AMMONIA HANDLING STRATEGIES OF AN ASYMBIOTIC SEA ANEMONE, *NEMATOSTELLA VECTENSIS*

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ABSTRACT

Although many animals have evolved mechanisms to tolerate and regulate ammonia, the ammonia tolerance and detoxification mechanisms of *Nematostella vectensis* (the starlet sea anemone) remain poorly understood. Unlike many cnidarians, the starlet sea anemone lacks a symbiotic relationship with microbes that detoxify ammonia. This study examines the effect of ammonia on survival, metabolic rate, and ammonia-detoxifying enzyme activity in two anemone populations (Nova Scotia and New Hampshire). The results indicate that ammonia caused a general decrease in survival. Ammonia exposure reduced survival in both populations, although the magnitude of the response differed between populations and time points. Enzyme activities (glutamine synthetase and glutamate dehydrogenase) showed population-specific responses. Metabolic patterns across populations were similar; both populations showed that fed and fasted individuals had comparable metabolic rates under high ammonia conditions. Collectively, these findings indicate that geographically distinct populations of *N. vectensis* differ in ammonia tolerance and in physiological, biochemical, and metabolic responses. This study provides insight into ammonia handling and metabolism strategies in an asymbiotic cnidarian species, establishing a foundation for future investigations into the mechanisms underlying ammonia tolerance in the starlet sea anemone.

DEDICATION

I would like to dedicate this thesis to my grandma and grandpa, who passed away before I started my master's. I know you both would have been so proud. I know you were with me through every step, rooting for me and giving me the strength to push through. This is for you! Forever my most beautiful angels. <3

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LIST OF ABBREVIATIONS

ADP	Adenosine 5′-Diphosphate
AFLP	Amplified Fragment Length Polymorphism
ALT/GPT	Alanine Aminotransferase/Glutamic-Pyruvic Transaminase
AMTs	Ammonia Transporters
ANOVA	Analysis of Variance
AST/GOT	Aspartate Aminotransferase/Glutamic-Oxaloacetic Transaminase
ASW	Artificial Seawater
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
BMR	Basal Metabolic Rate
BSA	Bovine Serum Albumin
ED	Effective Dose
EDTA	Ethylenediaminetetraacetic Acid
emmeans	Estimated Marginal Means
$FeCl_3$	Ferric Chloride
GDH	Glutamate Dehydrogenase
GS	Glutamine Synthetase
HCl	Hydrochloric Acid
HEA	High Environmental Ammonia
HEPES	4-(2-Hydroxyethyl)Piperazine-1-Ethanesulfonic Acid
LC	Lethal Concentration
LDH	Lactate Dehydrogenase

Lme4	Linear Mixed-Effects Model
MDH	Malate Dehydrogenase
mM/mmol L^{-1}	Millimolar/Millimoles Per Litre
MMR	Maximum Metabolic Rate
$MnCl_2$	Manganese (II) Chloride
MO_2	Metabolic Rate
mRNA	Messenger Ribonucleic Acid
Na^+/K^+-ATPase (NKA)	Sodium-Potassium Adenosine Triphosphate Pump
$Na_2HAsO_4 \bullet 7H_2O$	Sodium Arsenate Dibasic Heptahydrate
NAD^+/NADH	Nicotinamide-Adenine Dinucleotide
NH	New Hampshire
NH_3	Ammonia (gas)
NH_4^+	Ammonium (ion)
NH_4Cl	Ammonium Chloride (referred to as “ammonia” in this study)
NS	Nova Scotia
p.p.m.	Parts Per Million
p.p.t.	Parts Per Thousand
PLP	Pyridoxal 5’ Phosphate
qPCR	Quantitative/Real-Time Polymerase Chain Reaction
Rh	Rhesus
RMR	Routine Metabolic Rate
SEM	Standard Error of the Mean
SDA	Specific Dynamic Action

