Understanding the physiological effects of nitrate pollution on upland bully (*Gobiomorphus breviceps*) in Canterbury, New Zealand

A thesis submitted in partial fulfilment of the requirements for the Degree of Master of Science at the University of Canterbury

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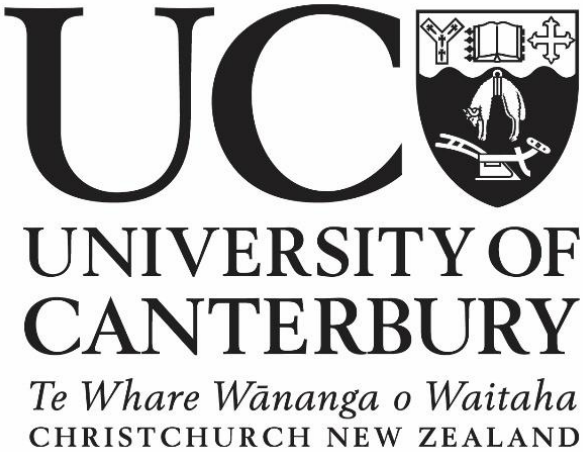

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Abstract

Nitrate pollution is a pervasive threat to freshwater ecosystems worldwide. In Canterbury, New Zealand (NZ), intensive agricultural activities have resulted in large quantities of nitrate (NO_3^-) leaching into and contaminating freshwater streams and rivers. Exposure to elevated nitrate concentrations can cause adverse effects on aquatic life and is thought to be linked to declines in freshwater fish. To address this issue, the NZ National Policy Statement for Freshwater Management has mandated a new national bottom line of 11 mg L^{-1} NO_3^- in rivers (MfE, 2020). However, this limit is primarily based on laboratory-based data collected on non-native fishes, with little consideration of how native freshwater fish species cope with nitrate.

To assess this pressing knowledge gap, I investigated the physiological effects of nitrate exposure on upland bully (*Gobiomorphus breviceps*), an endemic species inhabiting streams in Canterbury, NZ. The energetic costs associated with living in nitrate-contaminated streams were assessed by measuring fish body condition and organosomatic indices along a nitrate contamination gradient (spanning 3 – 51 mgL^{-1} NO_3^-). In the field (Chapter 2). I found non-significant associations between habitat nitrate concentration and fish body condition and organosomatic indices, suggesting living in nitrate-contaminated habitats was not energetically expensive. I further investigated the energetic costs of exposure to nitrate pollution in *G. breviceps*, under tightly controlled conditions, by exposing fish to one of three ecologically relevant nitrate concentrations in the laboratory (0, 11, or 50 mgL^{-1} NO_3^-) for a maximum of 66 days (Chapter 3). Following exposure, aerobic scope (maximum – standard metabolic rate) and performance measures (growth, body condition and organosomatic indices) were measured. Aerobic scope and performance indicators were not compromised in *G. breviceps* exposed to elevated nitrate concentrations (11 and 50 mgL^{-1} NO_3^-), suggesting these fish maintain aerobic performance in the face of nitrate contamination.

Lastly, I investigated the effect of nitrate exposure on the susceptibility of *G. breviceps* to elevated temperatures, because species are often exposed to multiple stressors in current-day freshwater environments. The upper thermal tolerance limits (measured as critical thermal maxima; CTmax) were measured in *G. breviceps* exposed to nitrate (0, 11, or 50 mgL^{-1} NO_3^-) for 56 days. CTmax of *G. breviceps* was unaffected by exposure to nitrate. Taken together, the results of this thesis suggest that *G. breviceps* can maintain growth, performance, and heat tolerance up to concentrations as high as 50 mgL^{-1} NO_3^-. The new national bottom line of 11 mg L^{-1} NO_3^- appears to be sufficient to protect *G. breviceps*, however, future studies should assess these limits for other, potentially more susceptible freshwater species using the framework developed in this thesis.

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Chapter One - General introduction

Nitrate pollution in freshwater environments

Globally, freshwater ecosystems are under threat due to human activities (Dudgeon, 2019). Mass land-use change, increased cycling of nutrients, biodiversity loss, human-facilitated species invasions and climate change are all factors contributing to freshwater ecosystem degradation (Craig et al., 2017; Dudgeon, 2019). Freshwater ecosystems are highly valued in New Zealand for their ecosystem services, economic and social value, and cultural importance (Collier, 2014; Moore, 2014). As freshwater habitats continue to degrade, it is vital for freshwater managers and scientists to understand how anthropogenic stressors impact the health of freshwater systems and the species within them (Ormerod, 2010; Collier, 2014;). One of the most pervasive threats to freshwater ecosystems that has emerged over recent decades is nitrate pollution (Carmago et al., 2005; Opinion et al., 2020).

Nitrate pollution refers to excessive amounts of nitrate leaching into and contaminating water bodies caused primarily by agricultural activities (Zhang et al., 2018). Nitrate (NO_3^-) is the most abundant form of inorganic nitrogen found in freshwater environments (Soucek and Dickinson, 2016). For clarity in this thesis, nitrate will be expressed as mgL^{-1} NO_3^- (milligrams per litre of nitrate). Nitrate occurs naturally in freshwater environments at low concentrations (~0-5 mgL^{-1} NO_3^-), but in highly polluted areas, nitrate concentrations can exceed 100 mgL^{-1} NO_3^- (Gomez Isaza et al., 2020a). Nitrate pollution in freshwater habitats has increased rapidly over the last few decades due to mass fertiliser application, animal waste, sewage runoff, mining, and other anthropogenic activities (Mahvi et al., 2005; Zhang et al., 2014; Gomez Isaza et al., 2020a). Nitrate pollution enters waterways from surrounding areas and can cause eutrophication, toxic algal blooms, and direct physiological stress to aquatic organisms (Carpenter et al., 1998). Intensive agricultural activities are widely

regarded as the largest source of nitrate pollution; remarkably, nitrate fertiliser application now exceeds natural inputs into the global nitrogen cycle (Tilman, 1998).

Nitrate pollution in the Canterbury plains

In New Zealand, intensive agricultural activities, including increased stocking rates and high fertilizer use, have resulted in marked increases in nitrate leaching into our freshwater environments (Joy et al., 2019). Over 70% of New Zealand's total river length now has nitrate levels above what would be expected under natural conditions (Stats NZ, 2020). The Canterbury Plains is one of the most intensively farmed areas in New Zealand (Foote et al., 2015; Joy et al., 2022). Mass land-use intensification from natural areas to highly productive pastures has resulted in some of the highest levels of nitrate contaminating freshwater systems worldwide (Joy et al., 2019). In Canterbury, irrigated dairy farms are the most significant source of nitrate pollution (Foote et al., 2015; Joy et al., 2022). Nitrate from nitrogen-based fertilisers and livestock waste leach into groundwaters and flow into and pollute freshwater habitats (Foote et al., 2015; Death et al., 2018; Joy et al., 2019). When excessive amounts of nitrate contaminate freshwater environments, it can have various adverse indirect and direct effects on aquatic species (Camargo and Alonso, 2006; McDowell et al., 2006; Gomez Isaza et al., 2020a).

Indirect effects of nitrate pollution

One of the most problematic issues of nitrate pollution is eutrophication (Smith and Schindler, 2009; Jenny et al., 2016). Eutrophication results from freshwater habitats becoming enriched with nutrients, such as nitrate and phosphorus, causing significant increases in aquatic macrophyte and algal growth (Smith et al., 2006; McDowell et al., 2009). This large increase in plant growth can clog waterways and strip the water of oxygen (hypoxia or anoxia) at night when plants respire (Jenny et al., 2016; Death et al., 2018).

Further anoxia can occur during the die-off of blooms, where microbial decomposition reduces dissolved oxygen levels (Mhlanga et al., 2006). Hypoxic episodes can result in mass die-offs such as 'fish kills' (Rodgers et al., 2021) and can significantly alter freshwater community assemblages (Death et al., 2018). For example, hypoxic conditions can result in more sensitive fish species becoming eliminated from the system or restricted to more oxygenated areas, such as riffles, while more tolerant species are favoured and can dominate the system, significantly changing community dynamics (Pollock et al., 2007).

Direct effects of nitrate

The most common method used to assess the direct effects of nitrate on aquatic organisms has been with short-term, lethal toxicity tests (i.e. LC_{50} test, Romano and Zeng, 2013). These tests determine the concentration of nitrate which kills half the population (Romano and Zeng, 2013). This method has been widely used to inform nitrate guidelines and regulations (Camargo et al., 2005; Hickey and Martin, 2009; Hickey, 2013; Gomez Isaza et al., 2020a). Lethal toxicity tests have shown that in most cases, only extremely high nitrate concentrations (> 1500 mgL^{-1} NO_3^-) are lethal to freshwater species (Edwards and Hamlin, 2018). One weakness of these studies is that they do not represent sub-lethal effects (e.g., reductions in growth, energy and locomotor performance) that can occur at much lower nitrate concentrations and are not representative of nitrate concentrations in natural freshwater environments (Gomez Isaza et al., 2020b). Furthermore, acute toxicity effects rarely match what is observed in the wild, where confounding effects of other contaminants often create different results (Opinion et al., 2020). Studies which assess sublethal effects are, therefore, much more informative because effects are tested at more ecologically relevant nitrate concentrations (Guillette and Edwards, 2005; Camargo and Alonso, 2006).

Many aquatic organisms likely incur sublethal effects following exposure to high nitrate, as seen in aquatic invertebrates and crustaceans. Alonso and Carmago (2013) found reduced locomotion in the New Zealand mud snail (*Potamopyrgus antipodarum*) following exposure to 191 mgL^{-1} NO_3^- and reproduction (number of live offspring) was diminished following exposure to 94 mgL^{-1} NO_3^- for 35 days. Romano and Zeng (2013) found that in freshwater crustacea, exposure to elevated nitrate concentrations (>150 mgL^{-1} NO_3^-) caused tissue damage, gill fouling and reduced lipid storage in the hepatopancreas. Although adverse physiological effects have been detected in invertebrates and crustaceans at relatively low nitrate concentrations, fish are typically more vulnerable to elevated nitrate because nitrate continuously seeps into their bodies via the gills and enters the circulatory system (Carmargo et al., 2005; Gomez Isaza et al., 2020a).

Freshwater fish are more susceptible to nitrate than marine fish because there is a higher saturation of chloride ions (Cl^-) in salt water, and chloride ions behave as a competitive inhibitor of nitrate/nitrite ion uptake across the gills (Camargo et al., 2005). Therefore, this reduces the amount of nitrate entering the blood (Jensen, 2003). The first indication that exposure to low nitrate concentrations might harm freshwater fish came from Kincheloe et al. (1979). Exposure to low concentrations of nitrate (<11 mgL^{-1} NO_3^-) reduced the survival of fry and the embryos of Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) (Kincheloe et al., 1979).

A later study by Guillette and Edwards (2005) found that exposure to ecologically relevant nitrate concentrations can cause endocrine disruption in freshwater vertebrates through *in-vivo* conversion to nitric oxide, which is involved in many metabolic pathways. Fish exposed to nitrate suffered altered thyroid hormone synthesis and subsequently had irregular development and growth (Guillette and Edwards, 2005). Similarly, Kellock et al. (2018) found that fathead minnows (*Pimephales promelas*) exposed to ecologically relevant

concentrations of nitrate (50 and 250 mgL^{-1} NO_3^-) experienced altered hormone synthesis (estrogen) and modified fish reproductive physiology (increased induction of vitellogenin in males), further suggesting that nitrate acts as an endocrine disruptor. Exposure to elevated nitrate concentrations has been shown to adversely affect reproduction in salmonids. Lake trout (*Salvelinus namaycush*) exposed to relatively low concentrations of nitrate (28 mgL^{-1} NO_3^-) suffered alterations to development and growth.

The main toxic action of nitrate on fish is the conversion of oxygen-carrying pigments (haemoglobin) into forms that cannot carry oxygen (i.e., methaemoglobin), thus reducing the blood oxygen-carrying capacity of the individual (Yang et al., 2019; Gomez Isaza et al., 2020b; Gomez Isaza et al., 2021a). In fish, nitrate continually enters the circulatory system due to passive diffusion across the gills (Camargo and Alonso, 2006). Inside the blood, nitrate is converted from nitrate to nitrite and then oxidises haemoglobin to methaemoglobin resulting in reduced blood oxygen-carrying capacity (Gomez Isaza et al., 2020b). This reduction in blood oxygen-carrying ability can manifest as a reduction in whole-animal aerobic capacity (i.e., aerobic scope; Gomez Isaza et al., 2020a) and cause cascading, adverse effects on aerobically supported performance traits (e.g., growth, locomotion, development, reproduction, predation, and predator avoidance) (Gomez Isaza et al., 2020b; Baker et al., 2017; Opinion et al., 2020). These effects would suggest that it is energetically expensive for fish living in nitrate-polluted habitats.

Reductions in aerobic (maximum metabolic rate (MMR)- standard metabolic rate (SMR)) can be caused by reductions in MMR or increases in SMR (Clark et al., 2013). Exposure to elevated nitrate can cause decreases in MMR due to reductions in blood oxygen carrying and can cause increases in SMR due to the stimulation of energetically demanding detoxifying mechanisms (e.g., methaemoglobin reductase system) (Gomez Isaza et al., 2020b; Gomez Isaza et al., 2020c; Opinion et al., 2020). For example, spangled perch (*Leiopotherapon*

unicolor) exposed to nitrate (50 and 100 mgL^{-1} NO_3^-) suffered reduced functional haemoglobin levels (blood oxygen supply), and this translated to a lowered aerobic scope and reduced growth (Gomez Isaza et al., 2020b). Ultimately, the magnitude and nature of the response of freshwater species to elevated nitrate exposure depends on a range of factors such as species, life-history stage, size, sex, reproductive state, and the dose and duration of nitrate exposure (Camargo et al., 2005; Camargo and Alonso, 2006). The toxic effects of nitrate on freshwater species can also be affected by interactions between nitrate and other stressors (Opinion et al., 2020; Gomez Isaza et al., 2020a; Gomez Isaza et al., 2020b; Gomez Isaza et al., 2021a).

Interactions between nitrate and other stressors

Cumulative impacts of multiple stressors (extreme environmental changes) are now common in many ecosystems (Gomez Isaza et al., 2020a). The combined effect of various stressors on an ecosystem or population can often act in a non-additive manner (Jackson et al., 2016; Opinion et al., 2020). Non-additive interactions between stressors can be antagonistic (impact is less than the sum of both stressors individually) or synergistic (impact is greater than the sum of both stressors separately) (Todgham and Stillman, 2013; Piggott et al., 2015). Freshwater ecosystems are especially sensitive to the non-additive effects of multiple stressors (Jackson et al., 2016). Interactions between nitrate and other stressors have been shown to produce unpredictable outcomes for freshwater species. For example, a study by Gomez-Isaza et al. (2020b) found that simultaneous exposure to elevated nitrate (0, 50 or 100 mgL^{-1} NO_3^-) and low pH reduced the blood oxygen-carrying capacity, the aerobic scope, and the swimming performance of spangled perch (*Leiopotherapon unicolor*). However, a similar experiment by Gomez Isaza et al. (2020c) found that simultaneous exposure to elevated nitrate (0, 50 or 100 mgL^{-1} NO_3^-) and high temperatures (heat stress) did not reduce the aerobic scope or swimming performance of silver perch (*Bidyanus bidyanus*). Silver perch

exposed to high temperatures and high nitrate were able to maintain their aerobic scope and swimming performance better than fish only exposed to nitrate (Gomez Isaza et al., 2020c).

Initial exposure to elevated nitrate has also been shown to increase the susceptibility of an organism to a subsequent stressor (cross-susceptibility) or increase an organism's tolerance to another stressor (cross-tolerance) (Sinclair et al., 2013; Todgham and Stillman, 2013). Nitrate is expected to increase the susceptibility of fish species to other stressors due to reductions in blood oxygen-carrying capacity and which manifests as a reduction in available energy to cope with other stressors (Camargo et al., 2005; Sokolova, 2013; Gomez Isaza et al., 2020b; Opinion et al., 2020). For example, Rodgers et al. (2021) found that exposure to nitrate (50 and 100 mgL^{-1} NO_3^-) increased the susceptibility of a freshwater salmonid (*Thymallus thymallus)* to heat stress. *T. thymallus* exposed to nitrate had a significantly lower thermal tolerance (critical thermal maxima; CTmax) than fish not exposed to nitrate. This finding is particularly concerning for freshwater fish species in New Zealand exposed to high levels of nitrate pollution. Nitrate pollution and water temperatures are expected to increase with climate change (Ficke et al., 2007; Dokulil and Teubner, 2011; Rodgers et al., 2021), threatening the persistence of freshwater fish in New Zealand.

National nitrate limits

In New Zealand, 76% of our native fish species are listed as 'threatened' or 'at risk' of extinction (Stats NZ, 2021), and it is thought that nitrate pollution from intensive agricultural activities may be a major contributing factor to these declines (Allibone et al., 2010; Joy et al., 2019). To address the issue of nitrate pollution in surface waters, the National Policy Statement for Freshwater Management has mandated a new national bottom line of 11 mgL^{-1} NO_3^- in rivers (MfE, 2020). This limit is intended to protect 95% of freshwater species from toxic effects (MfE, 2020). However, data from fish used to derive this limit primarily comes

from laboratory studies of large-bodied salmonids and lacks essential data on the effects of nitrate on native New Zealand fish species (Hickey and martin, 2009; Hickey, 2013; Joy et al., 2022). Moreover, this bottom-line threshold does not account for interactions between nitrate and other environmental stressors (e.g., water temperature) which may exacerbate negative impacts on freshwater species (Rodgers et al., 2021; Joy et al., 2022).

Thesis structure and aims

Nitrate pollution is a major issue in Canterbury, New Zealand, yet the effects of nitrate on native fish remain largely unknown. As such, the overarching aim of this thesis was to investigate the physiological effects of nitrate exposure on upland bully (*Gobiomorphus breviceps*), an endemic species inhabiting streams in Canterbury, New Zealand. Here I used a combination of field surveys (Chapter 2) and laboratory studies (Chapters 3 and 4) to provide a holistic understanding of the physiological effects of nitrate pollution on *G. breviceps.*

Chapter aims:

1. To determine the effect of nitrate on the physiological condition (e.g., body condition, organosomatic indices) of *G. breviceps* living in nitrate-polluted waters under natural field conditions (Chapter 2).
2. To investigate the energetic costs of exposure to elevated nitrate concentrations on *G. breviceps* using measurements of aerobic scope and complementary performance indicators (growth, body condition, organosomatic indices) (Chapter 3).
3. To assess how exposure to nitrate pollution affects the susceptibility of *G. breviceps* to elevated water temperatures (heat stress) (Chapter 4).

The results of this study provide insight into the effects of nitrate pollution on freshwater fish in New Zealand and provide evidence towards the suitability of the national bottom line (11

mg L^{-1} NO_3^-) to protect native fish species in New Zealand. Moreover, this study establishes a nitrate assessment framework for future studies to build upon.

Study Species

Upland bully (*Gobiomorphus breviceps*) is a common non-diadromous endemic freshwater fish species found in the lower North Island and throughout the South Island of New Zealand (McDowall, 1990; Jowett and Richardson, 1995; Smith et al., 2005). *G. breviceps* was chosen as an ideal study species because it is a major inhabitant of lowland streams where nitrate concentrations tend to be highest (Hickey, 2013; Ramezani et al., 2016). *G. breviceps* also differs morphologically and physiologically from large-bodied salmonids, which most nitrate toxicity tests have been conducted to date (Hickey and martin, 2009; Hickey, 2013; Joy et al., 2022). *G. breviceps* also shares similar morphological and physiological traits with two 'at risk' and declining species inhabiting the Canterbury plains; the redfin bully (*Gobiomorphus huttoni*) and bluegill bully (*Gobiomorphus hubbsi*) (Allibone et al., 2010) So results from this study may aid management decisions regarding the conservation of these declining species. Finally, G. *breviceps* have also been used successfully in lab experiments in the past (Hamilton et al., 1997; Hamilton and Poulin, 1999; Hamilton and Poulin, 2001)

Figure 1. A male upland bully (*Gobiomorphus breviceps*) photographed during laboratory experiments. Photographer: Charlie Barker

Chapter two - The physiological condition of upland bully (Gobiomorphus breviceps) in nitrate contaminated waterways

Introduction

Aquatic ecosystems throughout the world are under threat due to nutrient pollution (Carpenter et al., 1998). In particular, agricultural activities and land use intensification are the major causes of excess nutrients, such as nitrate and phosphorus, reaching waterways (Craswell, 2021). Some authors suggest nitrate pollution is one of the most pervasive threats to the health of freshwater systems globally (Carmago et al., 2005; Craswell, 2021).

In New Zealand, dairy farming is the biggest cause of nitrate pollution affecting freshwater environments (Foote et al., 2015). A comprehensive study on water quality in New Zealand found that high-producing pastures (used to feed dairy cows) had the greatest negative impact on river water quality (Julian et al., 2017). The Canterbury Plains is one of the most intensively dairy-farmed areas in New Zealand (Foote et al., 2015). Dairy farming started in Canterbury over 150 years ago and has been increasing rapidly ever since (Greenwood et al., 2012). In particular, over the last three decades, Canterbury has undergone rapid land-use intensification to support high dairy production (Joy et al., 2022; Snelder et al., 2017). From 1990 to 2017, the number of dairy cows in Canterbury increased from 113, 000 to 1.3 million in 2017 (Joy et al., 2022). To support this high density of dairy cows, irrigation is increased, and large amounts of nitrogen-based fertiliser are required to support high-producing pastures (Julian et al., 2017). As a result, excess nitrate from fertilisers and nitrate from animal waste leach into freshwater environments (Joy et al., 2019). Research estimates that nitrate leaching into the environment in Canterbury, New Zealand, has increased from 15,000 tonnes in 1990 to 33,000 tonnes in 2017 (Joy et al., 2022). Despite large amounts of nitrate leaching into waterways in Canterbury (Foote et al., 2015; Snelder et al., 2017), no published studies have

investigated the effects of nitrate pollution on native New Zealand fish species (Hickey and Martin, 2009; Hickey, 2013).

