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Chronic nitrate enrichment decreases severity and induces protection against an infectious disease

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ABSTRACT

Excessive fertilisation is one of the most pernicious forms of global change resulting in eutrophication. It has major implications for disease control and the conservation of biodiversity. Yet, the direct link between nutrient enrichment and disease remains largely unexplored. Here, we present the first experimental evidence that chronic nitrate enrichment decreases severity and induces protection against an infectious disease. Specifically, this study shows that nitrate concentrations ranging between 50 and 250 mg NO_3^-/l reduce *Gyrodactylus turnbulli* infection intensity in two populations of Trinidadian guppies *Poecilia reticulata*, and that the highest nitrate concentration can even clean the parasites from the fish. This added to the fact that host nitrate pre-exposure altered the fish epidermal structure and reduced parasite intensity, suggests that nitrate protected the host against the disease. Nitrate treatments also caused fish mortality. As we used ecologically-relevant nitrate concentrations, and guppies are top-consumers widely used for mosquito bio-control in tropical and often nutrient-enriched waters, our results can have major ecological and social implications. In conclusion, this study advocates reducing nitrate level including the legislative threshold to protect the aquatic biota, even though this may control an ectoparasitic disease.

1. Introduction

Humans have substantially altered nutrient cycles over the last two centuries through coal combustion, intensive farming and urban sewage discharge (Grizzetti et al., 2012; Whitehead and Crossman, 2012). In aquatic ecosystems, excessive nutrient input causes eutrophication, one of the most profound forms of ecological change (Smith and Schindler, 2009; Woodward et al., 2012). Besides reducing water quality, eutrophication alters trophic interactions including host-parasite relationships with major implications for ecosystem function and disease emergence (Smith and Schindler, 2009; Vega Thurber et al., 2014). Whilst the direct link between eutrophication and disease remains largely unexplored, pathogen numbers and virulence may be affected in either direction (Johnson et al., 2010; Lafferty, 2014) but often with a positive effect on parasites with direct life-cycles (McKenzie and Townsend, 2007). Considering forecasted agricultural intensification will promote eutrophication (Millennium Ecosystem Assessment, 2005), a better understanding of how environmental nutrient levels and disease interact is essential in order to control disease outbreaks and conserve natural resources.

The effect of nutrient enrichment on host-parasite systems can be explained by several mechanisms that are not mutually exclusive. Nutrients may indirectly benefit parasites because the associated increased productivity can increase host abundance and/or quality (e.g. Coyner et al., 2003; Smith et al., 2005; Johnson et al., 2007). Nutrients, including nitrate, can also increase pathogen virulence; this occurs in corals with the fungus *Aspergillus* (see Bruno et al., 2003; Vega Thurber et al., 2014), possibly because the fungus can use nitrate as a nutrient (Olutiola and Cole, 1977). Nitrate can, however, also be toxic for aquatic organisms but its effects under chronic exposure are still poorly studied (Guillette and Edwards, 2005; Hickey and Martin, 2009). Impaired host resistance is a likely mechanism affecting parasitic infection under nitrate pollution (see Hrubec et al., 1997; Rodríguez-Estival et al., 2010); parasites may, however, be more sensitive to nitrate than their hosts, as reported for *Saprolegnia* oomycete infections on tadpoles (Romansic et al., 2006). Given the complexity of host-parasite relationships, identifying which types of parasite-host-environmental combinations are likely to promote pathogenesis with nutrient enrichment becomes a research priority of disease ecology under global change (Johnson and Carpenter, 2008).

The monogenean gyrodactylids are well-known pathogens with direct-life cycles that cause mass mortalities on wild and captive fish populations (Bakke et al., 2007). A good example is *Gyrodactylus salaris* that was introduced into Norway in the 1970s and has devastated Atlantic salmon (*Salmo salar*) stocks (Olstad, 2013). Gyrodactylids can survive in eutrophic waters (e.g. Valtonen et al., 1997; Maceda-Veiga et al., 2013a), and there is correlative evidence of positive (Lafferty, 1997) or neutral effects of eutrophication on monogenean abundance

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(Vidal-Martínez et al., 2010; Palm, 2011). It is unknown, however, if either trend is associated with a particular nutrient or parasite given the diversity of this parasite group (Poulin, 2002) and the complex nutrient mixture that can lead to eutrophication (Smith et al., 1999). In natural environments, the effects of nutrients on monogeneans can also be masked by other factors associated with eutrophication, such as water turbidity that affects fish shoaling behaviour (Kelley et al., 2012) and hence parasite transmission (e.g. Hockley et al., 2014a, 2014b). Thus, experimental evidence linking nutrient enrichment and specific infections is required to assess the risk of gyrodactylid infections in nutrient-enriched waters.

The present experimental study tests the hypothesis that nutrient enrichment enhances *Gyrodactylus* infections in two populations of Trinidadian guppies, *Poecilia reticulata*. Specifically, our experiment examines how nitrates drive this host-parasite interaction and modulates fish susceptibility to infection after a chronic exposure to three ecologically relevant nitrate concentrations. It is hypothesised that nitrate would increase *Gyrodactylus* infections in fish exposed to nitrate by enhancing parasite proliferation and facilitating parasite attachment on fish skin, defined by the loss of host epidermis structure. If nitrate acts as an immune-suppressor, it is also predicted that fish susceptibility to *Gyrodactylus* infections would increase in fish pre-exposed to nitrate.