SMR	Standard Metabolic Rate
TCA	Trichloroacetic Acid
UV	Ultraviolet
α-ketoglutarate	Alpha-Ketoglutarate
µL	Microlitre

Chapter 1: General Background

1.1 *Nematostella vectensis* as a model organism

The phylum Cnidaria comprises jellyfish, hydra, coral, and sea anemones (Elran et al., 2014). The group Cnidaria possess some of the simplest animals, including *Nematostella vectensis*, in which its tissue organization has two germ layers: an outer ectoderm and an internal endoderm (also known as the entoderm, endomesoderm, or gastrodermis), which is bifunctional (Layden et al., 2016; Park et al., 2012; Putnam et al., 2007) (Figure 1).

Nematostella vectensis, commonly known as the starlet sea anemone, was originally used in evolutionary developmental research as a cnidarian model to compare the development of bilaterian animals and is considered a sister group to bilaterians (Layden et al., 2016). The burrowing starlet sea anemone lives in estuarine environments, including salt marshes and brackish water pools, where it is exposed to various changes in its environment, including fluctuations in temperature, salinity, oxygen concentration, pH, and many other anthropogenic environmental factors (Klein et al., 2021; Layden et al., 2016; Stefanik et al., 2013). This tolerance to a wide range of environmental changes can be attributed to its sessile lifestyle, which allows for physiological plasticity (Fraune et al., 2016). A key feature of the starlet sea anemone is that it can reproduce both sexually and asexually, which is done through the regeneration process (Layden et al., 2016). Regeneration in the starlet sea anemone can be induced naturally or experimentally, through physal pinching or through bisection of the animal, where it is cut into two halves, allowing for asexual reproduction (Darling et al., 2005; Hand & Uhlinger, 1992, 1994; Layden et al., 2016). The starlet sea anemone's ability to reproduce asexually makes it an ideal and accessible organism to maintain in the lab and to use for exposure experiments, as it can readily spawn under laboratory conditions (Al-Shaer et al., 2023).

The starlet sea anemone has a wide distribution, giving rise to various populations. This species populates estuarine areas in Nova Scotia, New Hampshire, England, Georgia, the North American Atlantic coast, Florida, Louisiana, the Gulf of Mexico, California, Washington, and the Pacific coast (Hand & Uhlinger, 1992; Heard, 1982; Kneib, 1985). It has been suggested that the distribution of anemone populations in salt marshes across different geographical areas leads to a lack of genetic similarity, as populations adapt to their local areas (Fraune et al., 2016). For instance, local adaptability has been observed in other sea anemone species, such as *Actinia tenebrosa*, in which adult clones adapted to their local environment (Ayre, 1985). The population, genetic structure, and dispersal of the starlet sea anemone are influenced by anthropogenic factors and its reproductive plasticity (Darling et al., 2004). Based on the distribution of the starlet sea anemone, populations are isolated, meaning minimal gene flow (Darling et al., 2004). Therefore, many starlet sea anemone populations are genetically distinct, with many different genotypes coexisting in a population, except for those populations in the United Kingdom, where a singular clonal genotype is dominant (Darling et al., 2004). In other words, it has been found that in northeastern United States populations, different sites or salt marshes are unique in their genetic makeup and are not dominated by a singular clone (Darling et al., 2004). This asexual (clonal) and sexual species, living in highly fluctuating environments, makes it an ideal model organism for assessing ammonia tolerance.

