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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