2. Materials and methods

2.1. Host and parasite origins

The guppies used in this study (n = 360) were naïve lab-bred juveniles (SL range = 4–10 mm) randomly selected from stock populations kept at Cardiff University. We used two fish populations, originally from the Lower Aripo River (n = 180) and Tacarigua River (n = 180), Trinidad, brought to Cardiff in 2003 and 2007, respectively. Fish were kept in mixed sex stocks 100 l tanks (9 tanks per population) provided with an under gravel filter and artificial plants and flowerpots for refugia. Fish were maintained under 24 ± 1 °C and 12 h light:12 h dark cycle, and fed twice daily with AQUARIAN® tropical fish flakes and weekly with frozen bloodworms. Chemical water properties were determined as follows using the Sera® colorimetric test kits previously used in our laboratory (Maceda-Veiga et al., 2015a): pH = 7, general water hardness (dGH) = 6, carbonate water hardness (dKH) = 5, [ammonia] < 0.5 mg/l, [nitrite] < 0.5 mg/l and [nitrate] < 10 mg/l). To detect a possible bias in nitrate readings, a linear regression was used to determine the relationship between nitrate values measured using the test kit and standard spectrophotometric procedure. Results indicated that the bias was negligible on the nitrate concentrations tested in the present study ($R^2 = 0.95$, slope = 1.00, $P < 0.01$). As the highest nitrate level was out the range of the test kit, we diluted tested solutions with ultrapure Milli-Q® water. The *Gyrodactylus turnbulli* strain LA utilised in experiments was a wild gyrodactylid strain isolated from the Lower Aripo River (see Cable and Van Oosterhout, 2007).

2.2. Experimental infections and screening procedure

Each fish was inoculated with two individual gyrodactylids following a well-established procedure (e.g. Cable and Van Oosterhout, 2007; Faria et al., 2010). Briefly, each experimental fish was anaesthetised with 0.02% tricaine methanesulfonate (MS222) and placed in a Petri dish containing clean dechlorinated tap water along with a fish parasite donor previously euthanized with an overdose of MS222. Their tails were brought into contact under a dissecting microscope with fibre-optic-epi-illumination to allow the transfer of gyrodactylids. The same equipment was employed to monitor daily parasite numbers on each fish. Anaesthesia was not used in monitoring gyrodactylid infections because it is unknown how MS222 might influence nitrate toxicity. During screening each fish was immobilised in a minimal volume of water in a crystallising dish; this allowed the fish to be gently manipulated so that all fish surfaces were viewed. To detect a possible bias in parasite counts, the relationship between parasite counts from anaesthetised and un-anaesthetised juvenile fish (n = 22) from our parasite cultures with similar parasite numbers to the experimental fish was determined using a linear regression. Results indicated that such bias was negligible on these small, easily manipulated fish ($R^2 = 1.00$, slope = 1.01, $P < 0.01$).

2.3. Experimental nitrate concentrations and exposure conditions

Three nitrate concentrations were considered (<10, 50 and 250 mg NO_3^-/l) and potassium nitrate (KNO_3) was used as nitrate source to test two hypotheses (see Experiments 1 and 2 below). The lowest nitrate concentration represents laboratory dechlorinated tap water, the intermediate is the current safety nitrate threshold established by legislation (Legislative value; European Union Nitrates Directive, 1991; European Groundwater Directive, 2006) and the highest level is within the range reported in rivers in European designated 'Nitrate Vulnerable Zones' (European Environment Agency, 2009) and tropical, nitrate-polluted countries (WRA/MIN, 2002; WHO, 2004). Fish were exposed to the experimental nitrate solutions individually in 1 l containers, and the solutions were fully renewed every 3 days on a balance between minimising fish stress associated with handling and maintaining the experimental environmental conditions. Water samples randomly collected from the different treatments and analysed as detailed above indicated that experimental conditions remained constant through the experiment.

Nitrate solutions were hand-made before each water change to further guarantee the accuracy of experimental nitrate concentrations.

2.4. Experiment 1: direct effect of nitrates on the host-parasite system

To assess direct effects of nitrate on the host and the host-parasite system, uninfected ($N_U = 90$) and infected ($N_I = 90$) fish with two worms were exposed to the three experimental nitrate concentrations, <10 mg/l ($N_U = 30$; $N_I = 30$), 50 mg/l ($N_U = 30$; $N_I = 30$) or 250 mg/l ($N_U = 30$; $N_I = 30$). The experiment was terminated when 80% of infected guppies at 250 mg/l were parasite-free. This allowed a representative number of fish (n = 10) from each treatment to be sampled for histological examination (see below).

2.5. Experiment 2: effect of chronic nitrate exposure on fish susceptibility to infection

Naïve fish (n = 60/treatment) were kept at the three experimental nitrate concentrations for 34 days. Any host mortality was recorded. After 34 days, 10 fish per treatment were processed for histology and the remainder were transferred to clean water. These fish were then infected with two gyrodactylid worms, and parasite intensity was monitored daily. The experiment was terminated when the parasite load increased above 40 individuals per fish following animal welfare standards for fish of this size range (8 days old).

2.6. Fish size and histology

On termination of both Experiments 1 and 2, the standard length of all fish was measured after anaesthesia with MS222. A sub-sample of 10 fish per treatment was then sacrificed using an overdose of MS222, and each caudal peduncle was processed for histology. The remaining individuals were treated to remove gyrodactylid infections (Schelkle et al., 2009) and/or transferred to clean water to recover from nitrate. For histology, samples were fixed in 10% neutral buffered formalin, dehydrated in ethanol, cleared in xylene and embedded in paraffin wax following similar studies (e.g. Gheorghiu et al., 2007; Maceda-Veiga et al., 2013b). Sections (5 μm thick; n = 30 per host) were cut at the same position of the caudal peduncle and stained with conventional

Hematoxylin and Eosin. Photographs were taken from each histological slide using 1000× magnification (Zeiss Axioskop) and a digital camera (Nikon Coolpix E4500; 2272 × 1704 pixels). The epidermal thickness was measured and epidermal cell numbers were counted along ten 0.1 mm length skin sections of each fish using ImageJ® (National Institute of Health, U.S.A.).