A number of native freshwater fish species are declining in Canterbury, New Zealand, and nitrate pollution is likely a major contributing factor (Allibone et al., 2010; Joy et al., 2019). Laboratory studies overseas have reported that freshwater fish are particularly vulnerable to the effects of nitrate as it enters the body through the gills and can cause a range of adverse developmental, morphological, behavioural, and physiological effects (Carmargo et al., 2005; McGurk et al., 2006; Gomez-Isaza et al., 2020a; Opinion et al., 2020). The primary toxic effect of nitrate on freshwater fish is thought to be the conversion of functional haemoglobin to methaemoglobin which manifests as a reduction in whole-animal aerobic capacity (i.e., aerobic scope; Gomez Isaza et al., 2020a; Gomez Isaza et al., 2020b). Reduction in aerobic capacity can cause cascading effects on aerobically supported performance traits such as growth, foraging ability, reproduction, and development (Gomez Isaza et al., 2020b; Baker et al., 2017; Opinion et al., 2020).

To protect species from the toxic effects of nitrate pollution, the National Policy Statement for Freshwater Management has mandated a new national bottom line of 11 mgL^{-1} NO_3^- in rivers (MfE, 2020). However, there is a paucity of studies that support this value as it is primarily based on laboratory studies conducted overseas with little robust science on its relevance to native fish species from New Zealand (Hickey, 2013; Joy et al., 2022).

Typically, most studies have investigated the effects of nitrate exposure on freshwater fish by measuring short-term lethal tolerance limits ($LC_{50,}$ lethal concentrations which kill half of the population) under controlled laboratory settings (Gomez Isaza, 2020). However, the application of physiological tools such as measures of metabolism, body condition and organosomatic indices offers a more informative way to investigate the sublethal effects of

freshwater pollutants because effects can be measured at ecologically relevant concentrations (McKenzie et al., 2007). Moreover, physiological tools provide mechanistic insight into '*how*' species are affected by contaminants and how these effects may scale up to threaten the persistence of natural populations (Zachariassen et al., 1991; McKenzie et al., 2007). Measurements of body condition such as the scaled mass index (SMI; Peig and Green, 2009) are good indicators of animal fitness because they are indicative of organismal health, reproductive capacity, and energy capital available for growth (Peig and Green, 2009; Maceda-Veiga et al., 2014)

Organosomatic indices describe the ratio of organs to body weight (Geode, 1990). The measurement of Organosomatic indices such as relative liver mass (RLM), relative ventricular mass (RVM), relative spleen mass (RSM) and relative gonad mass (RGM) are useful because they provide important information about health status and energy stores and can be used to determine the effects of pollutants on target organs (Maxwell and Duta, 2005; Giullo and Hinton, 2008; Nagrodski et al., 2013). RLM has been described by Singh and Canario (2004) as an important biomarker for detecting sublethal effects of pollutants due to the liver's role in detoxifying pollutants. Enlargement of the spleen (RSM) can indicate blood oxygen problems or endocrine disruption due to contaminants (Opinion et al., 2020). For example, Akani and Daka (2015) found that in African sharptooth catfish (*Clarias gariepinus*), the relative spleen size of fish increased when exposed to oilfield wastewater. Akani and Daka (2015) suggest this is a possible physiological response to increase red blood cells diminished by exposure to the pollutant. Organosomatic indices can also detect changes in energy status and nutritional information (Maxwell and Duta, 2005). For example, a decrease in liver size can indicate decreased lipid storage and, thus, decreased energy stores (Gabriel et al., 2010).

To date, most studies have investigated the physiological effects of nitrate on fish in controlled laboratory settings rather than in natural environments (Gomez Isaza et al., 2020a). A challenge of conducting experiments in the field is that many other environmental variables, such as water chemistry, might also impact fish's physiological condition. Temperature, pH, dissolved oxygen, conductivity, and alkalinity are all water quality parameters that can affect fish condition, growth, and reproduction (Moogouei, 2010; Maceda-Veiga et al., 2014). Temperature can influence body condition by influencing metabolic rates (Rummer et al., 2014). Energy can be redistributed away from growth at higher temperatures to meet maintenance metabolic rates (Rummer et al., 2014). Chabot and Dutil (1999) studied Atlantic cod (*Gadus morhua L.*) and found that food consumption was reduced as dissolved oxygen levels decreased, resulting in reduced growth and body condition. Numerous other habitat variables could influence fish conditions, such as prey abundance (Leeseberg and Keeley, 2014) and habitat quality and availability (Maceda-Veiga et al., 2014). For example, Oliva-Paterna et al. (2003) found that streams with higher habitat heterogeneity and more refugia produced fish (*Barbus sclateri*) in better conditions. Streams with better instream habitat quality also had increased food available to fish, thus indirectly benefitting the condition of fish (Oliva-Paterna et al., 2003).

Freshwater fish provide crucial functions in streams, such as critical food web linkages and nutrient cycling, thus contributing to healthy functioning aquatic ecosystems (Lamberti et al., 2010). Therefore, as aquatic ecosystems continue to degrade in Canterbury due to agricultural pollution (Foote et al., 2015; Julian et al., 2017), there is an urgent need to assess the impact of nitrate pollution on native fish populations and to determine if current national limits are suitable to protect native fish. This study aimed to determine the effect of nitrate on the physiological condition (e.g., body condition, organosomatic indices) of *Gobiomorphus breviceps* living in nitrate-polluted waters under natural field conditions. I predicted that *G.*

breviceps living in habitats with higher levels of nitrate pollution would be in poorer physiological conditions (poorer body condition, abnormal organosomatic indices) than those living in less nitrate-polluted habitats. This chapter aimed to provide insight into the effects of nitrate pollution on freshwater fish in New Zealand and provide evidence towards the suitability of the national bottom line to protect native fish species in New Zealand.

Methods

Site surveys

Over the summer of 2021 - 2022 (Nov-Jan), field surveys were conducted in Canterbury, New Zealand, to identify suitable streams and rivers to sample upland bully (*G. breviceps*). Appropriate field sites for sampling *G. breviceps* were identified through a combination of site visits, the New Zealand freshwater fish database (https://nzffdms.niwa.co.nz/search), and Land Air and Water Aotearoa (https://www.lawa.org.nz/).

A total of nine sites were sampled. At each site, the presence of upland bullies was confirmed by sampling with a hand net. Nitrate concentrations were measured using a portable nitrate meter (LAQUAtwin-NO3-11 meter, Horiba Scientific). Sites were classified as either low nitrate polluted sites (<11 mgL^{-1} NO_3^-, n = 3), moderately nitrate-polluted sites (11 < 40 mgL^{-1} NO_3^-, n = 4), or highly nitrate-polluted sites (> 40mg mgL^{-1} NO_3^-, n = 2; Figure 2). A mean habitat nitrate concentration was calculated from three different nitrate measurements recorded on different days. Two spot measurements were recorded on different days (first, during site surveys and the second, on the day of fish collection). Two water samples were also taken from each site and sent to Hill Laboratories to verify field site nitrate concentration via analytical methods (Appendix 1).

Figure 2. The nine streams and water races sampled for upland bully (*Gobiomorphus breviceps)* in the Canterbury Plains, New Zealand. (January-March, 2022). These locations were identified from previous studies, preliminary field surveys using the New Zealand freshwater fish database (https://nzffdms.niwa.co.nz/search), Land Air and water Aotearoa (https://www.lawa.org.nz/), and advice from local experts. Coloured pins represent low (green; <11 mgL^{-1} NO_3^-), moderately (yellow: 11<40 mgL^{-1} NO_3^-) and highly (red; > 40 mgL^{-1} NO_3^-) nitrate-polluted sites.

Environmental variables

In-stream habitat quality parameters

In-stream habitat quality parameters have previously been shown to influence the condition of freshwater fish (Oliva-Paterna et al., 2003). Several instream habitat quality parameters were measured at each site using methods adapted from the Environment Canterbury (ECAN) habitat assessment field sheet (Appendix 2). All instream habitat parameters were standardised to be measured on an ordinal scale from 1-10, with '1' being poor and '10' being optimal. Instream habitat roughness was measured by estimating the quality of cover for instream fauna. Substrate heterogeneity was measured by estimating the number of size classes of the benthic substrate. Embedded siltation was measured by estimating how much of the benthic substrate was surrounded by fine sediment. Emergent macrophyte presence was measured by estimating how much rooted plants that emerge from the water dominate the water channel (10 being largely absent). Submergent macrophyte presence was measured by estimating how much submergent rooted plants dominate the water channel (10 being largely absent).

Water chemistry

Water chemistry parameters were measured at each site when fish were collected using portable field meters; These included; Dissolved oxygen (mg/L) using a YSI Ecosense ODO 200, specific conductivity (μScm^{-1} at 25 °C), and temperature (°C) using a YSI Pro 1030. All sites were located between 3-350 m above sea level in the Canterbury Plains. All sites had relatively similar physical attributes (2-3m wide and had cobble and silt substrate)

Aquatic macroinvertebrate sampling

Aquatic macroinvertebrates were sampled using a kick net (0.5 mm mesh). At each site, sampling occurred within a reach of approximately 25 m. Macroinvertebrates were collected

using a kick-net (mesh 0.5mm) across three transects with approximately three minutes of sampling effort per transect (~9 min of sampling effort per site). Once collected, macroinvertebrate samples were preserved in the field in 70% denatured ethanol and transported back to the laboratory for analysis. In the laboratory, samples were washed through a 500 μm sieve, and taxa were identified to the lowest taxonomic group possible using standard keys developed by Winterbourn et al. (2006). During sorting and identification in the laboratory, macroinvertebrate abundances were estimated using coded abundance analysis following protocols by Stark et al. (2001). Coded abundance was used to estimate the total abundance of aquatic macroinvertebrates and the total abundance of (Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies) (EPT).

Fish collection

Fish sampling occurred over January-March (2022) to minimise seasonal effects on fish condition (Landman and Ling, 2011; Kortet et al., 2003). 11 upland bullies were collected using hand nets at each field site and placed in an aerated 20 l bucket. Hand netting was used instead of electrofishing methods because electrofishing can disrupt fish physiology, such as blood chemistry (Matsche et al., 2017), which may compromise the research objectives. Following capture, fish were transported to the University of Canterbury in aerated buckets. The journey typically lasted <2 h, during which the fish showed no adverse effects. This research was conducted with approval from Te Rūnanga o Ngāi Tahu and complied with the University of Canterbury animal ethics requirements (permit no. #202105R).

Body condition

Once in the laboratory, fish were euthanised using pH-buffered tricaine methanesulfonate (MS-222; 250 mg/l). After death had occurred (~3 mins), the wet mass of the fish was recorded (Sartorius TE153S model, Göttingen, Germany; to the nearest 0.001g), and the total

length was measured (mm). Body condition was then calculated using the scaled mass index (SMI) developed by Peig and Green (2009). SMI was chosen as a suitable body condition estimate because it includes a scaling mass factor (SMA) that accounts for the species-specific allometric relationship between mass and length to provide a condition index (Peig and Green, 2009). The scaled mass index (SMI) was calculated using the following equation (1):

$$\text{Scaled mass index (SMI)} = Wi\left[\frac{L_0}{L_i}\right]^{b_{SMA}} \quad (1)$$

Where W_i and L_i are each individual's mass (g) and length (mm), b_{SMA} is the scaling exponent estimated by the SMA regression of W on L. L_0 is the arithmetic mean of the length of all fish in the data set. L_0 for this study was 56.8 mm. b_{SMA} was calculated following procedures by Maceda-Veiga et al. (2014). In R studio (version 2022.12.0 + 353), the bivariate plot of W and length was log-transformed, and the slope of the fitted line b_{SMA} was calculated as 3.5.

Organosomatic indices

Following mass and length measurements, the liver, ventricle, spleen, and gonads were dissected from each fish, and the wet mass of each organ was measured (Sartorius CP225D model, Göttingen, Germany; to the nearest 0.00001g) following protocols by Todgham et al. (2005). Organosomatic indices (% body mass) were calculated using the following equation (2):

$$\text{Organosomatic indices (\% body mass)} = \frac{\text{wet mass of organ}}{\text{mass of fish}} \times 100 \quad (2)$$

Data analysis

Response variables

Before analyses were performed on R software (version 4.2.2) using the R-studio interface (version 2022.12.0 + 353), response variables were statistically transformed where necessary to improve normality and homoscedasticity for subsequent analyses. The relative liver mass (RLM) of *G. breviceps* was square root-transformed, the relative ventricle mass (RVM) and relative gonad mass (RGM) of *G. breviceps* were log-transformed, and the relative spleen mass (RSM) was fourth root-transformed.

Predictor variables

To determine the relationships between fish response variables and environmental predictor variables across a nitrate pollution gradient, a global mixed effects model was created for each of the response variables. The global models initially included eleven predictor variables: habitat nitrate concentration, instream habitat roughness, substrate heterogeneity, embedded siltation, emergent macrophytes, submerged macrophytes, water temperature, dissolved oxygen, specific conductivity, sex, the coded abundance of aquatic macroinvertebrates and the coded abundance of EPT aquatic macroinvertebrates.

These explanatory variables were then further reduced following methods described by Zuur et al. (2010) and Swain and Reynolds (2015) by examining multicollinearity among habitat variables using variance inflations (VIF). High colinearity (VIF>4; Swain and Reynolds, 2015) was found between instream habitat roughness, substrate heterogeneity and embedded siltation, so substrate heterogeneity and embedded siltation were excluded from the final model. Colinearity was also found between mean habitat nitrate concentration and conductivity, so conductivity was removed from the model. Similarly, emergent macrophytes and submerged macrophytes were highly correlated, so submerged macrophytes were

removed from subsequent analyses. Temperature and dissolved oxygen were initially included in the model but were later removed because these spot measurements were taken on different dates and times, and temperatures and dissolved oxygen levels have strong diel fluctuations, particularly in polluted streams (Franklin, 2014). The abundance of EPT aquatic macroinvertebrates was removed from the model assessing the effect of predictor variables on SMI due to analytical constraints (i.e., overfitting the model).

Linear mixed-effects models were constructed using the 'lmer' function of the 'lme4' package (R version 4.2.2). SMI predictor variables included habitat nitrate concentration, instream habitat roughness, emergent macrophytes, the coded abundance of aquatic macroinvertebrates, the coded abundance of EPT aquatic macroinvertebrates, and fish sex as predictor variables. Fish organosomatic response variables (RLM, RVM, RSM and RGM) included all of the predictor variables described above, with the addition of the coded abundance of EPT aquatic macroinvertebrates as a predictor variable. Site ID was included as a random effect in all models to account for pseudoreplication within sites.

Akaike's information criterion (AIC)

Linear mixed-effects models were constructed using the 'lmer' function of the 'lme4' package (R version 4.2.2). An information-theoretical model selection approach (AICc ; Akaike Information Criterion for small sample sizes) was used to evaluate the relative effects of nitrate and other explanatory variables on the SMI and organosomatic indices of *G. breviceps* (Burnham and Anderson, 2002; n= 9 streams). This created a series of candidate models with varying combinations of predictor variables explaining response variables (SMI and organosomatic indices). These competing models were then ranked according to their AIC_c value. Models were considered parsimonious if their ΔAIC_c value (AIC_c of the candidate model subtracted from the model with the lowest AIC_c) was less than two

($AIC_c \leq 2$; Burnham and Anderson 2002). Akaike weight (*w*; Burnham and Anderson, 2002) was also calculated for each parsimonious model. The Akaike weight (*w*) is the probability that a chosen model is the most parsimonious among all other candidate models. Finally, because AIC_c only ranks models relative to one another (Nakagawa et al., 2013), the proportion of variation explained by fixed effects (R^2m) and the proportion of variance explained by fixed and random effects (R^2c) was calculated to determine goodness of fit. The sum of Akaike weights ($\sum w$) was calculated to determine each predictor variable's relative importance in explaining each response variable and is interpreted as the probability that a predictor is included in the most parsimonious model (Giam and Olden, 2016).

To further assess the independent effect of each predictor variable on the SMI and organosomatic indices of *G. breviceps*, analysis of variance (ANOVA) was performed on each of the linear mixed-effects models containing all of the predictor variables (statistical significance was accepted at $P < 0.05$). Partial dependence plots were then created following methods from Crichton et al. (2023). Partial dependence plots show the independent effect of a predictor variable on the response when all other predictor variables are held constant (Elith et al., 2008).

Results

Akaike's information criterion (AIC)

The scaled mass index (SMI) of *G. breviceps* was explained parsimoniously ($\Delta AICc < 2$) by nine different models (Table 1). However, all nine models poorly explained variation in SMI ($R^2c \sim 0.11$). In-stream habitat roughness was the best explanatory variable of SMI being included in the top model (SMI_1; Table 1), as indicated by Akaike weights (0.11; Table 1) and having the highest relative importance value of (0.54; Table 2). Habitat nitrate concentration was an important variable in three of the nine parsimonious models (SMI_4,

SMI_5, SMI_6; Table 1) but had a weaker relative importance value (0.42; Table 2) than both in-stream habitat roughness and emergent macrophytes (Table 2).

In contrast, the relative liver mass (RLM) of *G. breviceps* was best explained by sex (RLM_1; Table 1) with a very high relative importance value of 1 (Table 2) and featuring in all five parsimonious models (Table 1). As a single predictor in the most parsimonious model (RLM_1), sex explained a similar amount of variation as other more complicated models (R^2c~0.66; Table 1). Habitat nitrate concentration poorly explained RLM (0.3; Table 2) and did not feature in any of the five parsimonious models (Table 1). Similarly, all other predictor variables poorly explained the RLM of *G. breviceps* having relative importance values <0.4 (Table 2).

The relative ventricle mass (RVM) of *G. breviceps* was explained parsimoniously by five models. Again, sex held the highest relative importance value (0.74; Table 2) in explaining RVM and was featured in all five parsimonious models. The most suitable model (RVM_1, w = 0.10; Table 1) included only sex as a predictor variable and explained a similar amount of variation compared to more complicated models (R^2c~ 0.18; Table 1). Streamnitrate concentration was not considered an important predictor of RVM ($\sum w$=0.25; Table 2) and featured in none of the parsimonious models (Table 1).

Emergent macrophytes and the coded abundance of (Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies) (EPT) aquatic macroinvertebrates were the best predictors in explaining the relative spleen mass (RSM) of *G. breviceps*. Both predictors had high relative importance values of 0.64 and 0.65 (Table 2), respectively, and together were included in the top model (RSM_1; Table 1) with an Akaike weight of 0.12 (Table 1). Habitat nitrate concentrations were less important than both of these predictors, having a relative

importance value of 0.45 but were still included as an important predictor in two of the five parsimonious models.

As expected, sex was an essential predictor in explaining the relative gonad mass (RGM) of *G. breviceps*. Sex had a high relative importance value of 1.0 (Table 2) and was featured as an essential predictor in all eight parsimonious models (Table 1). Habitat nitrate concentration and emergent macrophytes were also important predictors having relative importance values of 0.71 and 0.69, respectively (Table 2). Sex, habitat nitrate concentration and emergent macrophytes were all important predictors in the most parsimonious model (RGM_1) with an Akaike weight of 0.11 (Table 1). Habitat nitrate concentration featured in six of the eight parsimonious models, including the simplest model (RGM_5), which included only nitrate concentration and sex as predictors and together explained as much variation as the most parsimonious model (RGM_1, R^2c=0.54; Table 1)

Table 1. The top linear mixed-effects models (ΔAICc < 2) that explain variation in the scaled mass index (SMI), relative liver mass (RLM), relative ventricle mass (RVM), relative spleen mass (RSM), and relative gonad mass (RGM) of *G. breviceps* determined by Akaike's information criterion (AIC).