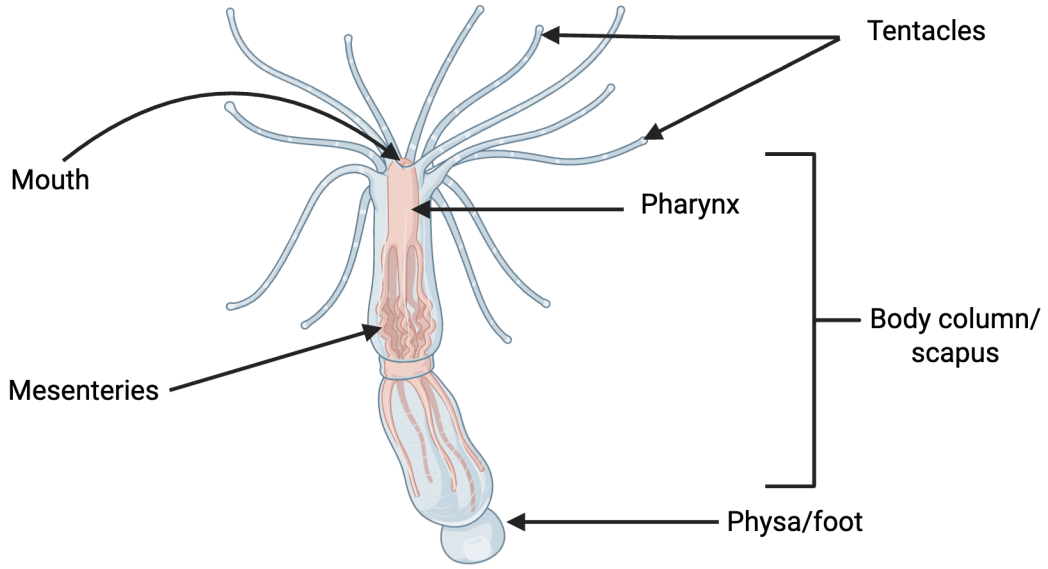

Figure 1. General anatomy of *Nematostella vectensis* (starlet sea anemone). Like many cnidarian species, the starlet sea anemone has a simple body plan. The main structures of the starlet sea anemone include: the tentacles, mouth, pharynx, mesenteries, physa or foot, and the body column or scapus. The tentacles are the primary structures to catch and paralyze prey or food that is fed to the mouth. The mesenteries act as digestive organs and possess many specialized cells. The physa or foot is used to anchor the anemones to sediment, which allows them to burrow themselves. Physal pinching occurs at the physa. When bisections are conducted, anemones are cut horizontally across the middle of the body column to create two halves. Image adapted from Stefanik et al., 2013, and created using BioRender.

1.2 An overview of ammonia

All animals rely on the ability to excrete and/or detoxify nitrogen (Wright, 1995). Macromolecules, such as nucleic acids and proteins, are essential for life; however, these macromolecules contain nitrogen (Terjesen, 2008). In turn, nitrogen is converted into nucleotides or amino acids (Terjesen, 2008). This nitrogen can be further degraded into other nitrogenous end-products, such as ammonia (Chew et al., 2005). In many cases, nitrogenous waste, such as ammonia, is mainly produced by the catabolism of amino acids (Chew et al., 2005) (Figure 2). When dietary proteins are ingested, they are catabolized into amino acids; in turn, these amino

acids are used to make new proteins, but when in excess, they are further catabolized into ammonia (Chew et al., 2005; Miramontes et al., 2020; Wright, 1995). Once ammonia is produced, it can be excreted as is, transported and/or detoxified, or converted to less toxic waste, such as urea or uric acid (Chew et al., 2005; Miramontes et al., 2020; Wright, 1995). Based on this pathway, there is a direct relationship between protein intake and ammonia excretion (Mommsen & Walsh, 1992). Apart from being produced through the catabolism of proteins, in aquatic environments, ammonia can be generated by decomposing organic matter, such as unconsumed feed and excreted wastes from living organisms, and decomposition of dead organisms (Chang et al., 2015). Although nitrogenous end-products are primarily in the form of ammonia at the start, they can be excreted and/or oxidized as ammonia, urea, or uric acid (Kroupina et al., 2022; Miramontes et al., 2020; Terjesen, 2008; Wright, 1995). Ammonia is less energetically expensive than uric acid and urea since converting ammonia to urea and uric acid requires substantial metabolic energy (Wright, 1995; Weihrauch & Allen, 2018). Despite the low energy requirement, ammonia requires a large volume of water for dissolution, making it a more suitable form of nitrogen excretion in aquatic environments (Wright, 1995). Therefore, aquatic animals, such as fish and certain invertebrates, excrete nitrogen as ammonia (Singer, 2003; Wright, 1995). It is important to note that ammonia occurs in two forms (two chemical species): non-ionic ammonia (NH_3), the gaseous form, and the ionic form, ammonium (NH_4^+) (Wright, 1995).