2.7. Statistical analysis

All statistical analysis was conducted using R version 2.15.1 (R development Core Team, 2008). Differences in *Gyrodactylus turnbulli* mean intensity (as defined by Bush et al., 1997) between nitrate conditions over time were analysed using a linear mixed model (GLMM, 'lmer' function in R) with Poisson distribution, followed by Tukey's honestly significant difference (HSD) multiple comparisons using the 'ghlt' function from the multcomp package (Hothorn et al., 2008). 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. Main and interactive effects of standard length with nitrate treatment were included to account for the effect of nitrate treatment on parasite intensity at different host standard lengths. Nitrate treatment and time were also included as interactive terms to determine the effect of nitrate treatment on intensity of parasites over time. Standard length and time were also introduced in the models as interaction terms to account for the effect of standard length on parasite intensity over time. Finally, fish population was included in the models but it was deleted in the final models due to the lack of significance (all $P > 0.10$). Significance of explanatory factors was tested using a likelihood-ratio test. Differences in fish survival between nitrate and parasite treatments over time were determined using the 'survit' function. Likewise, mean cell number and epidermis thickness were compared between these treatments using an Analysis of Variance (ANOVA) followed by Tukey HSD as a post-hoc test. Significance in all statistical procedures was reached at $P < 0.05$.

2.8. Ethical note

All of the work conducted in these experiments was covered by UK Home Office Licence (PPL 30/2876).

3. Results

3.1. Experiment 1: direct effect of nitrates on the host-parasite system

Nitrate-enrichment significantly prevented the mean intensity of *G. turnbulli* from increasing on guppies exposed to 50 mg NO_3^-/l (GLMM: $z = 4.45$, S.E. $= 0.28$, $P < 0.001$) and 250 mg NO_3^-/l (GLMM: $z = 8.85$, S.E. $= 0.30$, $P < 0.001$) compared to the controls at <10 mg NO_3^-/l (Table 1A, Fig. 1). In addition, fish size, ranging from 4 to 10 mm, influenced *G. turnbulli* intensity (Table 1A), with larger fish having lower parasite numbers in all three nitrate treatments (All $P < 0.001$). Skin epidermis was thicker at 250 mg NO_3^-/l than in the controls (Tukey HSD test, $P = 0.04$), but without significant changes in mean epidermal cell number (Tukey HSD test, $P > 0.05$) (Fig. 2A). Likewise, mean host epidermal cell number did not differ between nitrate treatments (Table 2), but was marginally significant between infected and uninfected fish (Table 2, Fig. 2A).

3.2. Experiment 2: effect of chronic nitrate exposure on fish susceptibility to infection

Guppies previously exposed to nitrate-enriched waters, which were then infected with *G. turnbulli*, had lower parasite intensities than controls (Table 1B), suggesting a host protective action of nitrate (Fig. 3). Such a positive effect was concentration-dependent, with lower parasite intensities on the fish from the 250 mg NO_3^-/l treatment (GLMM: $z = -2.95$, S.E. $= 0.26$, $P = 0.003$), compared to those at 50 mg NO_3^-/l (GLMM: $z = -6.78$, S.E. $= 0.26$, $P < 0.001$) and controls (Fig. 3). Besides reducing infection intensity, exposures of 50 mg NO_3^-/l and 250 mg NO_3^-/l were lethal to some fish, with higher survival at 50 mg NO_3^-/l (90%) compared to 250 mg NO_3^-/l (57%) NO_3^-/l (Fig. 4). No mortality occurred in controls throughout the entire study period (Fig. 4). As for Experiment 1, nitrate exposure increased fish epidermal thickness but differences occurred between the controls and the two nitrate treatments (all Tukey HSD, $P < 0.01$; Fig. 2B). Epidermal thickness in non-infected fish pre-exposed to nitrate decreased after 8 days in clean water (all Tukey HSD, $P < 0.01$), suggesting a quick fish recovery. Parasite intensity decreased with fish size after pre-exposure to 250 mg NO_3^-/l treatment (GLMM: $z = -4.90$, S.E. $= 0.02$, $P < 0.001$), whereas the opposite occurred in the controls and at 50 mg NO_3^-/l (GLMM: $z = 7.51$, S.E. $= 0.02$, $P < 0.001$). Infection increased mean epidermal cell number (Tukey HSD, $P < 0.01$), but without major differences between the nitrate treatments (Fig. 2B).

Table 1
GLMM output for guppy juveniles (*Poecilia reticulata*) simultaneously exposed for 7 days to *Gyrodactylus turnbulli* infection and three ecologically relevant nitrate concentrations (<10, 50 and 250 mg NO_3^-/l) (Experiment 1), and infected with *Gyrodactylus turnbulli* in clean water following exposure for 32 days to three ecologically relevant nitrate concentrations (<10, 50 and 250 mg NO_3^-/l) (Experiment 2).

Variables	χ^2	df	P value
Experiment 1			
Nitrate treatment	78.25	2	<0.001
Fish SL	3.15	1	0.076
Time	317.02	6	<0.001
Nitrate treatment: fish SL	162.40	2	<0.001
Nitrate treatment: time	78.31	12	<0.001
	Experiment 2		
Nitrate treatment	73,562	2	<0.001
Fish length	7.07	1	0.008
Time	1043.69	7	<0.001
Nitrate treatment: fish length	101.85	2	<0.001
Nitrate treatment: time	40.42	14	<0.001