Response *G. breviceps*	Model	Fixed effects	AIC_c	ΔAIC_c	w_i	R^2_m	R^2_c
Scaled mass index (SMI)	**SMI_1**	**HR**	**-30.28**	**0.00**	**0.11**	**0.06**	**0.11**
	SMI_2	EM+HR	-29.43	0.85	0.07	0.08	0.11
	SMI_3	EM	-29.21	1.08	0.06	0.05	0.11
	SMI_4	EM+N	-29.12	1.16	0.06	0.08	0.11
	SMI_5	HR+N	-28.92	1.36	0.05	0.07	0.11
	SMI_6	N	-28.91	1.37	0.05	0.04	0.10
	SMI_7	-	-28.83	1.45	0.04	-	0.11
	SMI_8	HR+S	-28.39	1.89	0.04	0.06	0.11
	SMI_9	HR+AM	-28.39	1.90	0.04	0.07	0.11
$\sqrt{\text{Relative liver mass (RLM)}}$	**RLM_1**	**S**	**-56.65**	**0.00**	**0.15**	**0.52**	**0.66**
	RLM_2	EM+S	-55.65	1.00	0.09	0.55	0.66
	RLM_3	EPT+S	-55.49	1.69	0.08	0.55	0.66
	RLM_4	EM+EPT+S	-54.95	1.71	0.06	0.58	0.67
	RLM_5	HR+S	-54.67	1.99	0.05	0.53	0.66
Log Relative ventricle mass (RVM)	**RVM_1**	**S**	**27.84**	**0.00**	**0.10**	**0.05**	**0.18**
	RVM_2	HR+S	28.34	0.50	0.08	0.10	0.19
	RVM_3	S+AM	28.87	1.03	0.06	0.08	0.18
	RVM_4	EM+S	29.33	1.48	0.05	0.07	0.18
	RVM_5	HR+S+AM	29.67	1.83	0.04	0.12	0.19
$\sqrt[4]{\text{Relative spleen mass (RSM)}}$	**RSM_1**	**EM+EPT**	**-280.4**	**0.00**	**0.12**	**0.19**	**0.23**
	RSM_2	EM+N	-279.0	1.48	0.06	0.17	0.23
	RSM_3	EM+EPT+N	-278.8	1.70	0.05	0.20	0.23
	RSM_4	EM+EPT+AM	-278.5	1.97	0.05	0.20	0.23
	RSM_5	EPT	-278.5	1.99	0.05	0.12	0.23
Log (Relative gonad mass (RGM)+1)	**RGM_1**	**EM+N+S**	**72.19**	**0.00**	0.11	**0.46**	**0.54**
	RGM_2	EM+EPT+N+S	72.71	0.52	0.09	0.48	0.53
	RGM_3	EM+EPT+N+S+AM	72.75	0.55	0.09	0.51	0.54
	RGM_4	EM+N+S+AM	72.87	0.68	0.08	0.48	0.54
	RGM_5	N+S	72.96	0.77	0.08	0.41	0.54
	RGM_6	EM+EPT+S+AM	73.06	0.86	0.07	0.48	0.54
	RGM_7	EM+EPT+S	73.24	1.04	0.07	0.44	0.53
	RGM_8	EPT+N+S	74.04	1.84	0.05	0.43	0.53

AICc is Akaike's information criterion values corrected for a small sample size. 'ΔAIC_c' is the difference between the top-ranked model and the candidate model. 'w_i 'is Akaike weight and represents the probability that a model is the most parsimonious among all the models. 'R^2m' is the marginal coefficient of determination, while 'R^2c' is the conditional coefficient of

determination. In-stream habitat roughness (HR) and Emergent macrophytes (EM) are habitat features measured on an ordinal scale from (1-10; Appendix 2). 'AM' is the total abundance of aquatic macroinvertebrates while 'EPT' is the total abundance of Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies) (EPT) aquatic macroinvertebrates measured using coded abundance protocols. 'N' is the mean habitat nitrate concentration ($mgL^{-1}NO_3^-$), and 'S' is fish sex. Models in bold were selected as the most suitable for predicting each response variable.

Table 2. The relative importance of predictor variables affecting the scaled mass index (SMI), relative liver mass (RLM), relative ventricle mass (RVM), relative spleen mass (RSM), and relative gonad mass (RGM) of *G. breviceps* in nine streams surveyed in Canterbury, New Zealand streams.

Response *G. breviceps*	**Fixed effects**	$\sum w$
Scaled mass index (SMI)	HR	0.54
	EM	0.49
	AM	0.28
	N	0.42
	S	0.31
$\sqrt{\text{Relative liver mass (RLM)}}$	HR	0.28
	EM	0.38
	AM	0.24
	EPT	0.44
	N	0.30
	S	1.00
Log Relative ventricle mass (RVM)	HR	0.44
	EM	0.57
	AM	0.34
	EPT	0.28
	N	0.25
	S	0.74
$\sqrt[4]{\text{Relative spleen mass (RSM)}}$	HR	0.29
	EM	0.64
	AM	0.31
	EPT	0.65
	N	0.45
	S	0.27
Log (Relative gonad mass (RGM)+1)	HR	0.25
	EM	0.69
	AM	0.41
	EPT	0.58
	N	0.71
	S	1.00

Each variable's relative importance ($\sum w$) was calculated by summing the Akaike weights (w) across the number of models in which the chosen variable appears. In-stream habitat roughness (HR) and Emergent macrophytes (EM) are habitat features measured on an ordinal scale from (1-10; Appendix 2). 'AM' is the total abundance of aquatic macroinvertebrates while 'EPT' is the total abundance of Ephemeroptera (mayflies), Plecoptera (stoneflies), and

Trichoptera (caddisflies) (EPT) aquatic macroinvertebrates measured using coded abundance protocols. 'N' is the mean habitat nitrate concentration (mgL^{-1} NO_3^-), and 'S' is fish sex.

With all other variables held constant, the SMI of *G. breviceps* was not significantly associated with instream habitat roughness ($F_{1,9.6}$=1.40, P=0.27; Figure 3A), emergent macrophytes ($F_{1,8.3}$=2.72, P=0.14; Figure 3B), the coded abundance of aquatic macroinvertebrates ($F_{1,8.7}$=0.75, P=0.41; Figure 3C), habitat nitrate concentration ($F_{1,9.1}$=1.17, P=0.31; Figure 3D), or sex ($F_{1,98.6}$=0.93, P=0.34; Figure 3E).

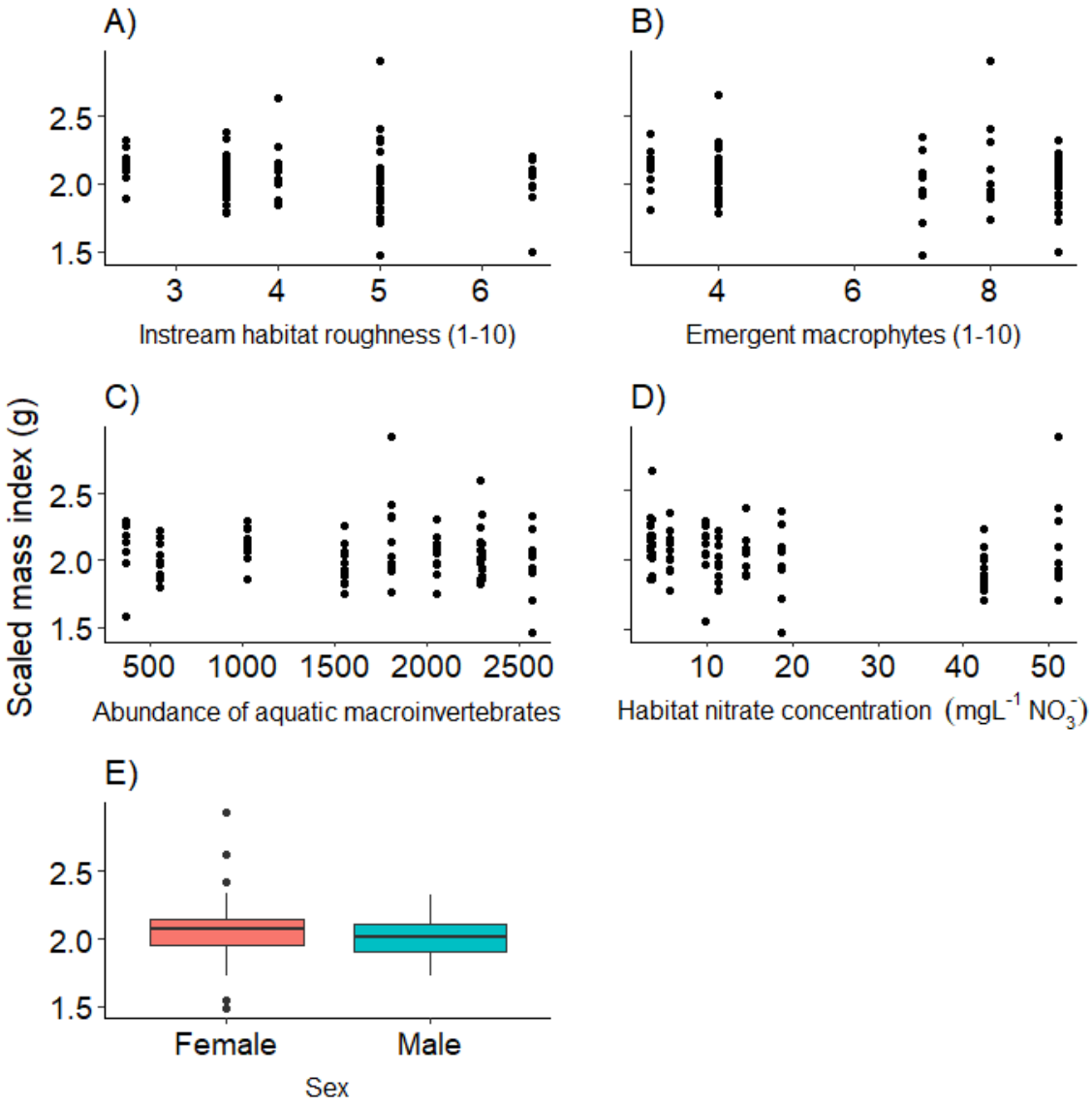

Figure 3. Independent effects of habitat characteristics and fish sex on the scaled mass index of 89 *G. breviceps*. Partial regression plots showing the independent effect of instream habitat roughness (A), emergent macrophytes (B), the coded abundance of aquatic invertebrates (C), habitat nitrate concentration and sex on the scaled mass index (SMI) of *G. breviceps*. Dots represent individual fish sampled within each of the nine field sites (n=89, ~10 fish per site).

Similarly, with all other variables held constant, the RLM of *G. breviceps* was not significantly associated with instream habitat roughness ($F_{1,8.7}$=0.26, P=0.62; Figure 4A), emergent macrophytes ($F_{1,8.7}$=1.25, P=0.29; Figure 4B), the coded abundance of aquatic macroinvertebrates ($F_{1,8.8}$=0.02, P=0.9; Figure 4C), the coded abundance of EPT aquatic macroinvertebrates ($F_{1,9.2}$=3.54, P=0.09; Figure 4D) and habitat nitrate concentration ($F_{1,8.2}$=1.04, P=0.34; Figure 4E). However, sex was very significant in the expression of

RLM, with females having significantly larger RLM values than males ($F_{1,82.6}$=120.2, P<0.001; Figure 4F).

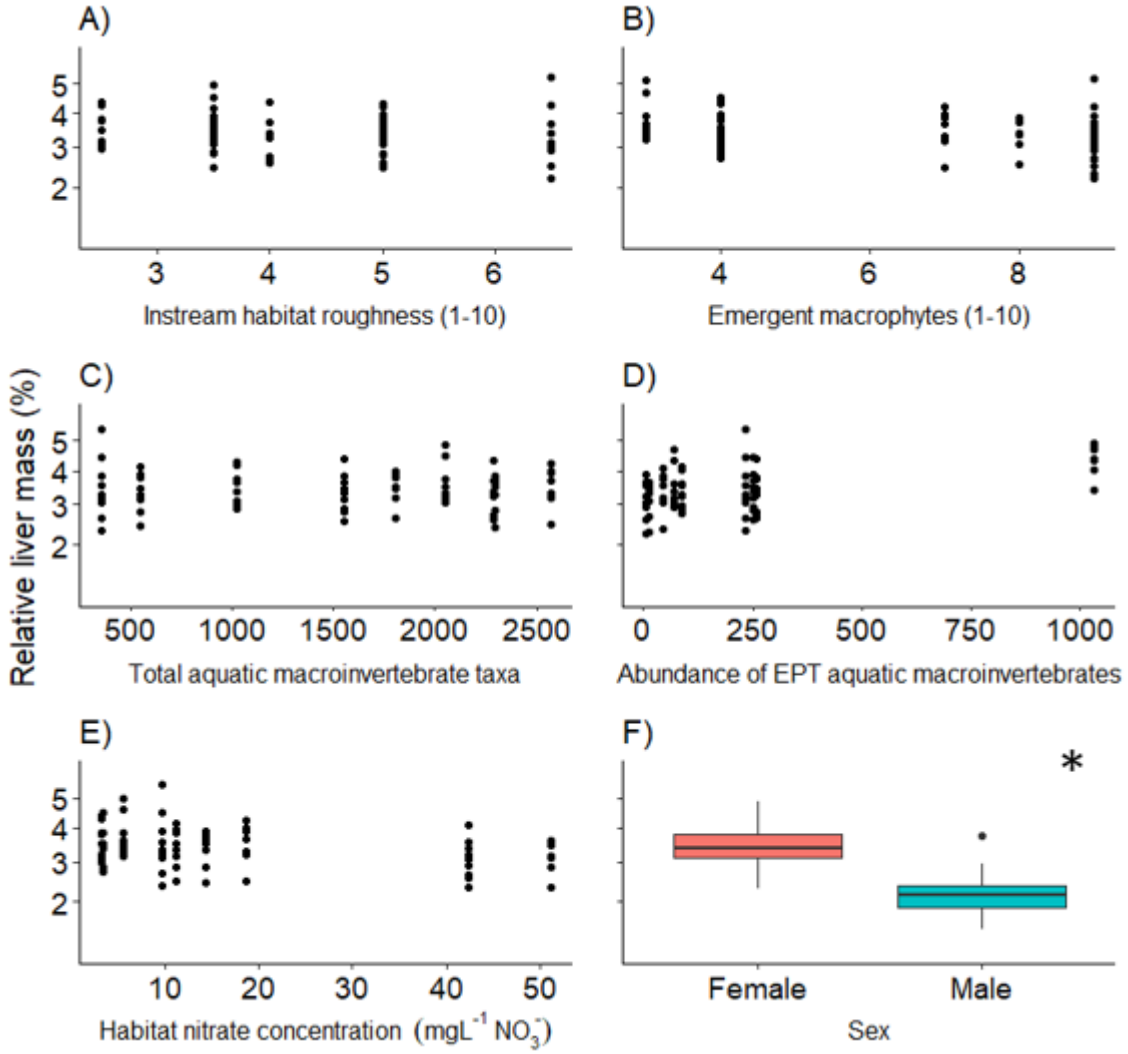

Figure 4. Independent effects of habitat characteristics and fish sex on the relative liver mass of 89 *G. breviceps*. Partial regression plots show the independent effect of instream habitat roughness (A), emergent macrophytes (B), the coded abundance of aquatic invertebrates (C), the coded abundance of Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies) (EPT) (D), habitat nitrate concentration (E) and sex (F) on the relative liver mass of *G. breviceps*. Note that the Y-axis is not linear as data were back transformed. Dots represent individual fish sampled within each of the nine sites (n=89, ~10 fish per site). Asterisks indicate statistically significant relationships.

With all other variables held constant, the RVM of *G. breviceps* was not significantly associated with in-stream habitat roughness ($F_{1,9.2}$=1.65, P=0.23; Figure 5A), emergent macrophytes ($F_{1,9.2}$=0.03, P=0.86; Figure 5B), the coded abundance of aquatic macroinvertebrates ($F_{1,9.3}$=0.76, P=0.41; Figure 5C), the coded abundance of EPT aquatic macroinvertebrates ($F_{1,10.1}$=0.63, P=0.44; Figure 5D) and habitat nitrate concentration ($F_{1,8.4}$=0.1, P=0.76; Figure 5E). Similar to the RLM results, females had significantly larger RVM values than males ($F_{1,84.7}$=4.13, P=0.04; Figure 5F).

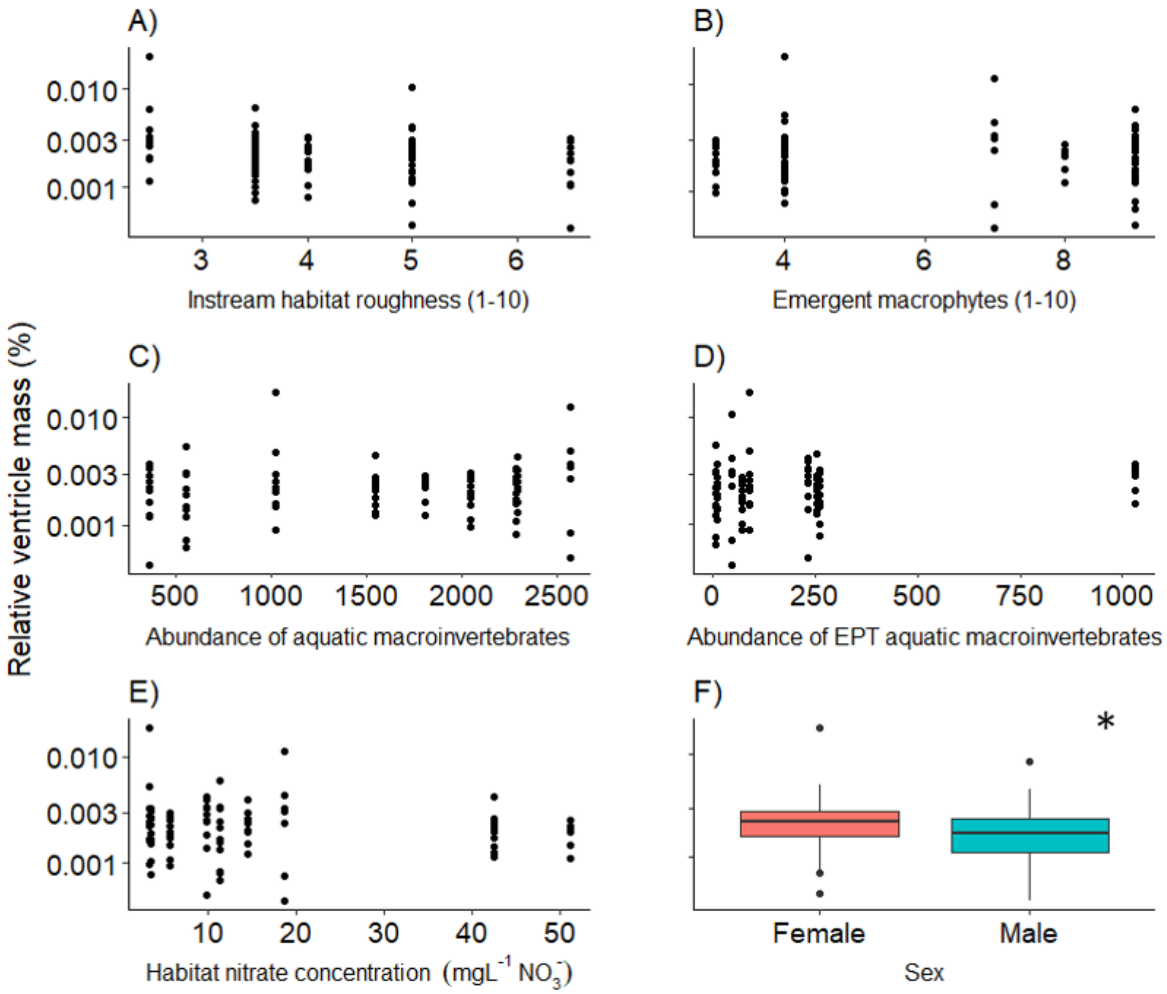

Figure 5. Independent effects of habitat characteristics and fish sex on the relative ventricle mass of 89 *G. breviceps*. Partial regression plots show the independent effect of instream habitat roughness (A), emergent macrophytes (B), the coded abundance of aquatic invertebrates (C), the coded abundance of Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies) (EPT) aquatic invertebrates (D), habitat nitrate concentration (E) and sex (F) on the relative ventricle mass of *G. breviceps*. Note that the Y-axis is not linear as data were back transformed. Dots represent individual fish sampled within each of the nine sites (n=89,~10 fish per site). Asterisks indicate statistically significant relationships.

With all other variables held constant, the RSM of *G. breviceps* was not significantly associated with instream habitat roughness ($F_{1,9.3}$=0.06, P=0.81; Figure 6A), emergent macrophytes ($F_{1,9.3}$=3.32, P=0.1; Figure 6B), the coded abundance of aquatic macroinvertebrates ($F_{1,9.5}$=0.56, P=0.47; Figure 6C), the coded abundance of EPT aquatic macroinvertebrates ($F_{1,10.7}$=2.0, P=0.19; Figure 6D), habitat nitrate concentration ($F_{1,8.2}$=0.76, P=0.41; Figure 6E), or sex ($F_{1,86.1}$=0.37, P=0.55; Figure 6F).

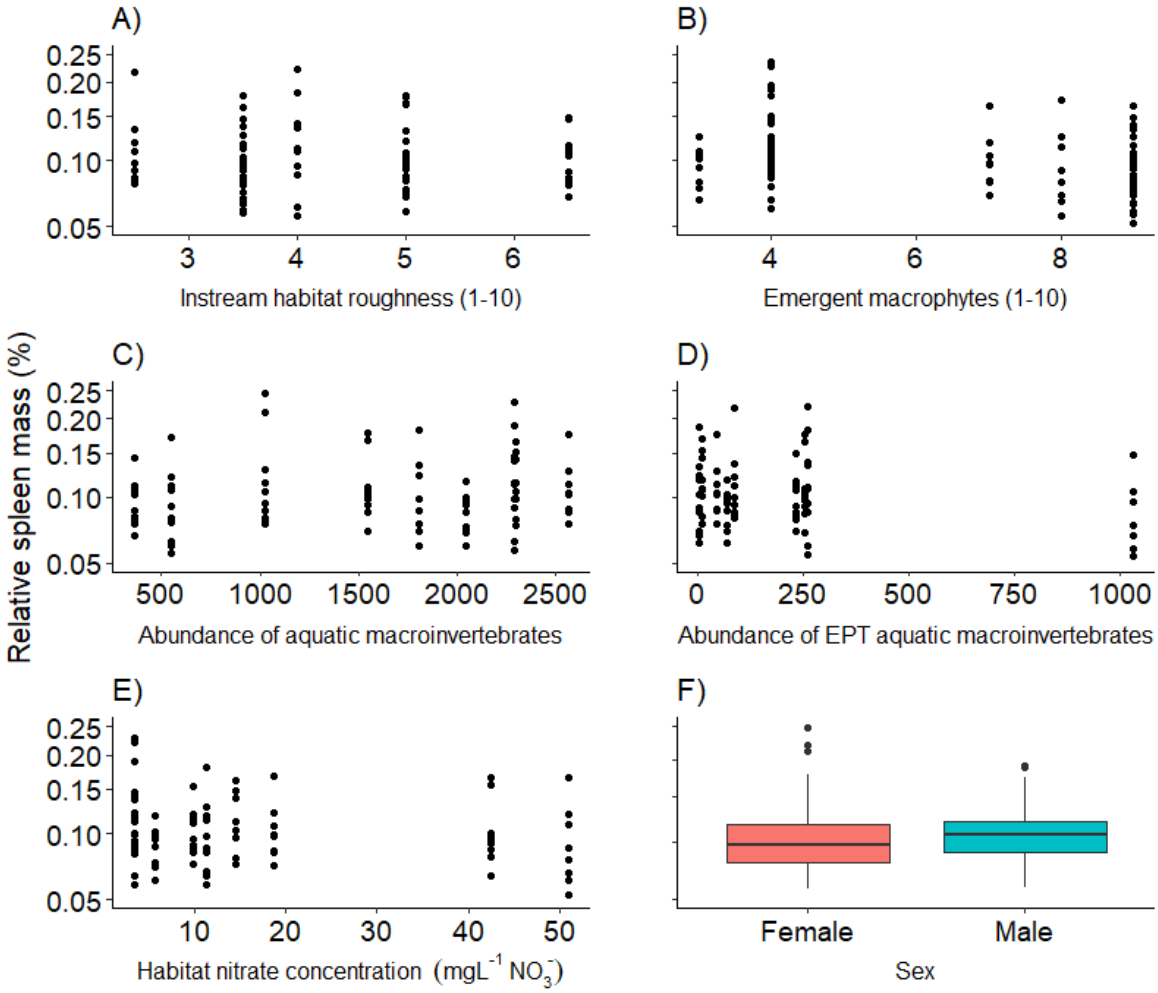

Figure 6. Independent effects of habitat characteristics and fish sex on the relative spleen mass of 89 *G. breviceps*. Partial regression plots show the independent effect of instream habitat roughness (A), emergent macrophytes (B), the coded abundance of aquatic invertebrates (C), the coded abundance of Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies) (EPT) aquatic invertebrates (D), habitat nitrate concentration (E) and sex (F) on the relative spleen mass of *G. breviceps*. Note that the Y-axis is not linear as data were back transformed. Dots represent individual fish sampled within each of the nine sites (n=89,~10 fish per site).