Despite ammonia being a natural by-product produced by many organisms, it is toxic, with both severe and lethal effects (Wright, 1995; Wright & Wood, 2012). The effects of ammonia in water and its aqueous equilibrium depend on the temperature and pH of the aqueous environment (Thurston et al., 1981). In other words, the form of ammonia that dominates depends on temperature and pH. For instance, an increase in pH and temperature causes an increase in NH_3

and a decrease in NH_4^+ due to the increased concentration of hydroxide ions (Thurston et al., 1981; Yan et al., 2019). The toxicity of NH_3 is significantly higher than the toxicity of NH_4^+ (Thurston et al., 1981; Yan et al., 2019). Due to its gaseous and uncharged nature, NH_3 can easily permeate biological membranes; however, NH_4^+ cannot do so because it is charged and requires transport proteins, demonstrating that NH_3 is more toxic (Ip & Chew, 2010; Randall & Tsui, 2002; Wilkie, 2002). The increase of ammonia in aquatic environments poses a problem for aquatic animals (Randall & Tsui, 2002). The rise of ammonia in aquatic environments is attributable to various factors, including anthropogenic factors, excretion from plants and animals, and decomposing matter (Edwards et al., 2024; Randall & Tsui, 2002; T. X. Zhang et al., 2023). Since ammonia is a widespread toxicant, it affects individuals, communities, populations, and ecosystems (Yun et al., 2026; T. X. Zhang et al., 2023). Collectively, the above factors, along with NH_3 and NH_4^+, can intoxicate aquatic animals, requiring direct excretion of ammonia and ammonia detoxification mechanisms that will prevent its toxic effects, making it of interest to examine how an asymbiotic cnidarian species, such as the starlet sea anemone, tolerates and regulates ammonia.