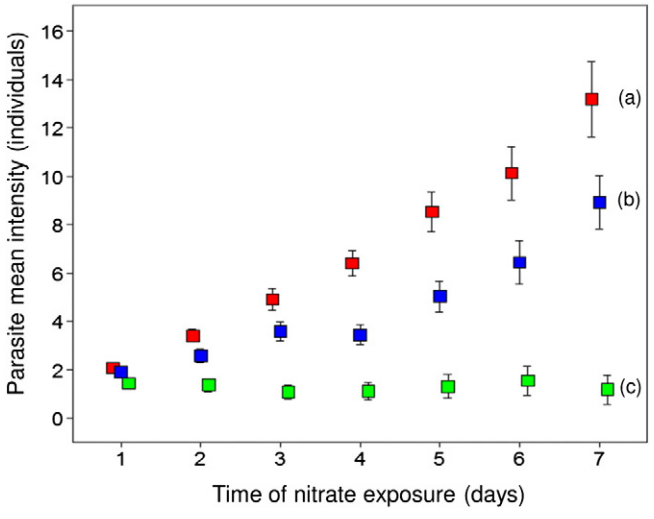

Fig. 1. Trends in *Gyrodactylus turnbulli* intensity (mean ± SEM) on guppies (*Poecilia reticulata*) exposed to three nitrate concentrations (Experiment 1): <10 mg NO_3^-/l (red, a), 50 mg NO_3^-/l (blue, b), and 250 mg NO_3^-/l (green, c). Note that the highest nitrate level prevented mean infection intensity from increasing on guppies.

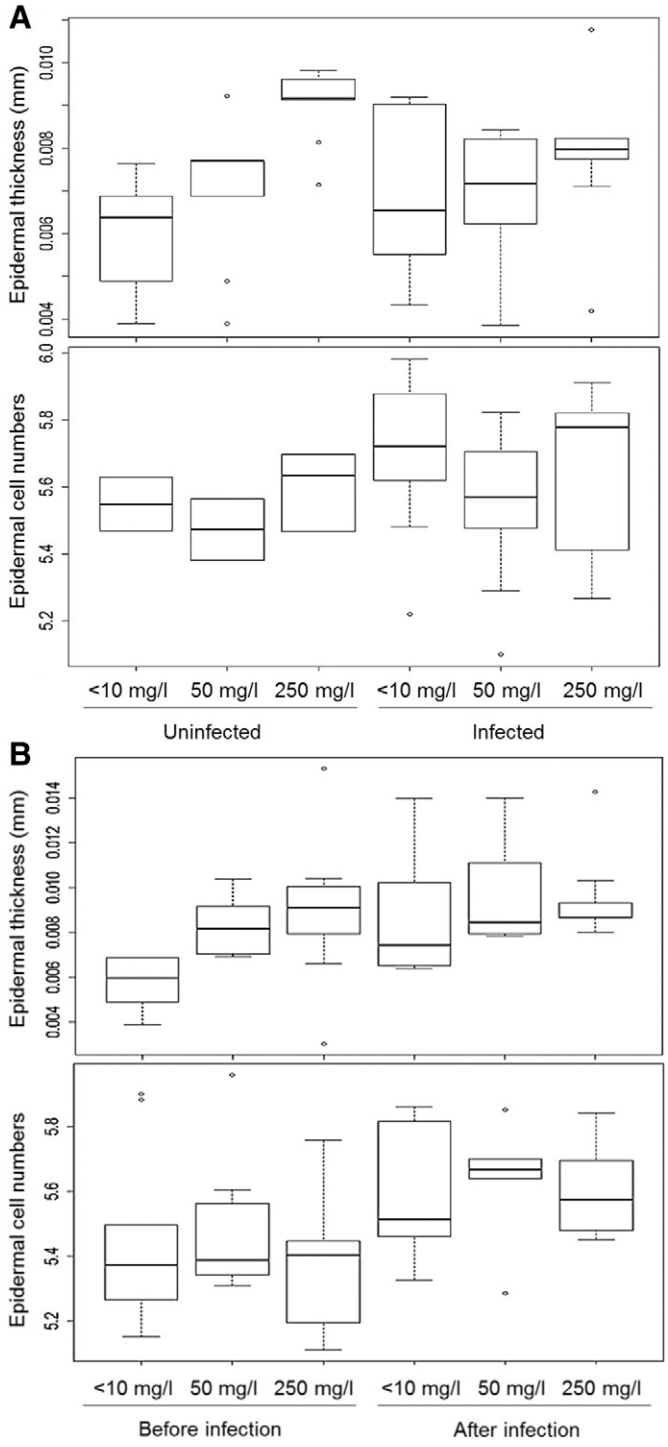

Fig. 2. Box-plots of changes in epidermal cell numbers and thickness in guppies infected with *Gyrodactylus turnbulli* exposed (A) and pre-exposed (B) to three ecologically relevant nitrate concentrations (<10, 50 and 250 mg NO_3^-/l). Each box corresponds to 25th and 75th percentiles; the dark line inside each box represents the median; error bars show the minima and maxima except for outliers shown as open circles.

4. Discussion

Nutrient enrichment can dramatically reduce the severity of an ectoparasitic fish infection. In particular, our results show that nitrate concentrations ranging between 50 and 250 mg NO_3^-/l reduce *Gyrodactylus turnbulli* infection intensity in guppies, and that the highest value can even clean the parasites from the fish. This, added to the fact that host nitrate pre-exposure reduced parasite intensity, suggests that nitrate did not act as an immune-suppressor. Given that ecologically relevant nitrate levels were used in this study, our results inform water agencies of the risk of current water management policies.

Table 2
ANOVA outputs for changes in mean epidermal cell numbers and thickness in guppies from Experiment 1 and 2 (see Materials and methods) infected and non-infected with *Gyrodactylus turnbulli* exposed to three ecologically relevant nitrate concentrations (<10, 50 and 250 mg NO_3^-/l).