With all other variables held constant, the RGM of *G. breviceps* was not significantly associated with instream habitat roughness ($F_{1,7.8}$=0.27, P=0.62; Figure 7A), the coded abundance of aquatic macroinvertebrates ($F_{1,7.9}$=2.51, P=0.15; Figure 7B), the coded abundance of EPT aquatic macroinvertebrates ($F_{1,8.7}$=3.26, P=0.11; Figure 7C) and habitat nitrate concentration ($F_{1,7}$=3.61, P=0.1; Figure 7D). However, RGM was positively

associated with emergent macrophytes ($F_{1,7.8}$=0.27, P=0.019; Figure 7E) and, as expected, sex was very significant in the expression of RLM with females having significantly larger RLM values than males ($F_{1,84.5}$=26.17, P<0.001; Figure 7F)

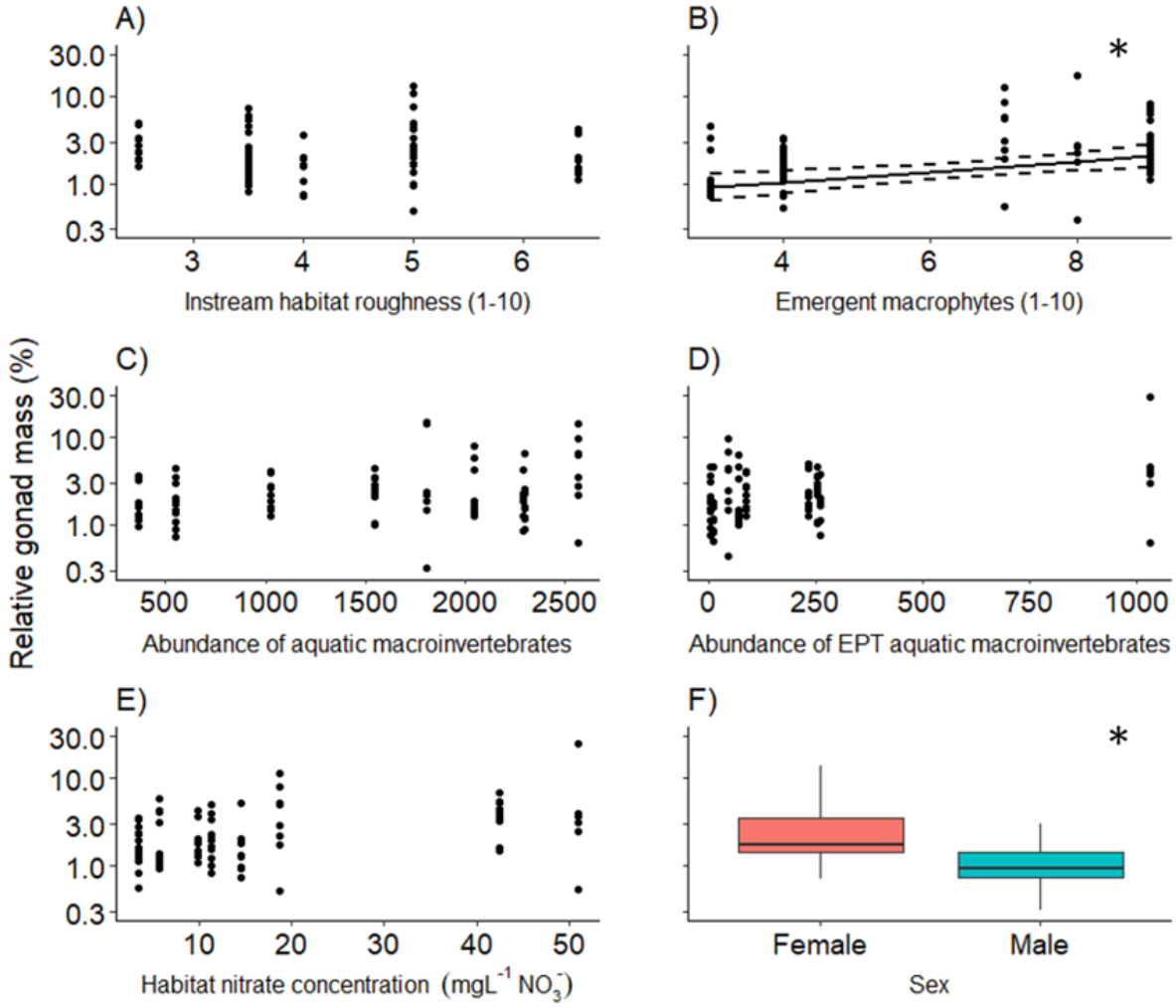

Figure 7. Independent effects of habitat characteristics and fish sex on the relative gonad mass of 89 *G. breviceps*. Partial regression plots show the independent effect of instream habitat roughness (A), emergent macrophytes (B), the coded abundance of aquatic invertebrates (C), the coded abundance of Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies) (EPT) aquatic invertebrates (D), habitat nitrate concentration (E) and sex (F) on the relative liver mass of *G. breviceps*. Note that the Y-axis is not linear as data were back transformed. Dots represent individual fish sampled within each of the nine sites (n=89, ~10 fish per site). A line of best fit is shown in B where a significant correlation was found (P < 0.05), and error bands show 95% confidence intervals determined from model fits. Asterisks indicate statistically significant relationships.

Discussion

Nitrate pollution from intensive agricultural activities is a major threat to freshwater fish species in Canterbury, New Zealand (Joy et al., 2019; Death et al., 2018). Recent laboratory studies on exotic fish species have determined that exposure to low (ecologically relevant) concentrations can adversely affect energy balance, physiological condition, and health in freshwater fishes (Carmargo et al., 2005; Gomez Isaza et al., 2020a; Opinion et al., 2020; Rodgers et al., 2021). However, it is unknown if these effects translate to native fish species in New Zealand or observations in the field. This research addressed this pressing knowledge gap by measuring the physiological condition (body condition and organosomatic indices) of a native fish species (upland bully; *Gobiomorphus breviceps*) across a nitrate pollution gradient in the field. Contrary to predictions, the results of this chapter revealed that the physiological condition (body condition and organosomatic indices) of *G. breviceps* was not influenced by increasing habitat nitrate concentrations. Sex was significant in explaining some organosomatic indices, with females having larger RLM, RVM and RGM values than males. Interestingly, RGM was also explained significantly by the independent effect of emergent macrophytes.

Effect of habitat nitrate concentration and other predictor variables on body condition

Nitrate was expected to reduce the body condition of *G. breviceps* due to reductions in blood oxygen-carrying capacity (methemoglobinemia), resulting in less available aerobic energy for growth and foraging for food. Such effects have been reported in a laboratory experiment by Gomez Isaza et al. (2020b), which found that exposure to elevated nitrate concentrations (50 and 100 mgL^{-1} NO_3^-) significantly reduced the body condition (SMI) of spangled perch (*Leiopotherapon unicolor*). However, this was not the case in this study. Nitrate was a weak predictor of body condition (SMI) and was non-significant in explaining SMI independently,

possibly suggesting living in nitrate-polluted waters (<51 mgL^{-1} NO_3^-) is not energetically costly for *G. breviceps*.

A similar study to the present study by Maceda-Veiga et al. (2014) found that the response of SMI in a freshwater fish (*Barbus haasi*) was significantly related to increasing nitrate concentration. Maceda-Veiga et al. (2014) suggested that increases in the SMI of wild-caught fish living in nitrate-polluted streams could be due to increases in the abundance of high-quality food items from nitrate-induced boosts in-stream productivity. In this study, I attempted to account for this potential effect by measuring variation in prey abundance (the abundance of freshwater macroinvertebrates) across streams. However, like nitrate, prey abundance was a weak predictor of body condition in this study. Importantly, the range of nitrate concentrations measured across streams was 5-12 mgL^{-1} NO_3^-, compared to 3-51 mgL^{-1} NO_3^- in the present study, which may explain the discrepancies between results.

One potential explanation for the weak influence of habitat nitrate concentration on *G. breviceps* observed in this study is that *G. breviceps* may be tolerant of anthropogenic disturbances such as nitrate pollution due to physiological acclimation or adaptation over many generations. Intensive agricultural practices, such as dairy farming, pollute streams with nitrate and have been prevalent in the Canterbury region for >15 years (Greenwood et al., 2012). Therefore, *G. breviceps* may have adapted physiological mechanisms to cope with nitrate pollution over many generations of elevated nitrate exposure.

Physiological adaptation to nitrite has been reported by Jensen et al. (2017), whereby clown knifefish (*Chitala ornate*) upregulate the methaemoglobin reductase enzyme, which reduces methaemoglobin (caused by nitrite exposure) back to functional haemoglobin. Jensen et al. (2017) suggested this mechanism may be likely responsible for the extremely high tolerances of *C. ornate* to nitrite exposure. Therefore, because nitrate exposure causes the same toxic

effect as nitrite exposure to fish (methemoglobinemia), *G. breviceps* may have physiologically adapted also to upregulate the methaemoglobin reductase enzyme as well, resulting in increased tolerance to nitrate. Further evidence that *G. breviceps* may be tolerant of nitrate pollution is suggested by Ramezani et al. (2016), they found that increasing dairying prevalence did not influence the density of *G. breviceps* in streams in Otago, New Zealand. Although, importantly, other native fish species did decrease in density, such as the Clutha flathead galaxiid (Galaxias Sp. D). This finding may be suggestive of species-specific tolerances to nitrate pollution. Indeed, a recent meta-analysis by Gomez-Isaza (2020a) on the effects of nitrate on aquatic species determined that the toxicity of nitrate to aquatic organisms is species-specific. Therefore, *G. breviceps* may simply be tolerant of nitrate pollution up to levels as high as 51 mgL^{-1} NO_3^-.

In-stream habitat roughness was the most important variable in explaining the SMI of *G. breviceps,* as indicated by the AIC analysis. As a single predictor, habitat roughness explained as much variation in SMI as other more complicated models. However, this association was non-significant when other environmental variables were accounted for. Instream habitat roughness measures the quality and availability of habitat for instream fauna such as fish (Appendix 2). Good quality instream habitat may influence the SMI of *G. breviceps* by providing refuge from predators (Jowett and Boustead, 2001) and providing optimal feeding patches (greatest energy return per unit effort) (Balcombe and Closs, 2016; De Silva and Hodson, 2021). My findings that habitat quality was the best predictor of SMI align with findings from Oliva-Paterna et al. (2003), which found that instream habitat quality was the most important driver of fish condition in a Mediterranean stream.

The presence of emergent macrophytes was not a significant factor in explaining body condition despite other studies reporting macrophytes provide refuge and optimal habitat for prey items such as *P. antipodarum* (Balcombe and Closs, 2016; White et al., 2021). Likewise,

the abundance of aquatic macroinvertebrates and sex had weak influences on body condition. It is important to recognise that a limitation of this study was that it was not possible to include every potentially important factor that could help explain variation in SMI due to logistical and analytical constraints. However, the fact that the most parsimonious models explained little variation ($R^2C < 0.11$) in the body condition of *G. breviceps* suggests other factors not included in this study may explain more variation than the predictors I investigated. Predation and conspecific competition may have indirectly influenced SMI. A study by James and Poulin et al. (1998) on *G. breviceps* found that the less energetically profitable prey items were chosen in the presence of predators and other upland bullies. Trout are common in New Zealand streams and are predators and superior competitors of native fish (Ramezani et al., 2016). Therefore, the abundance of trout and other predators may be important in explaining SMI. Intraspecific competition for food and resources is also presumed to affect the SMI of freshwater fish. For example, high fish density has been associated with decreased SMI of freshwater fish in the field (Maceda-Veiga et al., 2014).

Physiochemical water quality parameters such as dissolved oxygen (Franklin, 2014) and temperature (Culumber and Monks, 2014) have also been reported as important variables influencing the body condition of freshwater fish. In this study, logistical constraints meant I could not collect long-term measurements of dissolved oxygen and temperature (see methods). So, although spot measurements were taken, they were not included in analyses.

Effect of habitat nitrate concentration and other environmental variables on organosomatic indices

Organosomatic indices provide information about the energy stores of fish (Maxwell and Duta, 2005), the effects of pollutants on target organs (Giullo and Hinton, 2008), and provide information on the overall condition of fish (Nagrodski et al., 2013). Organosomatic indices

were only independently explained significantly by sex and emergent macrophytes. Females had larger relative liver masses (RLM), ventricle masses (RVM) and gonad masses (RGM) than male fish. An interesting result in this study was that reduced emergent macrophyte presence was positively associated with RGM.

Habitat nitrate concentration was not considered an important factor influencing organosomatic indices but was an important component of the top model explaining RGM. These results contrast with other studies which have found that exposure to environmental stressors can cause stressors or pollutants that can cause increases or decreases in relative organ sizes. For example, fish (*Brycon cephalus*) exposed to an insecticide (methyl parathion) experience significant reductions in RLM relative to control fish (Monteiro et al., 2006). Reductions in RLM can indicate reduced energy stores in fish (Gabriel et al., 2010). Therefore, the non-significant effect of nitrate on RLM observed in this study may provide further evidence that the energetics of *G. breviceps* is not affected by living in nitrate-polluted waters.

Other studies have reported increased RSM following nitrate exposure due to stressor-induced cardiorespiratory modelling (Gomez Isaza et al., 2021b; Opinion et al., 2020). Opinion et al. (2020) reported that European graylings exposed to nitrate (50 mgL^{-1} NO_3^-) experienced an 85% increase in RSM relative to control fish to maintain blood oxygen levels due to nitrate-induced methemoglobinemia (Opinion et al., 2020). However, in this study, cardiorespiratory remodelling of the ventricle or spleen was not evident in fish living in nitrate-polluted waters.

Independently RGM was not explained significantly by habitat nitrate concentration. Still, habitat nitrate concentration was included in the top model with emergent macrophytes and sex, which were significant in independently explaining RGM. The influence of habitat

nitrate concentration (although non-significant independently) on RGM may be attributed to reproductive endocrine disruption (Nagrodski et al., 2012) or reductions in energy (nitrate-induced methaemoglobinaemia; Gomez Isaza et al., 2020b) that reduce fish's ability to produce eggs or sperm.

The positive relationship between the reduced presence of emergent macrophytes and the RGM of *G. breviceps* may be explained by several reasons. Previous studies have reported that *G. breviceps* may be able to adjust its reproductive investment based on its current condition or the availability of resources and food (Hamilton and Poulin, 2001). Therefore, less emergent macrophytes may provide better in-stream habitat conditions for *G. breviceps* leading to greater investment in reproduction. *G. breviceps* also preferentially lay their eggs on the underside of rocks (Staples, 1975; Hamilton et al., 1997), so less rooted macrophytes dominating the water way may provide more suitable habitats to lay eggs.

Conclusion

In summary, the results of this study indicate that living in nitrate-polluted waters does not appear to influence the physiological condition (body condition and organosomatic indices) of *G. breviceps.* These results suggest that living in nitrate polluted waters may not be energetically costly for *G. breviceps* up to nitrate concentrations as high as 50 mgL^{-1} NO_3^-. Therefore, these results provide evidence that the national bottom line of 11mgL^{-1} NO_3^- may be sufficient to protect some native fish species in New Zealand. However, due to species-specific tolerances (Gomez Isaza et al., 2020a), further studies are required to fully evaluate the suitability of this bottom line to protect New Zealand's fish fauna.

Importantly, habitat nitrate concentrations' non-significant effect on physiological condition does not exclude the possibility that nitrate may have other physiological effects on *G. breviceps* not measured in this study. For example, exposure to nitrate has been shown to

cause a range of physiological effects on freshwater fish, such as alterations to osmoregulation and ionic composition (Edwards and Hamlin, 2018), endocrine disruption (Kellock et al., 2018), growth (Gomez Isaza et al., 2020a), gill histology (Rodgers et al., 2021) and changes to aerobic scope (Gomez Isaza et al., 2020a). Furthermore, an increasing quantity of literature describes interactions between nitrate and other stressors that produce negative outcomes on freshwater fish (Gomez Isaza et al., 2020a; Gomez Isaza et al., 2020b; Rodgers et al., 2021). For example, simultaneous exposure to elevated nitrate and temperature (Opinion et al., 2020) and elevated nitrate and low dissolved oxygen conditions (Gomez Isaza et al., 2021a) can have greater adverse effects on the physiology of fish than exposure to nitrate alone. Future research should include stressor interactions such as these where possible.

Finally, while, this study attempted to account for the effects of other potentially confounding variables that may influence the physiological condition of *G. breviceps*, due to the complicated multi-variate nature of field studies, isolating the individual effects of environmental variables is somewhat difficult. Therefore, to verify the findings of this chapter, the next study focuses on the physiological effects of nitrate pollution under laboratory conditions where environmental conditions can be kept constant.

Chapter three – Assessing the energetic costs of exposure to nitrate pollution in *G. breviceps*

Introduction

Nutrient pollution (i.e., nitrate and phosphorous inputs) from anthropogenic agricultural activities contributes to declines in freshwater species worldwide (Jenkins, 2003). Nitrate (NO_3^-) is the most prevalent form of nutrient pollution contaminating waterways (Gomez Isaza et al., 2018). Intensive agricultural activities such as dairy in lowland regions of New Zealand have resulted in large amounts of nitrate leaching into and contaminating freshwater environments (Foote et al., 2015). To attempt to regulate the amount of nitrate leaching into our freshwater environment and protect freshwater species, the National Policy Statement for Freshwater Management has mandated a new national bottom line of 11 mg L^{-1} NO_3^- in rivers (MfE, 2020). However, this bottom line has been developed with little robust science on how native fish species in New Zealand are affected by nitrate pollution (Joy et al., 2022). As nitrate concentrations in intensively farmed areas of New Zealand are predicted to increase (Foote et al., 2015; Joy et al., 2022), there is an urgent need to assess the effects of nitrate on native fish and determine if current nitrate limits are suitable for protection.

Freshwater fish are particularly vulnerable to nitrate pollution as nitrate continuously enters their body through the gills (Carmargo et al., 2005). Fish chronically exposed to elevated concentrations of nitrate can experience reduced reproductive success, diminished growth rates, reduced activity levels and stunted development, suggesting that exposure to nitrate pollution is energetically expensive (Gomez Isaza et al., 2020a; Gomez Isaza et al., 2020b; Opinion et al., 2020b). Physiological tools such as measures of aerobic scope offer a powerful way to assess the effects of pollutants such as nitrate on the energetics of fish (McKenzie et al., 2007).

Aerobic scope (maximum metabolic rate (MMR) – standard metabolic rate (SMR)) represents an animal's capacity to supply oxygen to support fitness-related activities beyond its standard ('basal') metabolic requirements (Sokolova et al., 2012; Pörtner et al., 2017; Opinion et al., 2020). Disruptions to the aerobic scope of fish can manifest as reductions in aerobically supported performance fitness traits such as growth, locomotion, reproduction, and the ability to cope with environmental stressors (Baker et al., 2017; Gomez Isaza et al., 2020a; Opinion et al., 2020). Therefore, measurements of aerobic scope can be useful to predict organismal fitness and ability to persist when exposed to an environmental pollutant or stressor (Claireaux and Lefrançois, 2007; Sokolova, 2013).

Nitrate exposure can cause reductions in aerobic scope by increasing standard metabolic rates (SMR) and by decreasing maximum metabolic rates (MMR) (Gomez Isaza et al., 2020b; Opinion et al., 2020; Figure 8). Increases in SMR are thought to be caused by increases in standard maintenance costs associated with the energetically expensive employment of detoxifying mechanisms to cope with nitrate (Gomez Isaza et al., 2020; Opinion et al., 2020). For example, the methaemoglobin reductase system is the main mechanism fish employ to cope with high nitrate conditions that raise standard metabolic rates (Jensen, 2003; Opinion et al., 2020). Increases in MMR are thought to be caused primarily by the nitrate-induced conversion of oxygen-carrying pigments (haemoglobin) into forms that cannot carry oxygen (i.e., methaemoglobin) that occurs when fish are exposed to elevated concentrations of nitrate (Gomez Isaza et al., 2020b; Gomez Isaza et al., 2021a; Opinion et al., 2020). This oxidation of haemoglobin to methaemoglobin reduces the blood oxygen-carrying capacity of the individual (Gomez Isaza et al., 2021a; Stormer et al., 1996; Yang et al., 2019), so the animal has less available aerobic energy, thus limiting maximal performance (Opinion et al., 2020). A reduction in MMR and/or an increase in SMR can manifest as a reduction in whole-animal aerobic capacity (i.e., aerobic scope; Gomez Isaza et al., 2020a). For freshwater fish, the

narrowing of aerobic scope can cause reduced fitness due to physiological trade-offs, whereby energy is directed away from fitness-related traits (e.g., reproduction, growth, locomotion) and instead towards maintenance costs (Camargo and Alonso, 2006; Claireaux and Lefrançois, 2007).