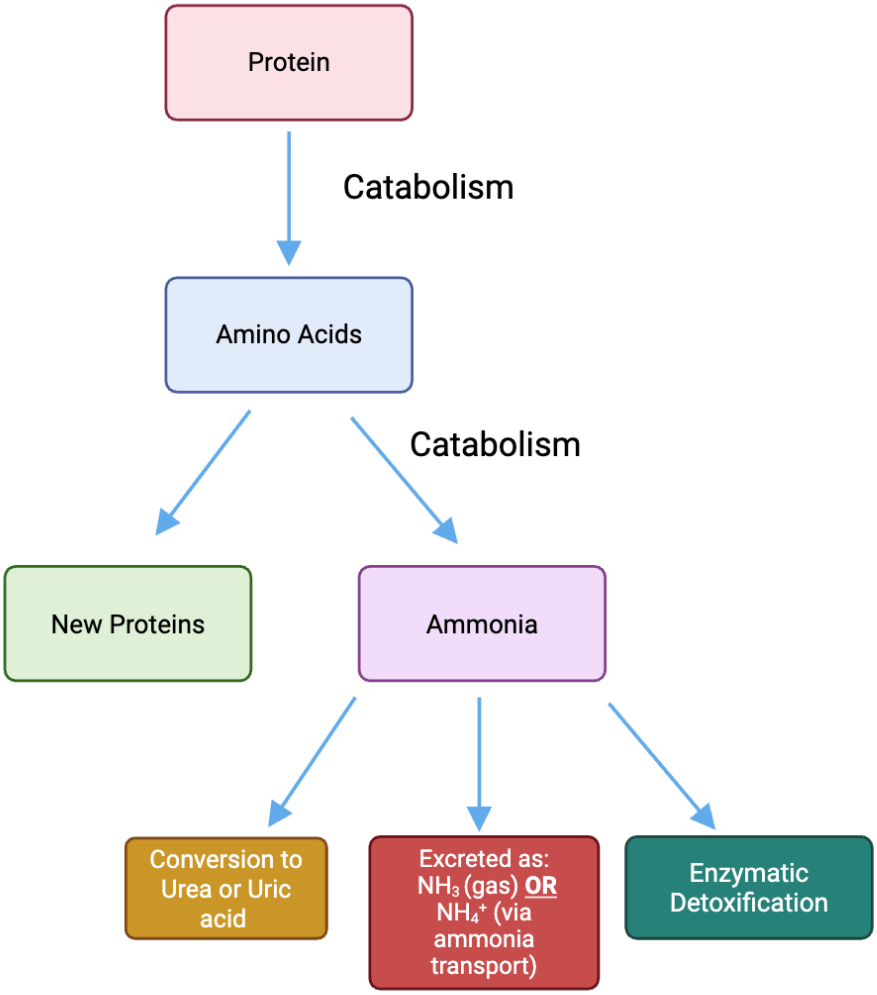

Figure 2. The production of ammonia. In animals, ammonia is primarily produced through the catabolism of amino acids. When protein is ingested through the diet, it is hydrolyzed into amino acids. These amino acids are used to make new proteins, but when in excess, they are catabolized into ammonia. Due to its toxicity, ammonia can be converted to less toxic nitrogenous waste, such as urea or uric acid, excreted as NH_3 or NH_4^+ (via ammonia transporters), or detoxified through enzymatic detoxification. Image adapted from Wright, 1995, and created using BioRender.

1.3 Ammonia detoxification and handling mechanisms

To mitigate ammonia intoxication, there are two options: enzymatic detoxification and transport.

A common pathway used for ammonia metabolism or detoxification is through the actions of key enzymes, referred to as ammonia-detoxifying enzymes in this study (Figure 3). Some of these

ammonia-detoxifying enzymes are glutamine synthetase (GS), glutamate dehydrogenase (GDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) (Miramontes et al., 2020; Qiu et al., 2018; Tng et al., 2008; Turner & Bucking, 2019). GS is a primary ammonia-metabolizing enzyme that synthesizes glutamine from glutamate and NH_4^+ using energy from adenosine triphosphate (ATP). Due to its role, GS is a key regulator of nitrogen metabolism, lowering the level of free ammonia in the body (Castegna & Menga, 2018; Tecson et al., 2025). GDH catalyzes a reversible reaction, where it can convert glutamate to alpha (α)-ketoglutatrate and NH_4^+, or convert α-ketoglutarate and NH_4^+ to glutamate. In its conversion of glutamate to α-ketoglutarate and NH_4^+, GDH reduces NAD^+ to NADH, using it as a coenzyme in this oxidative deamination reaction (Plaitakis et al., 2017; Smith et al., 2019). In this study, the amination pathway of GDH (α-ketoglutarate and NH_4^+ to glutamate) was observed. ALT and AST [formerly named glutamic-pyruvic transaminase (GPT) and glutamic-oxaloacetic transaminase (GOT), respectively] are transaminase enzymes that transaminate the amino acids alanine or aspartate to glutamate, a reaction dependent on pyridoxal 5'-phosphate (PLP) (Ellinger et al., 2011; X. J. Huang et al., 2006; Sookoian & Pirola, 2012; Toney, 2014). Similar to the reaction of GDH, the reactions of ALT and AST are reversible (Ellinger et al., 2011).