Variables	F	df	P value
Experiment 1			
Epidermal thickness			
Nitrate treatment	9.31	2,54	<0.001
Infection	0.07	1,54	0.797
Nitrate treatment: infection	2.87	2,54	0.066
Epidermal cell number			
Nitrate treatment	2.25	2,54	0.115
Infection	3.89	1,54	0.054
Nitrate treatment: infection	0.047	2,54	0.628
Experiment 2			
Epidermal thickness			
Nitrate treatment	5.25	2,54	0.008
Infection	6.48	1,54	0.014
Nitrate treatment: infection	1.38	2,54	0.259
Epidermal cell number			
Nitrate treatment	0.55	2,54	0.583
Infection	11.02	1,54	0.002
Nitrate treatment: infection	0.25	2,54	0.781

The reduced infection intensity of *G. turnbulli* on the guppy host in nitrate-enriched waters contrasts with reviews on the use of fish parasites as bioindicators reporting that eutrophication can have a positive (Lafferty, 1997) or neutral (Vidal-Martínez et al., 2010; Palm, 2011) effect on monogeneans. Our results, however, agree with Romansic et al. (2006) who found that nitrate kills the pathogenic water mold *Saprolegnia* on the northern red-legged frog (*Rana aurora*). Nitrate values in our study were, however, higher (50–250 mg NO_3^-/l) than those (5–20 mg NO_3^-/l) reported by Romansic et al. (2006), and nitrate also caused mortality to the host. Mortality also occurred in corals in nutrient-enriched waters (Vega Thurber et al., 2014), partly explained because nitrate enhances the virulence of the pathogenic fungus

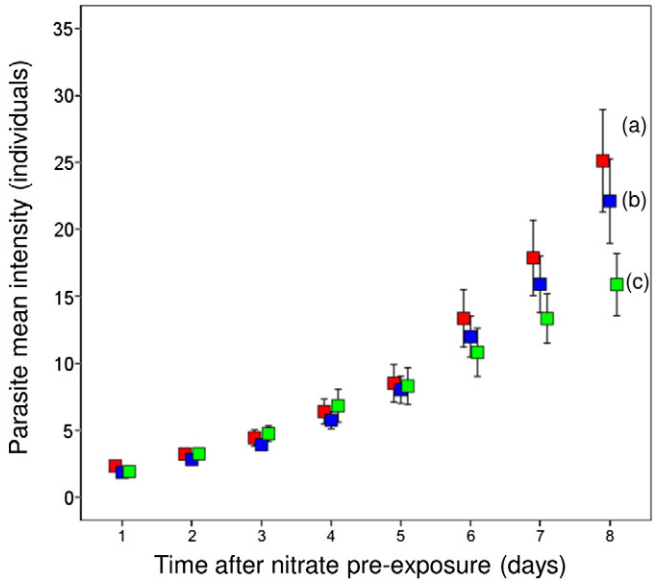

Fig. 3. Trends in *Gyrodactylus turnbulli* intensity (mean ± SEM) on guppies (*Poecilia reticulata*) infected in clear water following exposure to three ecologically relevant nitrate concentrations (Experiment 2): <10 mg NO_3^-/l (red, a), 50 mg NO_3^-/l (blue, b), and 250 mg NO_3^-/l (green, c). Note that mean infection intensity was markedly reduced in guppies pre-exposed to 250 mg NO_3^-/l. s.

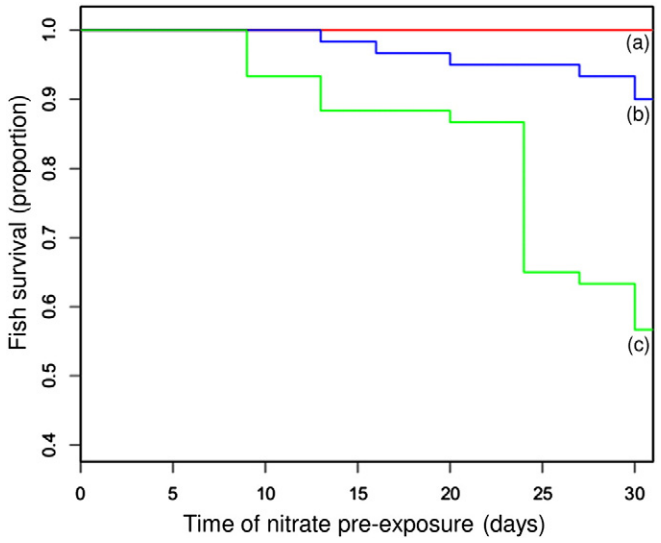

Fig. 4. Guppy (*Poecilia reticulata*) survival along a 32 day exposure to three ecologically relevant nitrate concentrations (Experiment 2): <10 mg NO_3^-/l (red, a), 50 mg NO_3^-/l (blue, b), and 250 mg NO_3^-/l (green, c).

Aspergillus spp. (see Olutiola and Cole, 1977). In our study, however, nitrate not only reduced the severity of *G. turnbulli* infection on the guppy host, but also made the fish less vulnerable to subsequent infection. These contrasting effects highlight the context-dependence of responses of host-parasite systems to nitrate, and the need of empirical studies to understand how ectoparasitic diseases thrive in nutrient-enriched waters.

The reduced *G. turnbulli* infection intensity on the guppy host in the nitrate treatment can be attributed to enhanced host immunity and/or to the direct toxic action of nitrate to the parasite or to the host. In fish, epidermis thickening is a typical innate response to the presence of ectoparasites and water quality hazards, including *G. turnbulli* infections and waterborne metals (Gheorghiu et al., 2007). Thus, the observed increase in epidermis thickness in non-infected guppies exposed to 250 mg NO_3^-/l can be understood as a protective mechanism being enhanced in the host. This is supported by the fact that the fish infected with *G. turnbulli* following a 30 days, nitrate exposure had lower parasite intensity than the controls. In contrast, Hrubec et al. (1997) reported immune-suppression in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) exposed for 28 days to 200 mg NO_3^- N/l. Such an effect has also been reported for other vertebrates, including humans (Ustyugova et al., 2002; Rodríguez-Estival et al., 2010). As far as we are aware, our study is the first to report a positive effect of pollutants on disease resistance, as opposed to examples of acquired tolerance of fish to toxics following pre-exposure to sub-lethal levels of the same or other pollutants (reviewed by Biagianti-Risbourg et al., 2013).