Studies that measure the aerobic scope of fish exposed to pollutants are often complemented by measures of fish performance, such as growth rates, body condition and locomotor capacity (Rodgers and De Boeck, 2019; Gomez Isaza et al., 2020c; Opinion et al., 2020). This is because, as discussed, functional performance traits are critically dependent on an organism's aerobic scope (Sokolova, 2013; Gomez Isaza et al., 2020b). For example, Lefevre et al. (2011) found that striped catfish (*Pangasianodon hypophthalmus*) exposed to nitrite experienced a 40% decrease in aerobic scope, translating into reduced swimming ability. Reductions in aerobic scope due to nitrate exposure have also been linked to reduced functional performance traits (Gomez Isaza et al., 2020b; Opinion et al., 2020; Opinion et al., 2021). For example, Gomez Isaza et al. (2020b) found that exposure to elevated nitrate concentrations (50, 100 mgL^{-1} NO_3^-) caused a narrowing of aerobic scope in spangled perch (*Leiopotherapon unicolor*) and resulted in reduced growth rates.

Organosomatic measurements (ratio of organs to body weight; Geode, 1990) can also detect changes in energy status in response to pollutants (Maxwell and Duta, 2005). For example, a decrease in relative liver mass can indicate decreased lipid storage and, thus, reduced energy stores (Gabriel et al., 2010). A reduction in gonad mass can indicate that energy has been redistributed away from reproductive investment in response to stress (Amat-Trigo et al., 2021). Exposure to nitrate can also induce remodelling of cardiorespiratory organs such as the gills, spleen and heart in freshwater fish, which can affect blood oxygen carrying capacity and thus influence aerobic scope (Gomez Isaza et al., 2020c; Opinion et al., 2020; Rodgers et al., 2021). For example, relative spleen mass (RSM) was 85% higher in European grayling

(*Thymallus thymallus*) exposed to nitrate (50 mgL^{-1} NO_3^-) relative to control fish (Opinion et al., 2020). This cardiorespiratory remodelling of the spleen by fish was expected to increase oxygenated blood supply to fish experiencing nitrate-induced methemoglobinemia and thus provide protection to reductions to aerobic scope (Opinion et al., 2020).

This study aimed to investigate the energetic costs of exposure to elevated nitrate concentrations on *Gobiomorphus breviceps* using measurements of aerobic scope and complementary performance indicators (growth, body condition, organosomatic indices). I predicted that exposure to increasing levels of nitrate pollution would cause fish to have higher energetic costs (indicated by a higher standard metabolic rate, lower maximum metabolic rate, and lower aerobic scope), reflecting a high cost of living. Following previous studies, I also predicted that higher energetic costs associated with nitrate exposure would result in reduced growth, body condition and abnormalities to organosomatic indices. The results of this chapter provide further evidence towards the suitability of the recently mandated national nitrate bottom line to protect native fish species in New Zealand.

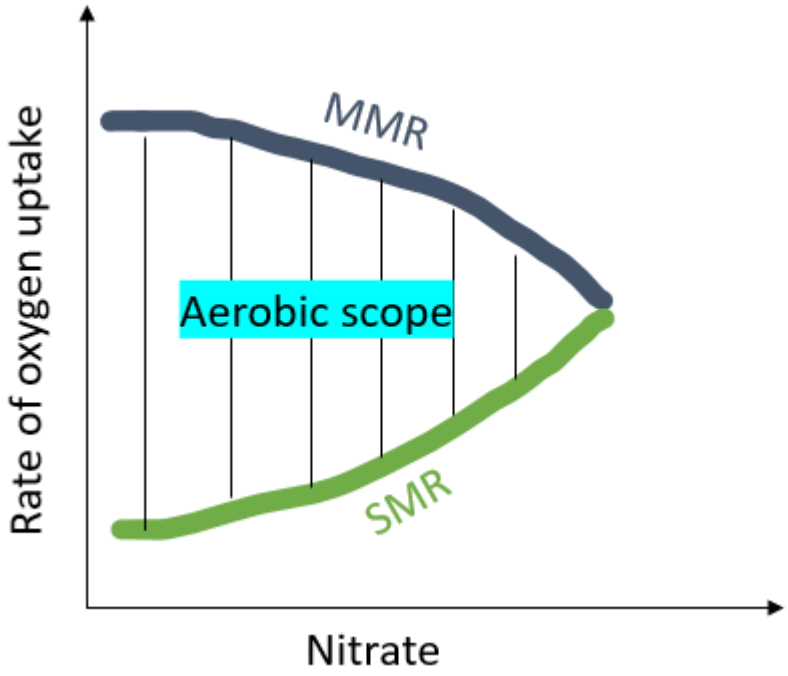

Figure 8. Conceptualised impact of nitrate exposure on the SMR, MMR and AAS of aquatic species. Adapted from Opinion et al. (2020). (A) The hypothesised reduction in maximum metabolic rate (MMR; solid grey line), increase in standard metabolic rate (SMR; solid green line) and subsequent change in aerobic scope (AS; dashed area) arising from exposure to nitrate.

Methods

Fish collection and husbandry

Sixty-three upland bullies (*G. breviceps*) were collected from Okeover stream (NZTM2000: E1566366.7, N5181109.3) that runs through the University of Canterbury over two days. Fish were collected using hand nets and once captured, were quickly transported to the laboratory in 20 L aerated buckets. In the laboratory, fish were weighed (Sartorius TE153S model, Göttingen, Germany; nearest 0.001g, wet weight), measured (total length) and randomly distributed among nine aerated 90 L plastic holding tanks (77 × 57 × 28 cm, l × w × h) with seven fish per tank. Tanks were maintained at a water temperature of 14°C (± 2°C), which

matched the temperature of the stream from which they were collected. Aquarium thermometers (Fluke 51 II Handheld Digital Probe Thermometer, WA, USA, 0.05 % + 0.3 °C) and a temperature logger (TinyTag; TG-4100) were used to monitor water temperature throughout the experiment. Each tank contained one aerator and sponge biofilter to ensure water filtration and oxygenation. Each tank also included four PVC pipes (3.2cm x 10cm; l x d), and a mesh hide to ensure that fish had places to shelter and help reduce stress levels.

Fish were exposed to 12-hour light and 12-hour dark photoperiod, with a 30-minute sunrise and sunset function. Partial water changes of at least 25% of the total water volume were completed every second day for all tanks (~25 L). Reverse osmosis (RO) water enriched with salts ($NaHCO_3$, $CaSO_4*2H_2O$, $MgSO_4*7H_2O$, KCL; Thermofisher, Australia) was used in the experiment. RO water was used to ensure control nitrate levels were kept as close to 0 mg L^- as possible. Basic water chemistry tests (nitrite and ammonia) were conducted bidaily using Aquarium Pharmaceuticals Ltd liquid test kits. If nitrite or ammonia levels exceeded 0.50 ppm, a larger water change was undertaken to maintain suitable levels.

Frozen blood worms were fed to fish daily until satiety, and chopped meal worms were fed biweekly. Faecal matter and uneaten food items were removed daily. Fish were also visually inspected daily. Fish showed no signs of infection, disease or injuries throughout this study. All experimental methods complied with the New Zealand Animal welfare act (1999) and were approved by the University of Canterbury's animal ethics committee (#202105R).

Nitrate treatments

Fish were given two weeks to habituate to the laboratory conditions before the start of the experiment. After two weeks, the nine tanks were randomly split into three nitrate treatment groups 0 [control], 11 and 50 mgL^{-1} NO_3^- (total = 63 fish, 21 fish/treatment, 3 tanks/treatment). This range of nitrate concentrations was chosen because 11 mgL^{-1} NO_3^- was

recently published as the new national bottom line for nitrate allowed in rivers in the 2020 national policy statement for freshwater management (MfE, 2020), and 50 mgL^{-1} NO_3^- was reflective of the highest level of nitrate where *G. breviceps* were found in natural field conditions (Chapter 2). Nitrate concentrations were maintained by dissolving sodium nitrate salt (ThermoFisher Scientific) in a 3 L bottle of RO artificial water and then slowly mixing it into the holding tank. Nitrate levels were checked daily using a portable nitrate meter (LAQUAtwin-NO3-11 meter, Horiba Scientific). Nitrate levels did not fluctuate from nominal levels by more than 10% for all treatment groups. Nitrate concentrations in control tanks were always maintained below the detection limit of the meter (< 5 mgL^{-1} NO_3^-). Fish were exposed to nitrate treatments for 40 days before metabolism measurements.

Metabolism

Respirometer design

Standard and maximum metabolic rates (oxygen uptake rates MO_2) were measured using intermittent respirometry (oxygen electrodes linked with a data acquisition system) (Clark et al., 2013). Four custom-built respirometers (10cm x 4.4 cm; l x d) were used, with a total volume of ~146 ml, including tubing (Figure 9). Each respirometer comprised of a clear cylindrical acrylic chamber and two circulation loops (Figure 9). The circulation loop attached to the chamber was fitted with an inline recirculation pump (Rule iL200 Inline/Submersible Pump 12.7LPM; Figure 9) that continuously mixed water within the chamber to eliminate water stratification inside the respirometer (Rodgers et al., 2016; Steffensen, 1989). Within this circulation loop, a FireSting optical oxygen probe (PyroScience GmbH, Aachen, Germany) was fitted to record changes in air saturation in the respirometers every second. The probe was linked to a PowerLab/4SP data acquisition system (ADInstruments, Dunedin, New Zealand) and LabChart V7 that recorded the data

from the probe. The probes were calibrated each day (by measuring barometric pressure, the vapour pressure of water, and water temperature) before starting each respirometry experiment. A second pump (AquaForce Aquatic TableTop Pump 480LPH; QD-1900 MV; Flush pump; Figure 9) connected to an automatic timer was attached to the chamber and flushed oxygenated water from the water bath into the chamber at 15-minute intervals (15 min on/off cycles). This ensured that the oxygen saturation within the chambers did not drop below 75% during trials. Fresh, oxygenated water pumped into the respirometer during off cycles was allowed to exit through an exit pipe that contained a one-way valve.

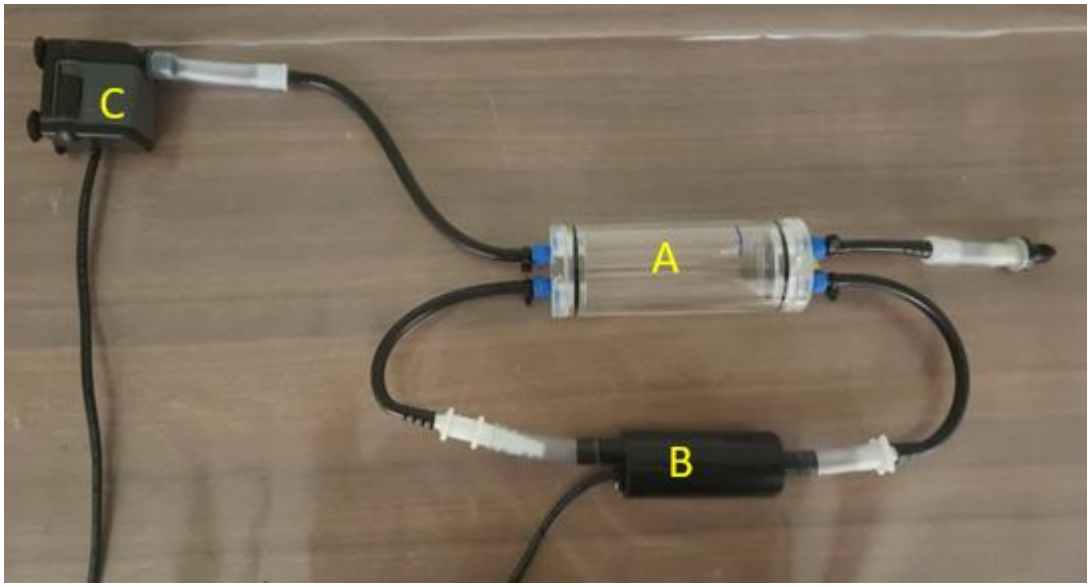

Figure 9. A photograph showing some aspects of the respirometer design. A is the acrylic respirometry chamber, B is the inline recirculation pump (Rule iL200 Inline/Submersible Pump 12.7LPM), and C is the flush pump (AquaForce Aquatic TableTop Pump 480LPH; QD-1900 MV).

SMR, MMR and Aerobic scope

Fish were exposed to nitrate treatment groups (0 [control], 11 and 50 mgL^{-1} NO_3^-) for 40 days before metabolism measurements. A total of 36 fish were used (12 fish/treatment, 4 fish/tank). Fish were fasted for at least 24 hours prior to measurements. This ensured the

possibility of measuring specific dynamic action (SDA) during experimental trials was minimised as fish were in a post-absorptive state (Chabot et al., 2016). Respirometers were submerged in two water baths (2/bath) set to a temperature of 14°C maintained by a water heater (Grant T-series Heated Circulator, Shepreth, Cambridgeshire). Both water baths contained an air stone that continuously aerated the water within the baths for the duration of the trials.

The first trials measured the standard metabolic rate (SMR) of *G. breviceps*. Water was changed in the water bath before the start of each metabolism trial, and nitrate concentrations were adjusted to reflect the respective treatment groups. For each trial, four fish were randomly selected from their holding tank and randomly placed inside each respirometer chamber. Air bubbles were carefully removed using a pipette. Oxygen uptake was then measured for 10 hours (15 min closed/open cycles, Δ% air saturation measured every second on LabChart V7). Black plastic bags were placed on top of the water baths to ensure fish were not disturbed during the trial.

After the SMR trials, maximum metabolic rate (MMR) trials began. Fish were individually removed from chambers and placed in a 20 l bucket filled (0.05m deep) with aerated water that matched the temperature (14°C) and nitrate treatment concentration. Fish were allowed to habituate to the bucket for 10 minutes before being chased vigorously with a net for three minutes (Norin and Clark, 2016). If the fish stopped swimming, the experimenter would gently touch its tail to keep it swimming. Exhaustive chase protocols (Norin and Clark, 2016) were used instead of critical swimming speed tests and burst-swimming protocols because *G. breviceps* have poor sustained swimming abilities (Boubee et al., 1998). After three minutes, fish were immediately placed in the respirometers, and MMR was recorded for 20 minutes. Upon completion of MMR trials, fish were weighed (Sartorius TE153S model, Göttingen, Germany; nearest 0.001g, wet weight) and returned to holding tanks. Microbial and bacterial

background respiration rates were accounted for by removing the fish and measuring the decline in oxygen consumption over two hours. Background respiration was then subtracted from MO_2 calculations, as shown below.

MO_2 calculations

Oxygen uptake rates ($\dot{M}O_{2SMR}$ and $\dot{M}O_{2MMR}$) were calculated following protocols from Opinion et al. (2020). For SMR trials, data acquired from the power lab over the 10 hours (15 min closed/open intervals) was analysed by calculating the average rate of change (slope) of oxygen saturation over time (Δ% air saturation s^{-1}) during closed respirometry cycles. Data collected in the first two hours were not analysed as fish were adjusting to conditions. Slope measurements were not analysed if raw traces were unclear. For MMR measurements, the maximum rate of change (slope) of oxygen saturation (Δ% air saturation s^{-1}) was calculated over the 20 minutes post exhaustive chase.

The average rate of change of oxygen saturation (Δ% air saturation s^{-1}) was first converted to the average rate of change of dissolved oxygen (Δ% DO m^{-1}) using the following equation (1):

$$(1)\ \Delta\%DO\ m^{-1} = 60 \times [(\Delta\%\ air\ saturation\ s^{-1}/100) \times \beta O2]$$

Where βO2 is the oxygen capacitance at the appropriate water temperature (14°C), in this case, 10.306 (Cameron, 1986).

Then metabolic rates (MO_{2SMR} and MO_{2MMR}) ($mgO_2\ g^{-1}\ h^{-1}$) were then calculated for each closed respirometry cycle as follows (2):

$$(2)\ MO_2 = -1 \times [(60 \times m \times v)/BM] - br$$

Where m = rate of change of oxygen saturation during the closed respirometry cycle (Δ% DO m^{-1}), v = volume of respirometer - fish body mass (assuming 1 g displaces 1 ml of water) (l); BM is body mass of the fish (g); and br is background respiration (mgO_2 h^{-1})

An estimate of each fish's standard metabolic rate (SMR) was then calculated using $MO_{2standard}$ values for each fish following methods from Chabot et al. (2016). Following recommendations from Chabot et al. (2016), the average of the lowest 20% quantile (q0.2) was calculated to estimate SMR. Absolute aerobic scope (AAS) and factorial aerobic scope (FAS) were calculated for each individual using the following equations (3, 4):

$$(3)\ AAS = MO_{2MMR} - MO_{2SMR}$$

$$(4)\ FAS = \frac{MO_{2MMR}}{MO_{2SMR}}$$

Growth rates

To determine the effects of nitrate exposure on growth rate, the mass and length of all individuals were measured before the nitrate treatments and at the end of the treatment period (66 days). Fish were weighed (Sartorius TE153S model, Göttingen, Germany; nearest 0.001 g, wet weight) and were measured using photographs of fish placed on a 1cm × 1cm grid (to the nearest mm). Tank averages were used to calculate growth rates. Specific growth rate (SGR; % day^{-1}) was calculated using the following equation (5):

$$(5)\ SGR(\%\ day^{-1}) = 100\ \times \frac{In(BMf) - In(BMi)}{t}$$

Where BMf is the final mass (g) per tank, BMi is the initial mass per tank, and t is the growth period (days).

Body condition

After 66 days of nitrate exposure, the body condition of all fish (n = 60) was calculated using the scaled mass index (SMI; Peig and Green 2009). SMI was calculated using the following equation (6):

$$\text{Scaled mass index (SMI)} = Wi\left[\frac{L_0}{L_i}\right]^{b_{SMA}} \quad (6)$$

Where W_i and L_i are each individual's mass (g) and length (mm), respectively. b_{SMA} is the scaling exponent estimated by the SMA regression of W on L. L_0 is the arithmetic mean of the length of all fish in the data set. L_0 for this study was 64.058. b_{SMA} was calculated following procedures by Maceda-Veiga et al. (2014). In R studio, the bivariate plot of W and length was log-transformed, and the slope of the fitted line b_{SMA} was calculated as 3.7

Organosomatic indices

At the end of the experiment (66 days), fish were euthanised using pH-buffered tricaine methanesulfonate (MS-222; 250 mg/l). After death had occurred (~3 mins), fish were weighed (Sartorius TE153S model, Göttingen, Germany; to the nearest 0.001g), and the total length was measured (mm). The liver, ventricle, spleen and gonads were dissected from each fish, and the wet mass of each organ was measured (Sartorius CP225D model, Göttingen, Germany; to the nearest 0.00001g) following protocols by Todgham et al. (2005). Organosomatic indices (% body mass) were calculated using the following equation (7):

$$\text{Organosomatic indices (\% body mass)} = \frac{\text{wet mass of organ}}{\text{mass of fish}} \times 100 \quad (7)$$

Data analysis

All analyses were performed using R software (version 4.2.2) using the R-studio interface (version 2022.12.0 + 353). Linear mixed effects models (LME) were created using the nlme

(linear and non-linear mixed effects models; https://CRAN.R-project.org/package=nlme) package to examine the effects of nitrate exposure on SMR, MMR, AAS, FAS, growth rates, body condition (SMI) and organosomatic indices. Fish body mass was included as a fixed covariate in all models except growth and body condition models, and tank number (Tank ID) was included as a random effect in all models except growth models. Sex was included as a fixed effect for organosomatic models. All models met assumptions of normality (Shapiro-Wilk test) and homoscedasticity (Bartlett's test). Analysis of variance (ANOVA) was performed on mixed effects models, and statistical significance was accepted at $P < 0.05$.

Results

Metabolism

The mean standard metabolic rate (SMR) of *G. breviceps* was not significantly different between nitrate treatment groups ($F_{1,7} = 1.50$, $P = 0.26$, LME, Figure 10A) after 40 days of nitrate exposure. Fish body mass was included as a covariate in the analyses and significantly affected SMR ($F_{1,26} = 31.39$, $P<0.0001$, LME). Tank ID was included as a random factor in the model but did not explain much variation ($R^2m \sim R^2c$)**.** The maximum metabolic rate (MMR) of *G. breviceps* was not significantly influenced by exposure to elevated nitrate concentrations ($F_{1,7} = 1.19$, $P = 0.31$, LME, Figure 10B). However, analogous to the SMR data, the mean MMR of *G. breviceps* was highest in the (control) mgL^{-1} NO_3^- treatment group (329.21 ± 11.75 mgO_2 g^{-1} h^{-1}) and lowest in the 50 mgL^{-1} NO_3^- treatment group (315.99 ± 12.56 mgO_2 g^{-1} h^{-1}). Mass was a significant factor in the expression of MMR ($F_{1,26}=162.22$, $P<0.0001$). The absolute aerobic scope (AAS) of *G. breviceps* was not significantly affected by exposure to elevated nitrate concentrations ($F_{1,7} = 0.002$, $P = 0.97$, LME, Figure 10C). Mass was a significant factor in the expression of AS ($F_{1,26}=64.68$, $P=<0.0001$). The factorial aerobic scope (FAS) of *G. breviceps* was not significantly

influenced by exposure to elevated nitrate concentrations ($F_{1,7}$ = 0.02, P = 0.89, LME/ANOVA, Figure 10D). Mass was not a significant factor in the expression of FAS ($F_{1,26}$ = 3.39, P = 0.08).