Despite having a role in ammonia detoxification, the above enzymes are multifunctional. GS plays a role in amino acid homeostasis and acts as a carbon and nitrogen donor (Ryu et al., 2026; Tecson et al., 2025). The pathway of GDH is also involved in acid-base equilibrium, redox homeostasis, lipid biosynthesis, lactate production, ATP production, and the tricarboxylic acid (TCA/Krebs) cycle (Plaitakis et al., 2013, 2017). Lastly, ALT and AST act as biomarkers for liver function (Lala et al., 2023). Overall, these enzymes are known to be multifunctional, in which they can be used for detoxifying ammonia or making amino acids. Hence, while important contributors

to ammonia handling, changes in these enzyme activities cannot be interpreted exclusively as evidence of ammonia detoxification because these enzymes participate in numerous metabolic pathways.

Along with enzymatic detoxification, cnidarians and aquatic invertebrates have shown expression of ammonia transporters used to regulate ammonia. These transport pathways include key transporters, such as Na^+/K^+-ATPase (NKA), Rhesus (Rh)-like glycoproteins, and ammonia transporters (AMTs) (C. H. Huang & Peng, 2005; Weihrauch & Allen, 2018; Wright & Wood, 2009) (Figure 4). These transporters are found in various aquatic invertebrate species and transport either NH_3, NH_4^+, or both (Weihrauch & Allen, 2018). These transporters can be found on the basolateral or apical membranes of invertebrate epithelium, creating a pathway for ammonia transport and excretion (Weihrauch & Allen, 2018). Specific to cnidarian species, studies in sea anemones *N. vectensis* and *Exaiptasia diaphana* have shown gene expression and protein localization of Rh-like glycoproteins and AMTs (Andrikou et al., 2019; Mashini et al., 2022). Transport mechanisms likely contribute substantially to ammonia handling but were beyond the scope of the present study. Enzymatic detoxification, direct excretion, and transport are all ways that help an organism tolerate ammonia and survive its toxic effects. The enzymatic reactions described above require energy from ATP hydrolysis and the oxidation or reduction of $NADH/NAD^+$. Additionally, transporters that eliminate excess ammonia require energy, which directly influences physiological processes such as metabolic rate and oxygen consumption.