Although our study suggests that nitrate pre-exposure may enhance guppy resistance to *G. turnbulli* infection, it also shows that nitrate exposure for >10 days to 50 or 250 mg NO_3^-/l can be lethal to the fish. Since we observed mortality at nitrate levels lower that the lethal concentration (71 h LC50 = 200 mg NO_3^- N/l.) reported by Rubin and Elmaraghy (1977), this highlights the risk of chronic exposure of fish to sub-lethal levels of nitrate. Fish size strongly affected *G. turnbulli* intensity on the guppy host and its vulnerability to nitrate, supporting previous data on other pollutants and ectoparasites including gyrodactylids (Poulin, 1999; Cable and Van Oosterhout, 2007). We, however, find a surprising negative relationship between fish size and parasite number in clean and nitrate-polluted treatments. Although we cannot determine the reasons behind this, such a pattern might be related to the small size range of fish examined in our study and the selective mortality of large, heavily infected fish. The latter, however, only applies to the nitrate treatments because no mortality occurred in the control.

The main toxic action of nitrate on aquatic animals is reportedly similar to nitrite in that it oxidises haemoglobin to methaemoglobin, which is incapable of carrying oxygen (Comly, 1945; but see Guillette and Edwards, 2005). This should have led to typical behavioural responses of fish to hypoxia (e.g. swimming close to the surface), but did not occur in our study (*authors' obs*). Another plausible explanation to fish mortality could be a compromised osmotic balance due to changes in water ionic composition and altered epidermal structure (Glover et al., 2013). Even though the effect of epidermis thickening on fish osmoregulation remains unknown for our study, it is unlikely to have been a major health issue because fish skin did not exhibit signs of disease (e.g. oedema, necrosis, and leucocyte infiltration) over the 32 days of our experiment. Also, since we used potassium nitrate as nitrate source, potassium could have led to pathological condition either in the guppy host or in the parasite caused by abnormal potassium levels (hyperkalaemia). Nonetheless, this seems to occur in fish under toxic levels of nitrogenous compounds regardless of potassium addition, as reported by Gisbert et al. (2004) in Siberian sturgeons (*Acipenser baerii*) after an acute exposure to sodium nitrite.

Since our study revealed fish mortality at 50 mg NO_3^-/l, the legislative safety NO_3^- concentration (EU Nitrates Directive), it supports previous data on other aquatic species recommending a reduction in the safety nitrate threshold (reviewed by Camargo et al., 2005). Although these studies cannot identify the toxic action of NO_3^-, they used environmentally relevant levels of nitrate mixtures with typical sources of nitrogen used in agriculture (sodium and potassium nitrate) (Smil, 2001). The latter seems to be more toxic than sodium nitrate to uninfected hosts (Camargo et al., 2005; Hickey and Martin, 2009). Host-parasite combinations do, however, often show different pathological outcomes under the same conditions (Marcogliese and Pietrock, 2011). Thus, more in-depth understanding of how nitrate affects hosts and parasites is needed, for instance, using in-vitro assays (e.g. Schelkle et al., 2013), blood analyses (e.g. Maceda-Veiga et al., 2015b) and traditional and/or -omic endpoints, including immunological assays (e.g. Boughton et al., 2011; Colin et al., 2016).

Overall, our results clearly show that the current nitrate level in some surface waters can threaten the biota, even though it may prevent an ectoparasitic disease from increasing. As nitrate is a major water quality hazard in Trinidadian waters (WRA/MIN, 2002), and guppies are the main top-down controllers of the aquatic community in some catchments (Walsh et al., 2011), their extirpation may threaten further the aquatic network stability, increasing multi-trophic impacts (Schwarzmüller et al., 2015) and mosquito-borne diseases (Rejmankova et al., 2006; Seng et al., 2008). Although the disease risk associated with nutrient enrichment is hard to predict due to the diversity and multi-factorial origin of diseases, this may be minimised if states reduce nutrient release into water bodies, particularly given the forecasted agricultural intensification to satisfy world population food demand.

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References

Bakke, T.A., Cable, J., Harris, P.D., 2007. The biology of gyrodactylid monogeneans: the "Russian-doll killers". Adv. Parasitol. 64, 161–376.