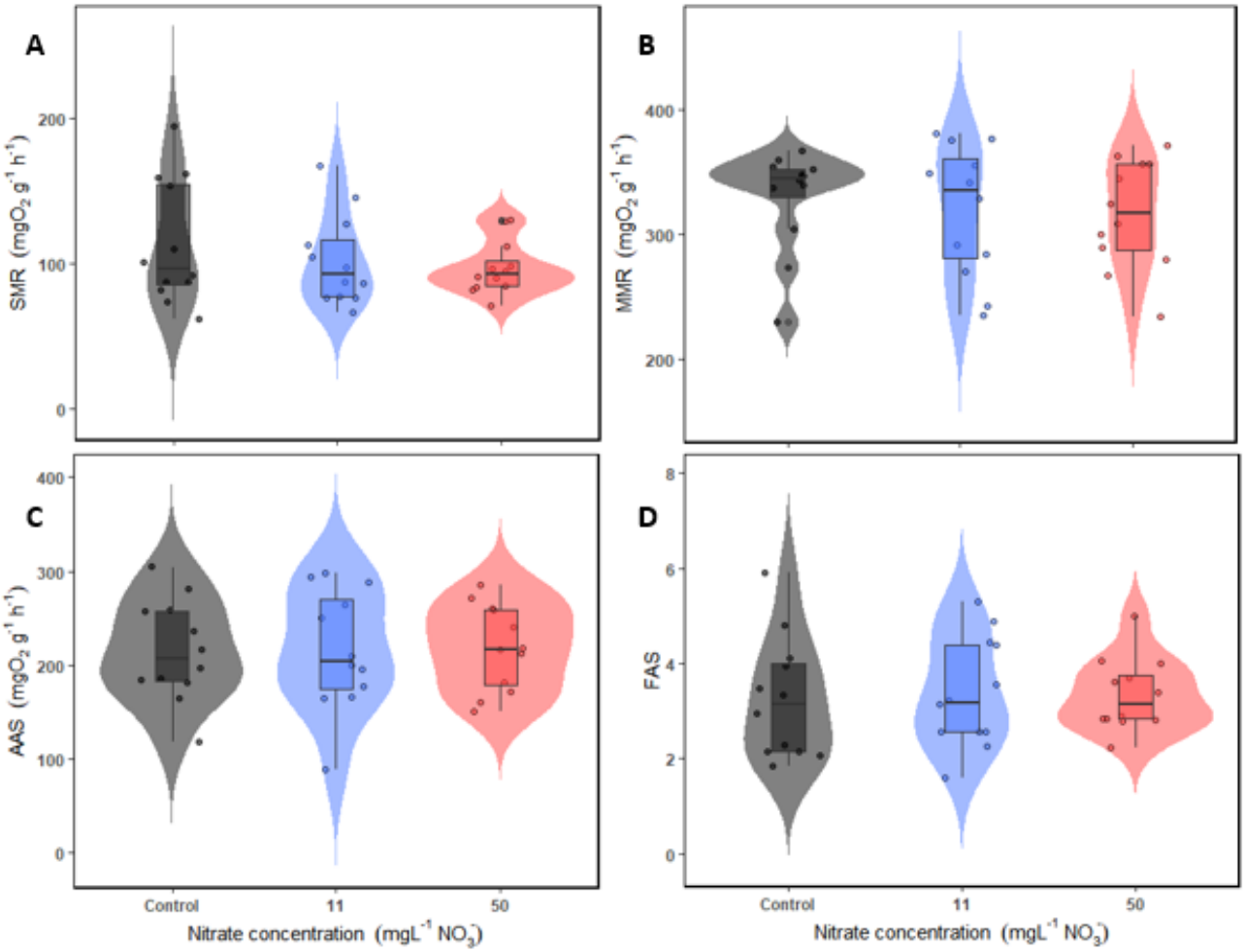

Figure 10. Oxygen uptake rates of upland bully (*Gobiomorphus breviceps*) exposed to different nitrate concentrations (0, 11 and 50 mgL^{-1} NO_3^-) for 40 days at 14°C. (A) Standard metabolic rate (SMR; mgO_2 g^{-1} h^{-1}). (B) Maximum metabolic rate (MMR; mgO_2 g^{-1} h^{-1}). (C) Absolute aerobic scope (AAS; mgO_2 g^{-1} h^{-1}). (D) Factorial aerobic scope (FAS). Data are presented as violin plots showing the distribution of data for each nitrate treatment group (thicker portions represent higher concentrations of values) and boxplots [minimum, first quartile (Q1), median, third quartile (Q3) and maximum] and raw data points (coloured points) are overlaid (n=12/treatment group)

Growth

Specific growth rate (SGR)

There was no significant difference in fish body mass ($F_{1,55}$ = 0.30, P = 0.59, ANOVA, Figure 11) between nitrate treatment groups at the start of the experiment (Table 3). The specific growth rate (SGR) of *G. breviceps* was not significantly influenced by exposure to elevated nitrate concentrations ($F_{1,6}$ = 0.80, P = 0.41, ANOVA). However, SGR of *G. breviceps* showed a decreasing trend with increasing nitrate concentration, with SGR declining in the 11 and 50 mgL^{-1} NO_3^- treatment groups (0.42 ± 0.03 and 0.36 ± 0.08% day^{-1}, respectively) compared to the control treatment (0.47 ± 0.12% day^{-1}).

Table 3. Initial mass, final mass, specific growth rate (SGR; % day^{-1}) and scaled mass index (SMI) of upland bully (*Gobiomorphus breviceps*) exposed to different concentrations of nitrate (0, 11 and 50 mgL^{-1} NO_3^-) for 66 days. Data are shown as mean ± s.e.m. See methods for calculations of SGR.

	Nitrate (mgL^{-1} NO_3):		
	0 (control)	**11**	**50**
Initial mass (g)	2.76 ± 0.36	2.69 ± 0.23	2.55 ± 0.22
Final mass (g)	3.80 ± 0.52	3.87 ± 0.41	3.27 ± 0.28
SGR (% day^{-1})	0.47 ± 0.12	0.42 ± 0.03	0.36 ± 0.08
SMI (g)	3.42 ± 0.075	3.33 ± 0.08	3.31 ± 0.08

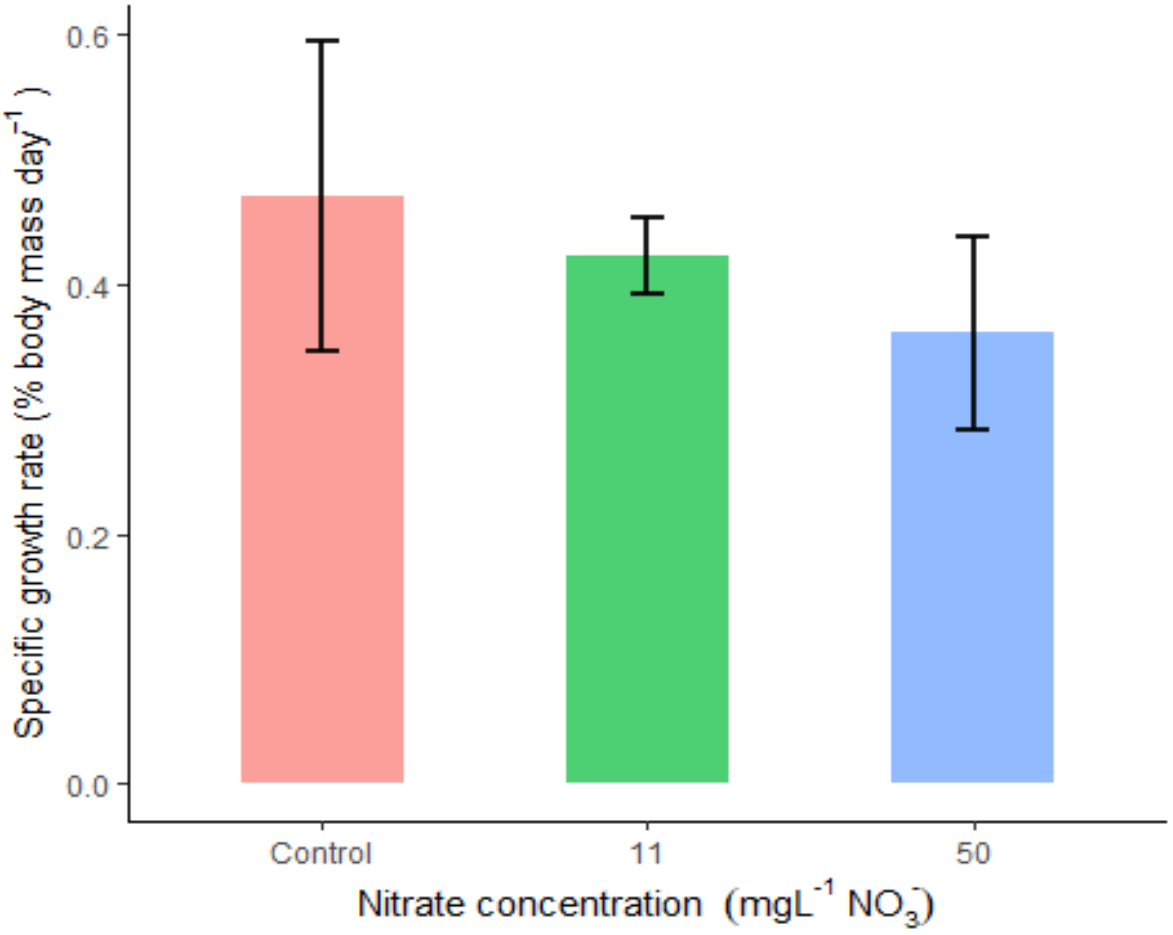

Figure 11. The specific growth rate (SGR; % body mass day^{-1}) of upland bully (*Gobiomorphus breviceps*) exposed to different concentrations of nitrate (0, 11 and 50 mgL^{-1} NO_3^-) for 66 days. Data are shown as mean ± s.e.m. No significant differences in SGR (% body mass day$^-$) were found between nitrate treatment groups ($F_{1,55}$ = 0.30, P = 0.59, ANOVA, n=3,2,3 tanks treatment^{-1}; control, 11 and 50 mgL^{-1} NO_3^-)

Scaled mass index (SMI)

The scaled mass index (SMI) of *G. breviceps* was not significantly different between fish exposed to different nitrate treatment groups ($F_{1,49}$ = 0.64, P = 0.43, LME, Figure 12) after 66 days of exposure.

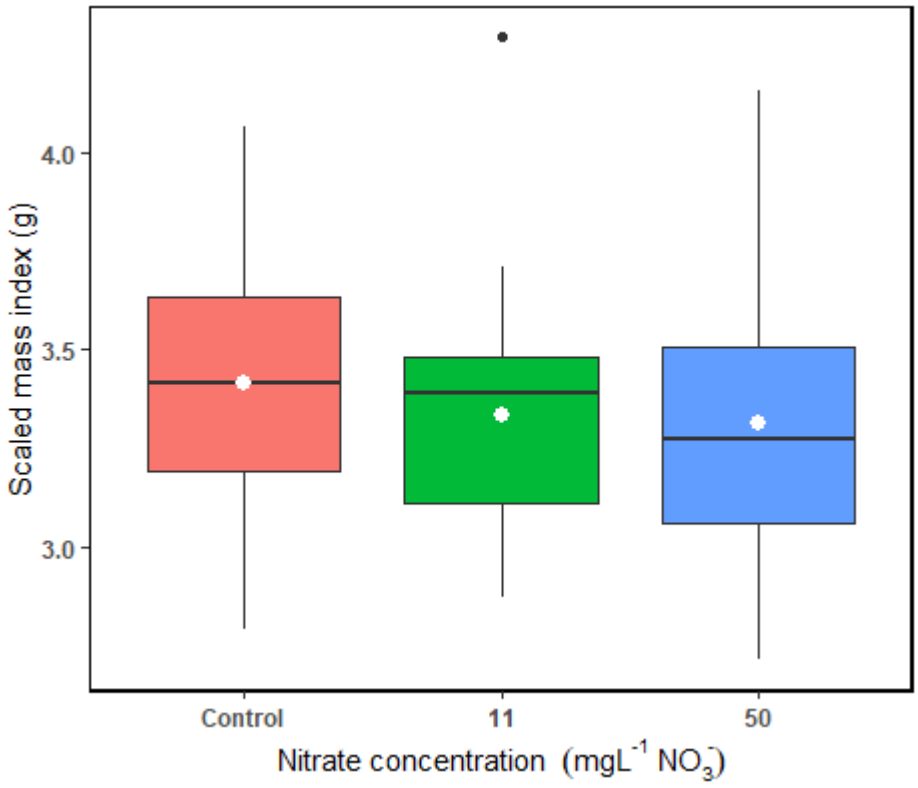

Figure 12. The scaled mass index (SMI; g) of upland bully (*Gobiomorphus breviceps*) exposed to different concentrations of nitrate (0, 11 and 50 mgL^{-1} NO_3^-) for 66 days. Data are shown as boxplots [minimum, first quartile (Q1), median, third quartile (Q3) and maximum] with white points representing group mean values. No significant differences in SMI (g) were found between nitrate treatment groups ($F_{1,49} = 0.64$, P = 0.43 LME, n = 21, 18, 20; control, 11 and 50 mgL^{-1} NO_3^-).

Organosomatic indices

The relative liver mass (RLM) of *G. breviceps* was not significantly different between fish exposed to different nitrate treatment groups ($F_{1,7} = 0.23$, P = 0.65 LME, Figure 13A). Similarly, relative ventricular ($F_{1,7}$=4.23, P= 0.078, LME, Figure 13B) and splenic ($F_{1,7}$=0.60, P=0.46 LME, Figure 13C) masses were unaffected by nitrate concentration treatment. Sex differences were observed for RLM and RVM, with females having larger RLM than males ($F_{1,37} = 44.86$, P <.0001, LME, Figure 13A) and also larger RVM values than males ($F_{1,37}$=5.06, P=0.031, LME, Figure 13B). However, there were no sex differences in the RSM of *G. breviceps.* The relative gonad mass (RGM) of male *G. breviceps* was not significantly

different between male fish exposed to different nitrate treatment groups ($F_{1,5}$=0.61, P=0.47, LME, Figure 13D). Similarly, RGM of female *G. breviceps* was not significantly different between female fish exposed to different nitrate treatment groups ($F_{1,7}$=0.01, P=0.91, LME, Figure 13D).

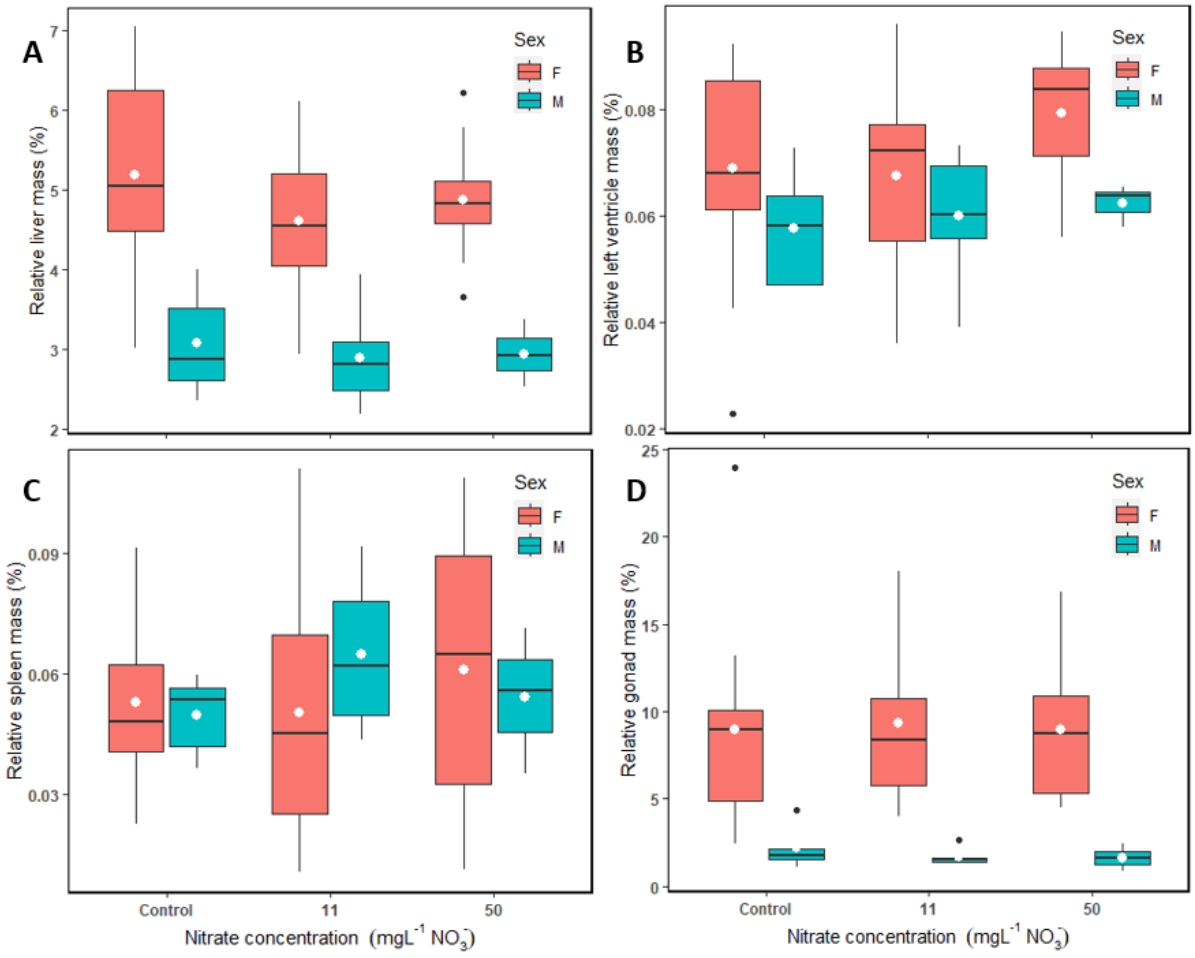

Figure 13. Organosomatic indices of upland bully (*Gobiomorphus breviceps*) exposed to different concentrations of nitrate (control, 11 and 50 mgL^{-1} NO_3^-) for 66 days. (A) Relative liver mass (RLM), (B) Relative ventricle mass (RVM), (C) Relative spleen mass (RSM) and (D) Relative gonad mass (RGM). Data are presented as boxplots [minimum, first quartile (Q1), median, third quartile (Q3) and maximum], and white points on boxes representing group mean values. Blue boxes representing male fish, and pink boxes representing female fish. (n=5, 6, 3; control, 11 and 50 mgL^{-1} NO_3^- for males and 11, 10, 12; control, 11 and 50 mgL^{-1} NO_3^- for females).

Discussion

Nitrate pollution is a pervasive stressor in freshwater environments that can cause adverse effects on freshwater species (Gomez Isaza et al., 2020a). To date, most studies assessing the effects of nitrate on freshwater species have focused on lethal toxicity tests (LC_{50}; Hickey and Martin, 2009). However, more recent studies (Gomez Isaza et al., 2020b; Opinion et al., 2020; Rodgers et al., 2021) have found that exposure to low nitrate concentrations can cause a range of sublethal physiological effects to aquatic organisms with potential negative ecological consequences. In this study, contrary to my predictions, *G. breviceps* did not experience adverse physiological effects when exposed to elevated nitrate concentrations. The aerobic scope, condition, growth and organosomatic indices of *G. breviceps* were unaffected by exposure to elevated nitrate concentrations (11, 50 mgL^{-1} NO_3^-). These results suggest that *G. breviceps* may tolerate nitrate at ecologically relevant concentrations.

Aerobic scope

An organism's aerobic scope (AS) represents its capacity to supply oxygen for fitness-related activities and protective stress responses (Piggott et al., 2015; Sokolova et al., 2012). According to Sokolva et al. (2012), freshwater pollutants such as nitrate can cause a narrowing of the aerobic scope of fish by increasing basal maintenance costs (i.e., SMR) and impairing the oxygen transport capacity needed for activities above maintenance. In this study, exposure to elevated nitrate concentrations (11, 50 mgL^{-1} NO_3^-) did not affect the aerobic scope of *G. breviceps.* The standard metabolic rate (SMR) and maximum metabolic rate (MMR) of *G. breviceps* were unaffected by exposure to elevated nitrate concentrations. The SMR results of this study align with Opinion et al. (2020) and Gomez Isaza et al. (2020b), which found that exposure to nitrate did not disrupt the SMR of European carp (*Cyprinus carpio*) or spangled perch (*Leiopotherapon unicolor*) respectively.

Studies on other aquatic species, such as the blueclaw crayfish (*Cherax destructor*, Gomez Isaza et al., 2018), did not detect an effect of nitrate on SMR. Conversely, other studies (Gomez Isaza et al., 2020c; Opinion et al., 2021) have reported a narrowing of aerobic scope in freshwater fishes following nitrate exposure. These studies attributed increases in SMR to be due to increases in basal metabolic costs associated with increased cellular maintenance costs (de Campos et al., 2014) or the employment of energy-demanding nitrate detoxifying mechanisms such as the methaemoglobin reductase system (Opinion et al., 2020; Opinion et al., 2021). The results of this study suggest that concentrations of ≤ 50 mgL^{-1} NO_3^- have little effect on cellular maintenance and the employment of detoxifying mechanisms for *G. breviceps*. Indeed, in European grayling (*Thymallus thymallus)* exposed to 0, 50 and 200 mgL^{-1} NO_3^- only fish exposed to 200 mgL^{-1} NO_3^- experienced significant increases in SMR compared to control fish (Opinion et al., 2020).

