). Hosts from barren tanks were significantly more susceptible to disease (n=26) compared to those from enrichment treatments (n=12).



506 **Figure 2**. (A) Hosts from barren tanks (n=40) had significantly higher peak parasite counts than
507 their enriched counterparts (n=40) and (B) peak parasite burdens occurred significantly later
508 (peak day) for hosts in barren tanks compared to those in enriched tanks.
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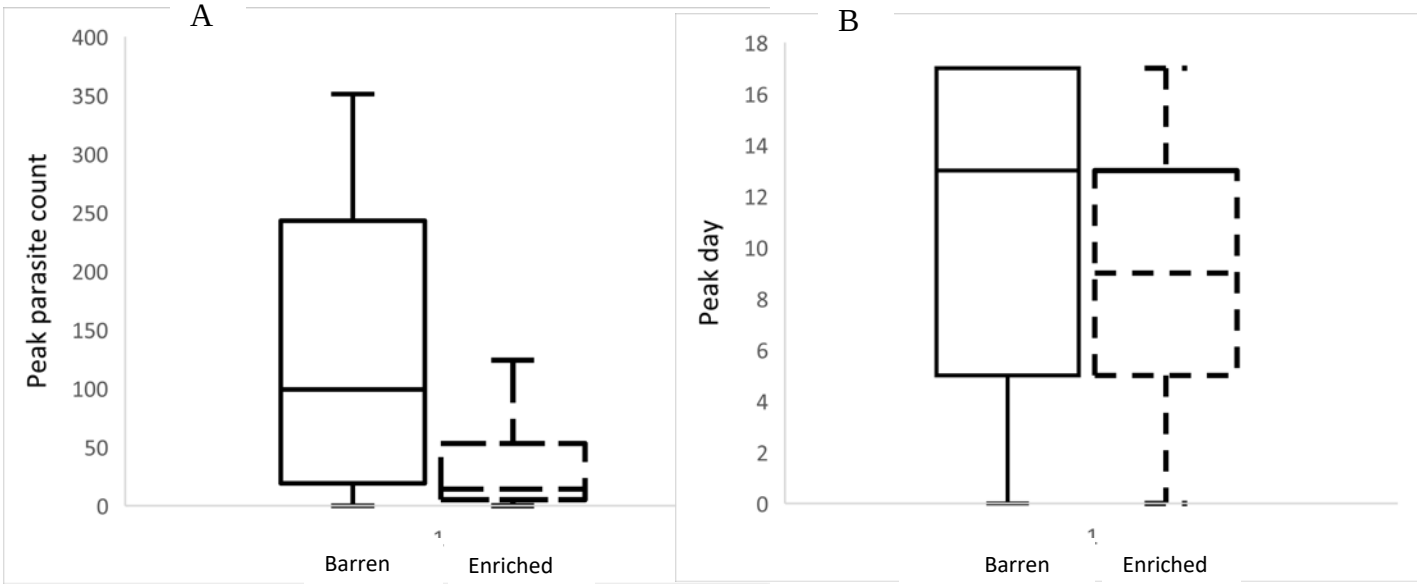

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Figure 3. Relationship between fish Standard Metabolic Rate (SMR, $mgO_2g^{-1}h^{-1}$), tank treatment (barren versus enriched) and infectious status. (A) No significant association was found between SMR and tank treatment (n= 29 barren and n=28 enrichment- no infections) but (B) fish that were infected (n=29) had significantly higher SMR compared to uninfected conspecifics (n=28). Moreover, (C) a significant proportion of SMR of infected hosts could be explained by parasite count.