Biagianti-Risbourg, S., Paris-Palacios, S., Mouneyrac, C., Amiard-Triquet, C., 2013. Pollution acclimation, adaptation, resistance, and tolerance in ecotoxicology. Encyclopedia of Aquatic Ecotoxicology. Springer, Netherlands, pp. 883–892.
Boughton, R.K., Joop, G., Armitage, S.A., 2011. Outdoor immunology: methodological considerations for ecologists. Funct. Ecol. 25, 81–100.
Bruno, J.F., Petes, L.E., Drew Harvell, C., Hettinger, A., 2003. Nutrient enrichment can increase the severity of coral diseases. Ecol. Lett. 6, 1056–1061.
Bush, A.O., Lafferty, K.D., Lotz, J.M., Shostak, A.W., 1997. Parasitology meets ecology on its own terms: Margolis et al. revisited. Int. J. Parasitol. 575–583.
Cable, J., Van Oosterhout, C., 2007. The impact of parasites on the life history evolution of guppies (*Poecilia reticulata*): the effects of host size on parasite virulence. Int. J. Parasitol. 37, 1449–1458.
Camargo, J.A., Alonso, A., Salamanca, A., 2005. Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. Chemosphere 58, 1255–1267.
Colin, N., Porte, C., Fernandes, D., Barata, C., Padrós, F., Carrassón, M., Monroy, M., Rocabayera-Cano, O., de Sostoa, A., Piña, B., Maceda-Veiga, A., 2016. Ecological relevance of biomarkers in monitoring studies of macro-invertebrates and fish in Mediterranean rivers. Sci. Total Environ. 540, 307–323.
Comly, H.H., 1945. Cyanosis in infants caused by nitrate in well water. J. Am. Med. Assoc. 129, 112–116.
Coyner, D.F., Spalding, M.G., Forrester, D.J., 2003. Epizootiology of *Eustrongylides ignotus* in Florida: transmission and development of larvae in intermediate hosts. J. Parasitol. 89, 290–298.
EEC, 1991. Council Directive 19/676/EEC of 12 December, 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources. Official Journal L 375, 1.
ECC, 2006. Directive 2006/118/EC of the European Parliament and of the Council of 12 December 2006 on the protection of groundwater against pollution and deterioration. Official Journal of the European Union, L 372.
EEA, 2009. Nitrate vulnerable zones in EU available at: http://www.eea.europa.eu/data-and-maps/figures/nitrate-vulnerable-zones-eu with nitrate value provided by the Catalan Water Agency in Catalonia, Spain.
Faria, P.J., van Oosterhout, C., Cable, J., 2010. Optimal release strategies for captive-bred animals in reintroduction programs: experimental infections using the guppy as a model organism. Biol. Conserv. 143 (1), 35–41.
Gheorghiu, C., Cable, J., Marcogliese, D.J., Scott, M.E., 2007. Effects of waterborne zinc on reproduction, survival and morphometrics of Gyrodactylus turnbulli (Monogenea) on guppies (Poecilia reticulata). Int. J. Parasitol. 37 (3), 375–381.
Gisbert, E., Rodrıguez, A., Cardona, L., Huertas, M., Gallardo, M.A., Sarasquete, C., et al., 2004. Recovery of Siberian sturgeon yearlings after an acute exposure to environmental nitrite: changes in the plasmatic ionic balance, Na+–K+ ATPase activity, and gill histology. Aquaculture 239, 141–154.
Glover, C.N., Bucking, C., Wood, C.M., 2013. The skin of fish as a transport epithelium: a review. J. Comp. Physiol. B. 183, 877–891.
Grizzetti, B., Bouraoui, F., Aloe, A., 2012. Changes of nitrogen and phosphorus loads to European seas. Glob. Chang. Biol. 18, 769–782.
Guillette Jr., L.J., Edwards, T.M., 2005. Is nitrate an ecologically relevant endocrine disruptor in vertebrates? Integr. Comp. Biol. 45, 19–27.
Hickey, C.W., Martin, M.L., 2009. A review of nitrate toxicity to freshwater aquatic species. Environment Canterbury, Canterbury, UK.
Hockley, F.A., Wilson, C.A.M.E., Graham, N., Cable, J., 2014a. Combined effects of flow condition and parasitism on shoaling behaviour of female guppies *Poecilia reticulata*. Behav. Ecol. Sociobiol. 68, 1513–1520.
Hockley, F.A., Wilson, C.A.M.E., Brew, A., Cable, J., 2014b. Fish responses to flow velocity and turbulence in relation to size, sex and parasite load. J. R. Soc. Interface 11, 20130814.
Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J. 50, 346–363.
Hrubec, T.C., Robertson, J.L., Smith, S.A., 1997. Effects of ammonia and nitrate concentration on hematologic and serum biochemical profiles of hybrid striped bass (*Morone chrysops* x *Morone saxatilis*). Am. J. Vet. Res. 58, 131–135.
Johnson, P.T., Carpenter, S.R., 2008. Influence of eutrophication on disease in aquatic. Infectious Disease Ecology: Effects of Ecosystems on Disease and of Disease on Ecosystems. 71.
Johnson, P.T., Chase, J.M., Dosch, K.L., Hartson, R.B., Gross, J.A., Larson, D.J., Carpenter, S.R., 2007. Aquatic eutrophication promotes pathogenic infection in amphibians. Proc. Natl. Acad. Sci. 104, 15781–15786.
Johnson, P.T., Townsend, A.R., Cleveland, C.C., Glibert, P.M., Howarth, R.W., McKenzie, V.J., et al., 2010. Linking environmental nutrient enrichment and disease emergence in humans and wildlife. Ecol. Appl. 20, 16–29.
Kelley, J.L., Phillips, B., Cummins, G.H., Shand, J., 2012. Changes in the visual environment affect colour signal brightness and shoaling behaviour in a freshwater fish. Anim. Behav. 83, 783–791.
Lafferty, K.D., 1997. Environmental parasitology: what can parasites tell us about human impacts on the environment? Parasitol. Today 13, 251–255.
Lafferty, K.D., 2014. Biodiversity loss and infectious diseases. Applied Ecology and Human Dimensions in Biological Conservation. Springer, Berlin Heidelberg, pp. 73–89.
Maceda-Veiga, A., Monroy, M., Salvado, H., Cable, J., de Sostoa, A., 2013a. Ectoparasites of native cyprinid *Barbus haasi*: first record of *Trichodina acuta* and *Trichodina fultoni* in Iberian catchments. Bull. Eur. Assoc. Fish Pathol. 33, 187–193.