Decreases in MMR were also hypothesised to contribute to reductions in the aerobic scope of *G. breviceps.* Previous studies have attributed reductions in MMR of fish following nitrate exposure to be caused by nitrate-induced methemoglobinemia, which results in reduced blood oxygen-carrying capacity (Opinion et al., 2020; Gomez Isaza et al., 2020b). For example, Gomez Isaza et al. (2020b) determined that decreased MMR of spangled perch (*Leiopotherapon unicolor*) following nitrate exposure was due to the oxidation of haemoglobin to methaemoglobin resulting in reduced blood oxygen-carrying capacity. Exposure to nitrite has been shown to decrease MMR in fish (Lefevre et al., 2011). Iridescent shark catfish (*Pangasianodon hypophthalmus)* exposed to nitrite experienced a 40% decrease in MMR relative to control fish (Lefevre et al., 2011). These results contradict the results of this study, which found no effect of nitrate exposure on the MMR of *G. breviceps.* However, the results of this study do align with those of Opinion et al. (2020), which found nitrate did

not affect the MMR of a freshwater salmonid, *Thymallus thymallus* exposed to elevated nitrate concentrations (50, 200 mg mgL^{-1} NO_3^-).

Species-specific differences in nitrate tolerance may explain the discrepancies observed between this study's results and previous studies' results. For example, Carmargo and Alonso (2006) determined that salmonid species were more sensitive to nitrate exposure than other freshwater species. Therefore, my results may suggest that *G. breviceps* is tolerant of elevated nitrate concentrations. However, the non-significant effect of nitrate on the aerobic scope of *G. breviceps* does not exclude the possibility that nitrate may interact with other stressors present in freshwater environments that could cause declines in aerobic scope. For example, Opinion et al. (2020) found that European graylings did not experience increased SMR when independently exposed to nitrate but did when exposed to elevated nitrate and elevated temperature, suggesting an interaction between these two stressors.

Growth and body condition

Physiological performance indicators such as growth and body condition provide important information about an organism's health, fitness, and energy available above maintenance (Sokolova, 2013). Measurements of growth and condition are important because in natural habitats, fast growth rates and good physiological conditions may provide fitness benefits such as a faster time to reproductive maturity (Alonzo and Camargo, 2013), competitive advantage for resources (Cutts et al., 1999) and predator avoidance (Ribeiro and Qin, 2015). In this study, the growth rate (Specific growth rate; SGR) and body condition (Scaled mass index; SMI) of *G. breviceps* was unaffected by exposure to elevated nitrate concentrations. These results are congruent with my aerobic scope results as reductions in aerobic scope are predicted to cause negative effects on performance traits such as growth (Claireaux and Lefrançois, 2007; Sokolova, 2013).

The non-significant effect of nitrate exposure on growth aligns with results from Davidson et al. (2014) which found that exposure to nitrate (133mgL^{-1} NO_3^-) over three months did not affect the growth of juvenile rainbow trout (Oncorhynchus mykiss). In contrast, a study by Learmonth and Carvalho (2015) found that Juvenile zebrafish (*Danio rerio*) exposed to nitrate showed decreased growth rates. However, in this study, fish were exposed to much higher nitrate concentrations (400 mgL^{-1} NO_3^-) which may explain discrepancies between results. Indeed, a meta-analysis by Gomez Isaza et al. (2020a) on the effects of nitrate on aquatic species concluded that effects of nitrate on growth were dependent on nitrate concentration and duration of exposure, with higher doses and durations resulting in greater effects on growth.

Organosomatic indices

Contrary to predictions, none of the organosomatic indices of *G. breviceps* were affected by nitrate exposure (RLM, RVM, RSM, RGM). Studies have reported that the plasticity of the cardiorespiratory system may mediate the negative effects of nitrate on oxygen-carrying capacity in fish and therefore be responsible for the observed maintenance of MMR (Opinion et al., 2020; Gomez Isaza et al., 2021b). For example, Opinion et al. (2020) attributed the lack of nitrate-induced change in AS/MMR due to the expansion of the spleen in fish exposed to elevated nitrate. The spleen plays an essential role in haemoglobin production, storage and circulation (Wells and Weber, 1990). So splenic expansion was thought to compensate for nitrate-induced methemoglobinemia by increasing blood production and supply, resulting in undifferentiated MMR results between nitrate-exposed fish and control fish (Opinion et al., 2020). In the present study, there was a slight but non-significant increase in RSM of *G. breviceps* exposed to 50 mgL^{-1} NO_3^-, so it is unlikely this mechanism was responsible for non-significant differences in MMR between nitrate-exposed fish and control fish.

Cardiac remodelling of the ventricle has been reported in rainbow trout (*Oncorhynchus mykiss*) in response to chemically induced (phenylhydrazine hydrochloride injection) reduced blood oxygen supply (Simonot and Farrell, 2007). Chemically induced fish increased their relative ventricle mass significantly (>15%) to increase cardiac output and compensate for anaemic *in-vivo* conditions. In the present study, no significant increase in RVM was observed in nitrate-exposed fish. These results suggest that *G. breviceps* does not compensate for nitrate-induced reduced blood oxygen supply by cardiorespiratory remodelling. These results, however, do not exclude the possibility that compensatory changes to physiological parameters not assessed in this study may have occurred in response to nitrate. For example, a study by Williams et al. (1997) found that *Cyprinus carpio* exposed to nitrite increased their ventilatory response to maintain aerobic capacity.

Conclusion

In summary, the results of this study suggest exposure to nitrate does not affect the energy homeostasis of *G. breviceps*. My inability to detect significant effects of nitrate (11, 50 mgL^{-1} NO_3^-) exposure on the aerobic scope, organosomatic indices, growth rates or condition of *G. breviceps* indicates that the national bottom line of 11 mgL^{-1} NO_3^- (MfE, 2020) may be sufficient to protect *G. breviceps* and other closely related *Gobiomorphus* species from adverse energetically related effects. However, it is important to note that this study was 'short' in duration, and because the duration of nitrate exposure affects physiological parameters (Gomez Isaza et al., 2020a), longer-term data is required for fish living in chronically elevated nitrate waters.

Moreover, due to species-specific tolerances to nitrate pollution (Carmargo and Alonso, 2006), further studies are needed on other potentially more sensitive species to fully evaluate the suitability of this regulatory limit to protect native fish. In line with Hickey and Martin

(2009), I recommend the suitability of regulatory guidelines be based on the sensitivity of the most sensitive species to nitrate pollution. Future studies would also benefit from measuring haemoglobin and methaemoglobin concentrations of fish exposed to nitrate to quantify the effect of nitrate exposure on oxygen-carrying capacity and discern if other compensatory mechanisms may be at play. Finally, I also recommend that future studies be conducted across multiple life history stages, as Gomez Isaza et al. (2020a) determined that the effects of nitrate on aquatic species are more prominent in larval and juvenile life history stages.

Chapter 4 -The effect of nitrate exposure on the heat tolerance of G. breviceps

Introduction

Nitrate pollution is a significant threat facing freshwater ecosystems and their biota (Carmago et al., 2005; Craswell, 2021). However, in modern freshwater environments, one stressor rarely acts alone upon an ecosystem and its biota (Sokolova et al., 2012). Today, aquatic species face a multitude of threats, such as eutrophication, pollution, introduced species, habitat degradation, hypoxia and rising water temperatures due to global warming (Jackson et al., 2016; Rodgers et al., 2021). Rising water temperatures pose a significant threat to freshwater species because, as ectotherms, temperature plays an important role in regulating the rate of physiological processes such as growth, development, reproduction and metabolism (Claireaux, 2000; Gomez Isaza et al., 2020c; Opinion et al., 2021). According to Ficke et al. (2007), global air temperatures are predicted to increase by around 5°C over the next century, with similar rises in water temperatures also expected (Reiger and Meisner, 1990). In New Zealand, the frequency and severity of heatwaves are also predicted to increase over the coming decades, likely pushing many freshwater species beyond their thermal tolerance limits (Stillman, 2019; Woolway et al., 2021).

Freshwater fish are vulnerable to the threat of rising water temperatures because fish physiology is intrinsically linked to temperature, and fish have evolved to function with specific temperature windows (Dahlke et al., 2020). Elevated water temperatures can cause changes to fish community composition and distribution, fish body size and biomass, and the diversity of fish communities (Perry et al., 2005; Ficke et al., 2007; Daufresne et al., 2009). The combined threat of rising water temperatures and other stressors, such as nitrate pollution, threaten freshwater fish species (Rodgers et al., 2021). Concerningly, nitrate

pollution is one threat foreseen to intensify as global temperatures rise and the frequency and intensity of heatwaves increase because elevated temperatures boost nutrient cycling rates (Ficke et al., 2007; Dokulil and Teubner, 2011). Together, forecasted rising water temperatures and increases in nitrate pollution are stressors that threaten the persistence of freshwater fish in New Zealand. It is, therefore, imperative to understand how these stressors may interact with one another to affect freshwater fish.

Individually, both elevated temperatures and nitrate pollution can cause adverse effects on fish, such as lowered fitness, disrupted energy homeostasis, and disrupted performance-related activities (de Campos et al., 2014; Lefevre, 2016; McArley et al., 2017; Gomez Isaza et al., 2018, Gomez Isaza et al., 2020; Opinion et al., 2020). However, when two or more stressors act on an animal, they can interact in a non-additive manner (Jackson et al., 2016). For example, one stressor may increase a species' susceptibility to another stressor (cross-susceptibility) or increase the species' tolerance to another stressor (cross-tolerance) (Sinclair et al., 2013; Todgham and Stillman, 2013). According to Jackson et al. (2016), freshwater species like fish are susceptible to the non-additive effects of multiple stressors. Some recent studies have investigated the non-additive effects of nitrate pollution and other stressors on freshwater fish species. For example, a study by Rodgers et al. (2021) found that exposure to nitrate (50 and 200 mgL^{-1} NO_3^-) decreased the thermal tolerance of a freshwater salmonid (*Thymallus thymallus),* demonstrating cross susceptibility. Similarly, Gomez Isaza et al. (2020c) also found that nitrate exposure (50 and 100 mgL^{-1} NO_3^-) reduced the heat tolerance of juvenile silver perch (*Bidyanus bidyanus*).

Predicting the responses of fish to the effects of multiple stressors requires a mechanistic understanding of how the initial stressor may modulate the animal's response to the second stressor (Gomez Isaza et al., 2021b). In natural environments, nitrate pollution is often the initial stressor as it is considered relatively stable and persistent in many polluted streams

(Rodgers et al., 2021). As discussed in chapters one and three, exposure to nitrate pollution causes the oxidation of haemoglobin to methaemoglobin, lowering the blood oxygen-carrying capacity and aerobic scope of freshwater fish (Camargo et al., 2005; Gomez Isaza et al., 2020b). This means that fish may be more susceptible to additional stressors, such as rapid increases in temperature, that require efficient oxygen uptake (Rodgers et al., 2021;) and are influenced by cardiorespiratory capacity (Eliason et al., 2013; Pörtner et al., 2017)

Here this research assesses how exposure to elevated nitrate concentrations (0, 11 or 50 mg L^{-1} NO_3^-) may affect the susceptibility of *G. breviceps* to elevated/extreme temperatures. *G. breviceps* is common in shallow agricultural streams and rivers throughout Canterbury. Streams are often highly nitrate-polluted and are likely to experience rising water temperatures now and into the future due to their shallow nature and because there is often little canopy cover providing shade (Collins et al., 2013). In this study, the heat tolerance of *G. breviceps* was assessed by measuring the critical thermal maxima (CT_{max}) of *G. breviceps* after exposure to elevated nitrate concentrations. CT_{max} is a common physiological tool for investigating ectotherm upper thermal tolerance levels (Vinagre et al., 2015). In fish, the CT_{max} is accepted as the temperature at which fish lose equilibrium and cannot maintain an upright position (Vinagre et al., 2015; Rodgers et al., 2021;). This endpoint is considered an ecologically relevant indicator of upper thermal tolerance because it represents the temperature at which fish cannot escape unfavourable environmental conditions or predators (Becker and Genoway, 1979; Rodgers et al., 2021; Desforges et al., 2013; Morgan et al., 2018). Measuring CT_{max} is also considered a good representation for determining an organism's ability to withstand extreme temperature events like heat waves (Åsheim et al., 2020; Rodgers et al., 2021).

Data regarding the vulnerability of native freshwater fish species to multiple stressors in New Zealand is extremely limited. Furthermore, national limits like the $11mgL^{-1}$ NO^{3-} bottom line

(MfE, 2020) do not account for interactions between nitrate and other stressors. As global warming increases water temperatures and the frequency of heat waves, it is important to understand how fish living in nitrate-polluted habitats will cope with the threat of multiple stressors. This chapter aimed to determine how exposure to nitrate pollution may increase the susceptibility of *G. breviceps* to elevated water temperatures (heat stress). I predicted that exposure to increasing levels of nitrate pollution would increase the susceptibility of *G. breviceps* to heat stress (indicated by fish losing equilibrium at lower temperatures).

Methods

Fish collection and husbandry

Fish collection and animal husbandry methods were the same as those outlined in Chapter 3. Sixty-three upland bullies (*G. breviceps*) were collected from Okeover stream (NZTM2000: E1566366.7, N5181109.3) that runs through the University of Canterbury in Christchurch, New Zealand, over two days. Fish were collected using hand nets and, once captured, were quickly transported to the laboratory in 20 L aerated buckets. In the laboratory, fish were weighed (Sartorius TE153S model, Göttingen, Germany), measured (total length) and randomly distributed among nine aerated 90 L plastic holding tanks (77 × 57 × 28 cm, l × w × h) with seven fish per tank. Tanks were maintained at a water temperature of 14°C (± 2°C), which matched the temperature of the stream from which they were collected. Aquarium thermometers (Fluke 51 II Handheld Digital Probe Thermometer, WA, USA, 0.05 % + 0.3 °C) and a temperature logger (TinyTag; TG-4100) were used to monitor water temperature throughout the experiment. Each tank contained one aerator and sponge biofilter to ensure water filtration and oxygenation. Each tank also included four PVC pipes (3.2cm x 10cm; l x d), and a mesh hide to ensure that fish had places to shelter and help reduce stress levels. Fish were exposed to 12-hour light and 12-hour dark photoperiod, with a 30-minute sunrise and

sunset function. Partial water changes of at least 25% of the total water volume were completed every second day for all tanks (~25 L). Reverse osmosis (RO) water enriched with salts ($NaHCO_3$, $CaSO_4*2H_2O$, $MgSO_4*7H_2O$, KCL; Thermofisher, Australia) was used in the experiment. RO water was used to ensure control nitrate levels were kept as close to 0 mg L^{-1} as possible. Basic water chemistry tests (nitrite and ammonia) were conducted bidaily using Aquarium Pharmaceuticals Ltd liquid test kits. If nitrite or ammonia levels exceeded 0.50 ppm, a larger water change was undertaken to maintain suitable levels. Frozen blood worms were fed to fish daily until satiety, and chopped meal worms were fed biweekly. Faecal matter and uneaten food items were removed daily, and fish were visually inspected daily. Fish showed no signs of infection, disease, or injuries throughout this study. All experimental methods complied with the New Zealand Animal Welfare Act (1999) and were approved by the University of Canterbury's Animal Ethics Committee (#202105R).

Nitrate treatments

Fish were given two weeks to habituate to the laboratory conditions before the start of the experiment. After two weeks, the nine tanks were randomly split into three nitrate treatment groups 0 [control], 11 and 50 mgL^{-1} NO_3^- (total = 63 fish, 21 fish/treatment, 3 tanks/treatment). This range of nitrate concentrations was chosen because 11 mgL^{-1} NO_3^- was recently published as the new New Zealand national bottom line for nitrate allowed in rivers in the 2020 National Policy Statement for Freshwater Management (MfE, 2020), and 50 mgL^{-1} NO_3^- was reflective of the highest level of nitrate where *G. breviceps* were found in natural field conditions (Chapter 2). Nitrate concentrations were maintained by dissolving sodium nitrate salt (ThermoFisher Scientific) in a 3 L bottle of RO artificial water and then slowly mixing it into the holding tank. Nitrate levels were checked daily using a portable nitrate meter (LAQUAtwin-NO3-11 meter, Horiba Scientific). Nitrate levels did not fluctuate from nominal levels by more than 10% for all treatment groups. Nitrate concentrations in

control tanks were always maintained below the detection limit of the meter (< 5 mgL^{-1} NO_3^-). Fish were exposed to nitrate treatments for 64 days before CT_{max} experiments.

Critical thermal maxima (CT_{max})

To determine if nitrate exposure affects the heat tolerance of *G. breviceps*, critical thermal maximum (CT_{max}; Beitinger et al., 2000; Morgan et al., 2018) was measured on 18 fish from each nitrate treatment group (total = 54, 5 - 7 fish/tank). Fish were fasted for 24 hours prior to experiments. Before starting each CT_{max} trial, six fish were transferred to an aerated water bath set to 14°C and nitrate concentration as their holding tank. Fish were given a 30-minute adjustment period to recover from handling stress before experimental trials began. The temperature was then increased constantly at a rate of ~0.3ºC/minute following recommendations from (Becker and Genoway 1979; Morgan et al., 2018) using water heaters (Grant T-series Heated Circulator, Shepreth, Cambridgeshire; Jebo 200 w submersible heater, China). The temperature was continuously measured every minute using a digital thermometer (Fluke 51 II Handheld Digital Probe Thermometer, WA, USA, 0.05 % + 0.3 °C) and a tiny tag (TG-4100). Fish were carefully observed for the duration of the trial, and CT_{max} was determined as the temperature that fish lost equilibrium (LOE; disorganised movement/ inability to right themselves in the water column) (Becker and Genoway, 1979; Rodgers et al., 2019). This endpoint is ecologically relevant because it is the temperature at which fish cannot escape predators and unfavourable conditions (Morgan et al., 2018). Once CTmax was reached for an individual, it was recorded, and then the fish was quickly placed in an aerated recovery container at 14°C. Fish were given ~15 min or as long as necessary to fully recover before being weighed and returned to holding tanks. Survival was high following CTmax trials, with only one fish failing to regain equilibrium. This individual was euthanised using MS222.

Results

Critical thermal maxima

Exposure to elevated nitrate concentrations for 64 days did not significantly affect the critical thermal maxima CT_{max} of *G. breviceps* ($F_{1,7}$ = 4.26, P = 0.08, LME, Figure 14). Although the mean CT_{max} of *G. breviceps* in the 0 (control) mgL^{-1} NO_3^- treatment group was 0.38°C higher than the 50 mgL^{-1} NO_3^- treatment group, with the 11 mgL^{-1} NO_3^- showing an intermediate response in CT_{max}. Fish body mass did not influence CT_{max} ($F_{1,40}$ = 0.96, P = 0.33, LME).

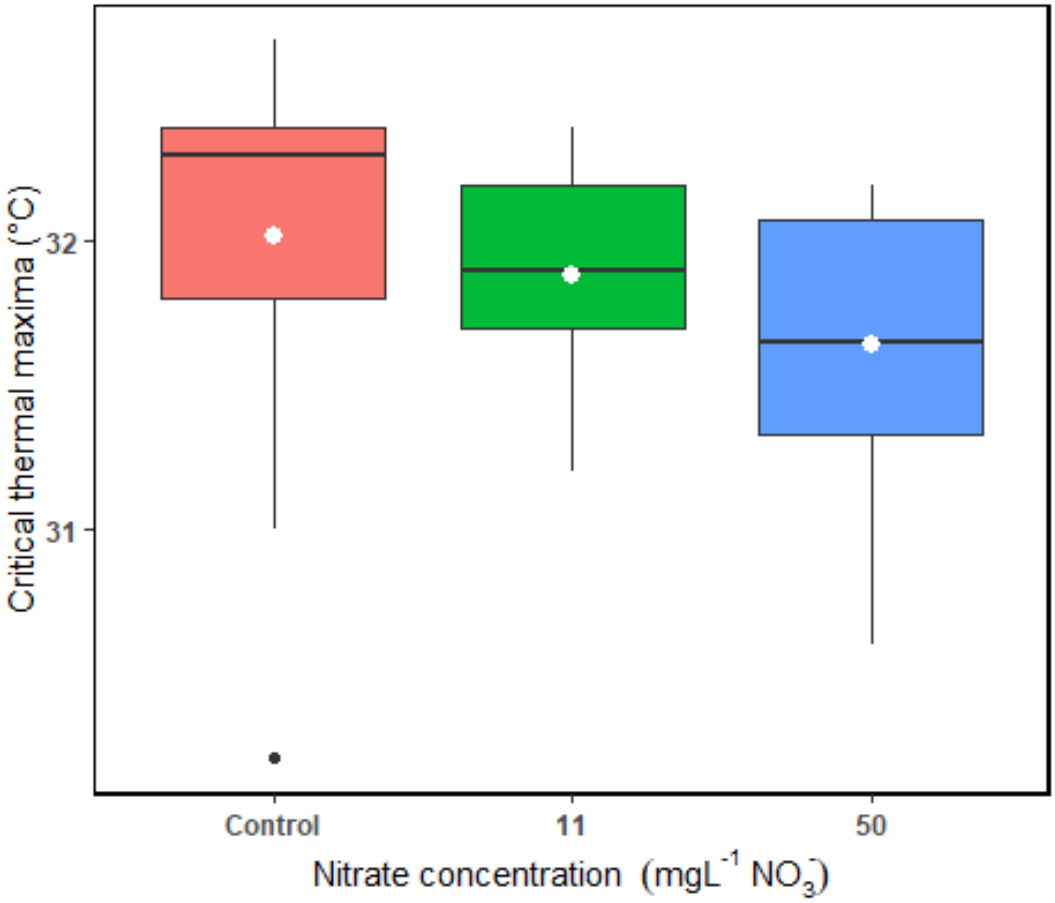

Figure 14. The critical thermal maxima (CT_{max};°C) of upland bully (*Gobiomorphus breviceps*) exposed to three different concentrations of nitrate (0, 11 and 50 mgL^{-1} NO_3^-) for 64 days. Data are presented as boxplots [minimum, first quartile (Q1), median, third quartile (Q3) and maximum] with white points representing group mean values. No significant differences in CT_{max} (°C) were found between nitrate treatment groups ($F_{1,7}$=4.26, P=0.078, LME, n=18, 14, 18; control, 11 and 50 mgL^{-1} NO_3^-)**.**

Discussion

Contrary to predictions, the results of this study suggest that nitrate exposure does not affect the thermal tolerance (critical thermal maxima; CT_{max}) of *G. breviceps.* However, a small (non-significant) reduction in CT_{max} (0.38°C) was observed in 50 mgL^{-1} NO_3^- nitrate-exposed fish compared to control fish. In line with my results, Opinion et al. (2021) observed no significant effect of nitrate exposure (0, 50, 200 mgL^{-1} NO_3^-) on the CT_{max} of common carp (*Cyprinus carpio*). In contrast, previous studies have reported significant decreases in the CT_{max} of freshwater fish exposed to nitrate (Gomez Isaza et al., 2020c; Rodgers et al., 2021) and nitrite (Rodgers and De Boeck, 2019).