Maceda-Veiga, A., Monroy, M., Navarro, E., Viscor, G., de Sostoa, A., 2013b. Metal concentrations and pathological responses of wild native fish exposed to sewage discharge in a Mediterranean river. Sci. Total Environ. 449, 9–19.
Maceda-Veiga, A., Figuerola, J., Martínez-Silvestre, A., Viscor, G., Ferrari, N., Pacheco, M., 2015a. Inside the Redbox: applications of haematology in wildlife monitoring and ecosystem health assessment. Sci. Total Environ. 514, 322–332.
Maceda-Veiga, A., Webster, G., Canals, O., Salvadó, H., Weightman, A.J., Cable, J., 2015b. Chronic effects of temperature and nitrate pollution on *Daphnia magna*: is this cladoceran suitable for widespread use as a tertiary treatment? Water Res. 83, 141–152.
Marcogliese, D.J., Pietrock, M., 2011. Combined effects of parasites and contaminants on animal health: parasites do matter. Trends Parasitol. 27, 123–130.
McKenzie, V.J., Townsend, A.R., 2007. Parasitic and infectious disease responses to changing global nutrient cycles. EcoHealth 4, 384–396.
Millennium Ecosystem Assessment, 2005. Millennium Ecosystem Reports. Available at www.maweb.org.
Olstad K. (2013): NOBANIS – Invasive Alien Species Fact Sheet – *Gyrodactylus salaris* – From: Online Database of the European Network on Invasive Alien Species – NOBANIS www.nobanis.org, Date of access 02/02/2016.
Olutiola, P.O., Cole, O.O., 1977. Some environmental and nutritional factors affecting growth and sporulation of *Aspergillus sydowi*. Physiol. Plant. 39, 239–242.
Palm, H.W., 2011. Fish parasites as biological indicators in a changing world: can we monitor environmental impact and climate change? Progress in Parasitology. Springer, Berlin Heidelberg, pp. 223–250
Poulin, R., 2002. The evolution of monogenean diversity. Int. J. Parasitol. 32, 245–254.
R Development Core Team, 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria 3-900051-07-0 URL http://www.R-project.org.
Rejmankova, E., Grieco, N., Achee, P., Masuoka, K., Pope, D., Roberts, D., Higashi, R.M., 2006. Freshwater community interactions and malaria. In: Collinge, S., Ray, C. (Eds.), *Disease Ecology*: *Community Structure and Pathogen Dynamics*. Oxford University Press, Oxford, UK, pp. 90–104.
Rodríguez-Estival, J., Martínez-Haro, M., Martín-Hernando, M.P., Mateo, R., 2010. Subchronic effects of nitrate in drinking water on red-legged partridge (*Alectoris rufa*): oxidative stress and T-cell mediated immune function. Environ. Res. 110, 469–475.
Romansic, J.M., Diez, K.A., Higashi, E.M., Blaustein, A.R., 2006. Effects of nitrate and the pathogenic water mold *Saprolegnia* on survival of amphibian larvae. Dis. Aquat. Org. 68, 235–243.
Rubin, A.J., Elmaraghy, G.A., 1977. Studies on the toxicity of ammonia, nitrate and their mixtures to guppy fry. Water Res. 11, 927–935.
Schelkle, B., Shinn, A.P., Peeler, E., Cable, J., 2009. Treatment of gyrodactylid infections in fish. Dis. Aquat. Org. 86, 65–75.
Schelkle, B., Snellgrove, D., Cable, J., 2013. In vitro and in vivo efficacy of garlic compounds against *Gyrodactylus turnbulli* infecting the guppy (*Poecilia reticulata*). Vet. Parasitol. 198, 96–101.
Schwarzmüller, F., Eisenhauer, N., Brose, U., 2015. 'Trophic whales' as biotic buffers: weak interactions stabilize ecosystems against nutrient enrichment. J. Anim. Ecol. 84, 680–691.
Seng, C.M., Setha, T., Nealon, J., Socheat, D., Chantha, N., Nathan, M.B., 2008. Community-based use of the larvivorous fish *Poecilia reticulata* to control the dengue vector *Aedes aegypti* in domestic water storage containers in rural Cambodia. Journal of Vector Ecology 33, 139–144.
Smil, V., 2001. Enriching the Earth: Fritz Haber, Carl Bosch, and the Transformation of World Food Production. Press, MIT.
Smith, V.H., Schindler, D.W., 2009. Eutrophication science: where do we go from here? *Trends Ecol. Evol.* 24, 201–207.
Smith, V.H., Tilman, G.D., Nekola, J.C., 1999. Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. Environ. Pollut. 100, 179–196.
Smith, V.H., Ii, T.P.J., Smith, M.S., 2005. Host nutrition and infectious disease: an ecological view. Front. Ecol. Environ. 3, 268.
Ustyugova, I.V., Zeman, C., Dhanwada, K., Beltz, L.A., 2002. Nitrates/nitrites alter human lymphocyte proliferation and cytokine production. Arch. Environ. Contam. Toxicol. 43 (3), 0270–0276.
Valtonen, E.T., Holmes, J.C., Koskivaara, M., 1997. Eutrophication, pollution and fragmentation: effects on parasite communities in roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*) in four lakes in central Finland. Can. J. Fish. Aquat. Sci. 54, 572–585.
Vega Thurber, R.L., Burkepile, D.E., Fuchs, C., Shantz, A.A., McMinds, R., Zaneveld, J.R., 2014. Chronic nutrient enrichment increases prevalence and severity of coral disease and bleaching. Glob. Chang. Biol. 20, 544–554.
Vidal-Martínez, V.M., Pech, D., Sures, B., Purucker, S.T., Poulin, R., 2010. Can parasites really reveal environmental impact? Trends Parasitol. 26, 44–51.
Walsh, M.R., Fraser, D.F., Bassar, R.D., Reznick, D.N., 2011. The direct and indirect effects of guppies: implications for life-history evolution in *Rivulus hartii*. Funct. Ecol. 25, 227–237.
Whitehead, P.G., Crossman, J., 2012. Macronutrient cycles and climate change: key science areas and an international perspective. Sci. Total Environ. 434, 13–17.
World Health Organization, 2004. Guidelines for drinking-water quality: recommendations (Vol. 1). World Health Organization.
Woodward, G., Gessner, M.O., Giller, P.S., Gulis, V., Hladyz, S., Lecerf, A., et al., 2012. Continental-scale effects of nutrient pollution on stream ecosystem functioning. Science 336, 1438–1440.
WRA/MIN, 2002. Water Resources Agency and Ministry of the Environment. National Report on Integrating the Management of Watersheds and Coastal Areas in Trinidad and Tobago.