According to Gomez Isaza et al. (2020c), reductions in thermal tolerance are likely attributed to a decrease in red blood cells (methemoglobinemia) that occurs when fish are exposed to nitrate. As discussed previously, a reduction in red blood cells manifests as diminished oxygen-carrying capacity (Gomez Isaza et al., 2020b). As thermal tolerance limits are hypothesised to be associated with oxygen supply (Pörtner, 2010; Pörtner et al., 2017), reduced oxygen-carrying capacity was suggested to be responsible for decreases in CT_{max} of nitrate-exposed fish (silver perch; *Bidyanus bidyanus*) (Gomez Isaza et al. 2020c). Indeed, experimentally induced reductions (phenylhydrazine injection) in blood oxygen-carrying capacity lowered the CT_{max} of European sea bass (*Dicentrarchus labrax*) (Wang et al., 2014).

The non-significant effect of nitrate exposure on the CT_{max} of *G. breviceps* observed in this study suggests that nitrate exposure does not alter the blood oxygen-carrying capacity of this species sufficiently to influence thermal tolerance. This conclusion is supported by the results of Chapter three of this thesis, in which the aerobic scope (associated with blood oxygen carrying capacity; Gomez Isaza et al., 2020b) of *G. breviceps* was unaffected by exposure to nitrate. However, my results may also be explained by the temperature at which the fish were

acclimated. For example, a study by Rodgers et al. (2021) found that cold-acclimated (18°C) European grayling (*Thymallus thymallus*) exposed to nitrate experienced significantly decreased CT_{max}, while warm-acclimated (22°C) fish experienced significant increases in CT_{max}. In my experiment, fish were kept at a constant temperature of 14°C, matching the temperature of the stream from which they were caught. Future studies may wish to consider the effects of nitrate exposure in both warm and cold-acclimated fish.

In summary, the results of this study suggest that exposure to ecologically relevant nitrate concentrations (≤50 mgL^{-1} NO_3^-) does not increase the susceptibility of *G. breviceps* to water temperature extremes. However, nitrate may increase the susceptibility of fish to other stressors not examined in this study, such as low dissolved oxygen (Gomez Isaza et al., 2021a) or low pH (Gomez Isaza et al., 2020b) resulting in negative synergistic effects. It is therefore recommended that future studies investigate the interactive effects of nitrate and other common stressors that are often present in streams draining agricultural areas in New Zealand, such as low dissolved oxygen, metal pollution, acidification, and turbidity (Ryan, 1991; Greig et al., 2010; Collins et al., 2013; Moore, 2014). This will open the door to flexible nitrate thresholds conducive to adaptive management under changing conditions.

Chapter five - General Discussion

Summary

Nitrate pollution is regarded as one of the most prevalent threats to freshwater systems worldwide (Carmago and Alonso, 2006; Dudgeon, 2019). In Canterbury, New Zealand, high agriculture intensity has resulted in large quantities of nitrate leaching into and contaminating freshwater streams and rivers (Foote et al., 2015; Joy et al., 2019; Joy et al., 2022). In New Zealand, 76% of freshwater fishes are listed as 'threatened' or 'at risk' of extinction (Stats NZ, 2021), with many threatened species occurring in Canterbury (Allibone et al., 2010). Nitrate pollution, primarily from dairy farming, is thought to be a major factor contributing to freshwater fish declines in New Zealand (Foote et al., 2015; Julian et al., 2017; Joy et al., 2019). Laboratory studies abroad have reported adverse physiological effects on species such as salmonids (McGurk et al., 2006; Opinion et al., 2020) and perch (Gomez Isaza et al., 2020b; Gomez Isaza et al., 2020c; Gomez Isaza et al., 2020a; Gomez Isaza et al., 2021b) following exposure to ecologically relevant nitrate concentrations (28-200 mgL^{-1} NO_3^-). However, very little is known about how native fish species in New Zealand respond to elevated nitrate concentrations (Hickey and Martin, 2009; Hickey, 2013) nor if results from laboratory experiments overseas translate to observations in the field (Gomez Isaza., 2020). To address this pressing knowledge gap, I investigated the physiological effects of nitrate exposure on upland bully (*Gobiomorphus breviceps*), a native fish species common in freshwater environments in Canterbury. Here, I discuss key findings, implications for management, limitations, and future directions.

The physiological effects of nitrate on upland bully (*Gobiomorphus breviceps*)

The first results of this thesis (Chapter 2) indicated that living in nitrate-contaminated waterways does not influence the physiological body condition (scaled mass index (SMI),

organosomatic indices) of *G. breviceps.* These results provide evidence that living in nitrate-contaminated waters does not compromise energy homeostasis in this species. However, while this work sought to account for confounding environmental variables previously reported to influence fish body condition (prey abundance and instream habitat), due to the multi-variate nature of field studies, it was not logistically or analytically possible to include all measured environmental variables that may influence fish condition in this assessment. For example, the presence of predators (James and Poulin, 1998) and high fish density (Maceda-Veiga et al., 2014) can decrease fish body condition in wild fish. Therefore, the results of chapter two should be interpreted with caution and may only tentatively be attributed to nitrate contamination effects.

To isolate the effect of nitrate alone on the physiology of *G. breviceps,* fish were exposed to one of three ecologically relevant nitrate concentrations in the laboratory (0, 11, or 50 mgL^{-1} NO_3^-) with other variables held constant (Chapter 3). The results of chapter three aligned with those of chapter two. Fitness correlates (aerobic scope, growth, body condition and organosomatic indices) of *G. breviceps* were unaffected by nitrate exposure at both concentrations (11, 50 mgL^{-1} NO_3^-). Together, the results of chapter two and chapter three indicate that *G. breviceps* is tolerant of nitrate exposure at concentrations not exceeding 50 mgL^{-1} NO_3^-. These results come despite other studies reporting significant physiological effects in other fish species exposed to 50 mgL^{-1} NO_3^- (Gomez Isaza et al., 2020b; Opinion et al., 2020; Gomez Isaza et al., 2021b).

The results of chapter four suggest that exposure to elevated nitrate concentrations does not affect the thermal tolerance of *G. breviceps,* indicating that nitrate does not cause cross-susceptibility to heat stress. This result is supported by the results of chapter three of this study. Aerobic scope, blood oxygen carrying capacity, and thermal tolerance (critical thermal maxima; CT_{max}) are thought to be closely linked (Gomez Isaza et al., 2020b; Opinion et al.,

2020). The energy-limited tolerance to stress hypothesis (ELTS; Sokolova, 2013) proposes that energy allocation towards dependent functions such as growth, reproduction, and the ability to cope with other stressors depends on organismal aerobic scope. In chapter three, no effect of nitrate exposure was observed on the aerobic scope of *G. breviceps.* Therefore, it is likely that blood oxygen levels, and thus energy to cope with heat stress, was maintained in nitrate-exposed fish resulting in undifferentiated thermal tolerances of nitrate-exposed fish relative to control fish.

The seemingly non-significant effect of nitrate exposure on *G. breviceps* observed in this study may be attributed to a number of reasons. First, because nitrate toxicity increases with longer exposure times (Gomez Isaza et al., 2020a), the duration of nitrate exposure in the lab experiments (Chapters 2 and 3) may not have been long enough to observe significant effects. Nitrate diffusion rate into the body can be very slow (Stormer et al., 1996). For example, rainbow trout (*Oncorhynchus mykiss*) fingerlings exposed to 75 mgL^{-1} NO_3^- for eight days had nitrate plasma concentrations significantly below ambient nitrate concentration, and this slow uptake rate translated to weak toxic effects (Stormer et al., 1996). Therefore, the duration of nitrate exposure in the laboratory experiments (aerobic scope: 40 days, Chapter 3, CT_{max}; 64 days, Chapter 4) days of exposure (metabolism experiment) may not have been enough time for plasma nitrate to accumulate in the body to cause physiological effects. However, Gomez Isaza et al. (2020b) observed significant reductions in spangled perch (*Leiopotherapon unicolor*) after just 28 days of nitrate exposure (50 mgL^{-1} NO_3^-), and Rodgers et al. (2021) found reductions in thermal tolerance (CT_{max}) after 56 days of nitrate exposure (50, 200 mgL^{-1} NO_3^-). The discrepancies between these results may indicate species-specific differences in nitrate tolerance.

Species-specific tolerances of nitrate exposure have been reported in previous reviews of nitrate toxicity to aquatic species (Carmargo and Alonso, 2006; Gomez Isaza et al., 2020a).

Species-specific differences in nitrate toxicity are likely due to differences in the detoxification capabilities of different fish (Gomez Isaza et al., 2020b). For example, clown knifefish (*Chitala ornate*) are thought to have a high tolerance to nitrite exposure due to their ability to upregulate the methaemoglobin reductase enzyme, which reduces methaemoglobin (caused by nitrite and nitrate exposure) back to functional haemoglobin (Gam et al., 2017). This physiological adaptation may also be responsible for the high tolerance of *G. breviceps* to nitrate, but further experiments are required to confirm this. Nitrate pollution has been prevalent in many waterways in Canterbury, New Zealand, for over a decade (Greenwood et al., 2012; Joy et al., 2019). Therefore, *G. breviceps* may have adapted physiological mechanisms such as upregulation of the methaemoglobin reductase enzyme to cope with nitrate-induced methemoglobinemia and thus reduce toxic physiological effects. However, upregulation of the methaemoglobin reductase enzyme is energetically demanding (Opinion et al., 2020), so increases in the SMR of *G. breviceps* may have been expected, but this was not observed in the present study. Instead, *G. breviceps* may employ other elimination, storage or detoxification capabilities to reduce nitrate toxicity. This is a potential avenue for further research.

Stress history may also be responsible for the non-significant effects of nitrate on *G. breviceps* observed in this study. Previous exposure to stressors in the past may result in cross-tolerance to stressors in the future (Rodgers and Gomez Isaza, 2022). The fish in this study were collected from the wild, so their stress history is unknown. Similar laboratory studies which have reported significant effects of nitrate on fish (Gomez Isaza et al., 2020b; Opinion et al., 2020; Gomez Isaza et al., 2021b) typically source their fish from hatcheries where fish are kept in stable conditions. The results of this study are unlikely to be attributed to pre-acclimation to nitrate at the collection site as nitrate concentration was low (11 mgL^{-1} NO_3^-; Appendix 1)

Management implications

The 2020 national policy statement for freshwater management (NPSFWM) recently implemented a new national bottom line of 11 mgL^{-1} NO_3^- allowed in rivers (MfE, 2020). This bottom line is intended to regulate nitrate contamination in freshwater systems to protect freshwater fish and aquatic invertebrates from direct toxic effects (MfE, 2020). A key objective of this study was to investigate the suitability of this national bottom line to protect native fish in New Zealand. Previous studies have expressed concern over the adequacy of nitrate limits to protect freshwater fish as most of the data that bottom line limits were created from comes from lethal toxicity tests and data from invasive salmonid species (Hickey and Martin, 2009; Hickey, 2013).

The results of this study determined that exposure to 11 mgL^{-1} NO_3^- does not pose a significant risk to *G. breviceps*. All fitness proxies assessed in this study (growth, body condition, aerobic scope, organosomatic indices) were unaffected in fish exposed to 11 and 50 mgL^{-1} NO_3^- relative to control fish (Chapter 2). These results suggest that *G. breviceps* is tolerant of nitrate. This conclusion may be supported by other studies that have suggested that *G. breviceps* is tolerant of low water quality (Richardson and Jowett, 2005) and adjacent agricultural land use (Ramezani et al., 2016). Hickey and Martin (2009) recommend nitrate guidelines should include the tolerance of the most sensitive species. Therefore, further studies that assess the effects of nitrate on other potentially more sensitive species are needed. Canterbury galaxias (*Galaxias vulgaris)* would be an ideal candidate for future studies as their conservation status is ‘at risk/declining’, and they are only found in Canterbury, New Zealand (Stats NZ, 2021).

This thesis provides a framework for assessing the effects of nitrate on native freshwater fish species in New Zealand. Nitrate pollution is predicted to increase due to continued dairy

intensification and climate change (Stevenson et al., 2010; Pangborn and Woodford, 2011; Rodgers et al., 2021), so it is crucial that data be collected on the tolerances of native fish species. Without a rapid assessment of the tolerances of native New Zealand fish species, shifts may occur in nitrate-contaminated streams towards more nitrate-tolerant species unnoticed by freshwater management.

Study limitations and future directions

Several areas of this research could be expanded to provide a greater understanding of the effects of nitrate on native fish in the future. A limitation of chapter two was that I could not collect reliable long-term data on dissolved oxygen levels (DO) or temperature due to logistical constraints. Recent studies have shown that both elevated temperatures (Opinion et al., 2020) and hypoxic conditions (low dissolved oxygen) (Gomez Isaza et al., 2021a) can interact with nitrate in a non-additive manner resulting in potentially negative synergistic effects on freshwater fish. Therefore, future studies investigating the effects of nitrate in the field should aim to include long-term DO and temperature data in analyses. Especially considering freshwater temperatures are expected to increase (Stillman, 2019) and hypoxic conditions often accompany areas contaminated by nitrate due to eutrophication (Rodgers et al., 2021).

Likewise, as discussed in chapter three, laboratory experiments conducted in future would benefit from including interactions between nitrate and other stressors such as DO, temperature, and pH, which have all been shown to affect the toxicity of nitrate to freshwater species (Hatch and Blaustein, 2000; Ortiz-Santaliestra and Marco, 2015; Opinion et al., 2020; Gomez Isaza et al., 2020b; Gomez Isaza et al., 2020c; Gomez Isaza et al., 2021a; Gomez Isaza et al., 2021b; Opinion et al., 2021). This would greatly improve understanding of

stressor interactions in natural freshwater environments which is an increasing ecological reality for biota within freshwater ecosystems (Gomez Isaza et al., 2020a).

It is important to recognise that the results of this study do not exclude the possibility that nitrate exposure caused other adverse physiological effects not measured in this study. For example, exposure to nitrate can cause endocrine disruption in freshwater species through *in-vivo* conversion to nitric oxide, which is involved in many metabolic pathways (Guillette and Edwards, 2005; Cano-Rocabayera et al., 2019). Kellock et al. (2018) reported endocrine disruption of reproductive hormones in nitrate-exposed fathead minnows (*Pimephales promelas*). Exposure to nitrate has also been shown to disrupt key sexual traits, including sexual behaviours (Secondi et al., 2009; Secondi et al., 2013), which were not measured in this study and could have negative implications for wild fish populations. Therefore, future studies may benefit by complementing measures of physiological traits with measures of behavioural traits.

Another limitation of this study was that the fish were too small to collect blood samples to measure haemoglobin and methaemoglobin concentrations. Therefore, although measurements of aerobic scope (closely associated with blood oxygen supply) and aerobically supported traits (growth, condition, organosomatic indices) were not affected in nitrate-exposed fish, the effect of nitrate-induced methemoglobinemia (Gomez Isaza et al., 2020b) was unable to be fully evaluated. To fully evaluate the effect of nitrate-induced methemoglobinemia in freshwater fish, future studies should include measures of methaemoglobin and haemoglobin concentrations where possible. Furthermore, measures of methaemoglobin reductase activity would be useful to assess detoxification rates.

This research only focused on the adult (>35mm; McDowall and Eldon, 1997) life stage of *G. breviceps*. A recent meta-analysis by Gomez Isaza (2020a) concluded that larval and juvenile

life history stages are more sensitive to nitrate exposure than other life history stages (adult and embryonic). For example, Cano-Rocabayera et al. (2019) showed female adult mosquito fish were more tolerant to nitrate exposure than juveniles. Therefore, I recommend future studies investigate the effects of nitrate at larval and juvenile stages in combination with adult assessments.

Conclusion

Nitrate is a major stressor to freshwater ecosystems in New Zealand, yet its effects on native freshwater fish in New Zealand are largely unknown. This research thesis aimed to understand the effects of nitrate pollution on *G. breviceps* by assessing the physiological effects of elevated nitrate exposure in naturally occurring populations and under controlled laboratory conditions. The potential for nitrate exposure to increase susceptibility to other stressors was also evaluated by measuring the heat tolerance of *G. breviceps* following nitrate exposure. The results of this thesis indicate that *G. breviceps* is tolerant of nitrate pollution not exceeding >50 mgL^{-1} NO_3^- as no significant effects were detected on ecologically relevant proxy measurements of fitness (growth, condition, organosomatic indices and aerobic scope). Nitrate exposure also did not influence the thermal tolerance of *G. breviceps*.

These results suggest that recently established national bottom lines (MfE, 2020) are sufficient to protect *G. breviceps*, but further studies on other, potentially more sensitive species (e.g., Canterbury galaxias (*Galaxias vulgaris*)) are required to evaluate this national bottom line's suitability fully. Furthermore, I recommend that future studies build upon the assessment framework established here by assessing nitrate exposure across fish life history stages (particularly larval and juvenile) and include interactions between nitrate and other stressors such as hypoxia and elevated temperatures.

Appendices

Appendix 1.

The calculation of mean habitat nitrate concentrations.

Site name	Nitrate measurement during site survey (mgL^{-1} NO_3^-)	Nitrate measurement on the day of fish collection (mgL^{-1} NO_3^-)	Hills laboratories results (mgL^{-1} NO_3^-)	Mean habitat concentration. (mgL^{-1} NO_3^-)
Surveyors road water race	47	51	55.11	51.04
Doyleston drain	23	20	12.84	18.61
Halkets road water race	5	5	0.19	3.40
Okeover stream	12	11	10.62	11.21
Ridgens road water race	6	6	4.67	5.56
Weedons road water race	5	5	0.01	3.34
Ballentines drain	19	16	8.23	14.41
Hawkins stream	12	11	6.42	9.81
Leeston road water race	50	48	29.22	42.41

Appendix 2.

Habitat assessment sheet adapted from Environment Canterbury (https://www.ecan.govt.nz)

Instream habitat quality parameters

Habitat Parameter	Category			
	Optimal	**Suboptimal**	**Marginal**	**Poor**
33. Instream habitat/ Roughness - Cover for instream fauna.	Greater than 70% of substrate favourable for faunal cover/ utilisation and fish cover – mixture of cobble, boulder, snags, undercut banks etc.	40-70% cover of suitable habitat including cobbles, boulders logs and snags	Only 20-40% cover is suitable habitat – habitat dominated by fine or unstable sediments, lack of instream cover features	Little stable cover or habitat, substrate open, fine, unstable.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1

Habitat Parameter	Category			
	Optimal	**Suboptimal**	**Marginal**	**Poor**
34. Substrate heterogeneity and quality	Wide range of substrate sizes (<4), of angular nature and well packed, no size class > 50%. Bedrock, Boulder (>25), Large cobbles (12-25), Small cobbles (6-12), Gravel (0.5-6), Sand (<0.5), mud/silt.	3-4 size classes, some interstitial spaces filled with silt, no size class > 50%.	2-3 size classes, interstitial spaces rare, usually dominated by > 50% one class	One or two cobble sizes dominate substrate, cobbles more rounded and loser packing, interstitial spaces rare
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1

Habitat Parameter	Category			
	Optimal	**Suboptimal**	**Marginal**	**Poor**
35 Embeddedness/Siltation	Gravel, cobble, and boulder particles are 0-25% surrounded by fine sediment. 5 10 15 20 25%	Gravel, cobble, and boulder particles are 30-50% surrounded by fine sediment. 30 35 40 45 50 %	Gravel, cobble, and boulder particles are 55-75% surrounded by fine sediment. 55 60 65 70 75 %	Gravel, cobble, and boulder particles are more than 75% surrounded by fine sediment. 80 85 90 95 100 %
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1

Habitat Parameter	Category			
	Optimal	**Suboptimal**	**Marginal**	**Poor**
38. Emerged Macrophyte presence	Rooted macrophytes largely absent (less 20 %) – stony substrate with periphyton or moss/bryophytes, not obstructing flow patterns	Small areas of rooted emerged macrophytes (20 to <50%) in flowing channel, not obstructing flow patterns	Significant (≥50 – 80 %) of bed or channel affected by emergent macrophytes on edges, reducing water velocities in places	Emerged macrophytes dominate channel and clogging waterway, 80 – 100%
SCORE ___	10 9	8 7 6	5 4 3	2 1

Habitat Parameter	Category			
	Optimal	**Suboptimal**	**Marginal**	**Poor**
39. Submerged Macrophyte presence	Rooted macrophytes largely absent (less 20%) – stony substrate with periphyton or moss/bryophytes, not obstructing flow patterns	Small areas of rooted submerged macrophytes (20 to <50%) in flowing channel, not obstructing flow patterns	Significant (≥50 – 80%) of bed or channel affected by submerged macrophytes in channel reducing water velocities in places	Submerged macrophytes dominate channel and clogging waterway, 80 – 100%
SCORE ___	10 9	8 7 6	5 4 3	2 1

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