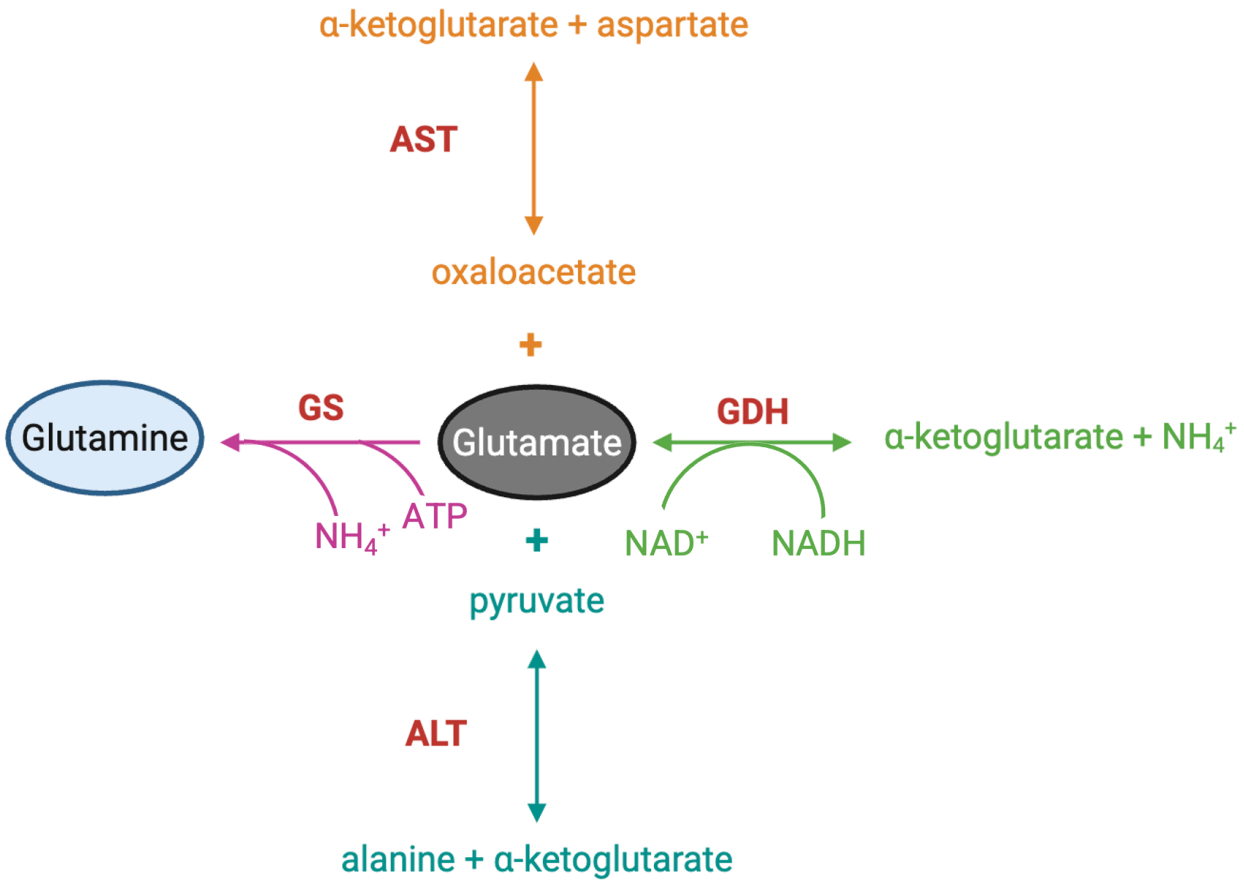

Figure 3. Enzymatic detoxification of ammonia. Enzymatic detoxification of ammonia is facilitated by various ammonia-detoxifying enzymes. Glutamine synthetase (GS) uses ammonium (NH_4^+) and glutamate to make glutamine, a less toxic product. Glutamate dehydrogenase (GDH) catalyzes a reversible reaction that can produce NH_4^+ or use NH_4^+ to make glutamate. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are aminotransferases that transaminate alanine and aspartate, respectively, to make glutamate. Image adapted from Wicks & Randall, 2002b and created with BioRender.

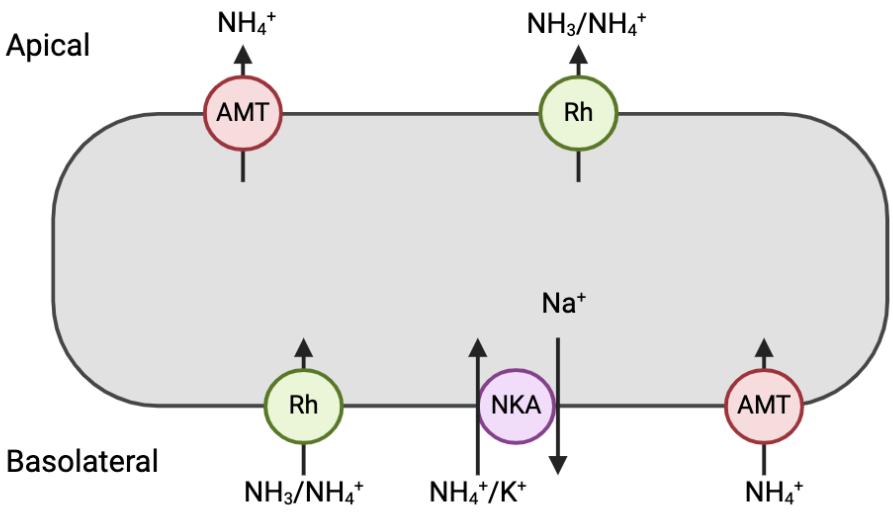

Figure 4. The transport of ammonia. Key transporters in invertebrates that transport ammonia include Rhesus (Rh)-glycoproteins, ammonia transporters (AMTs), and Na^+/K^+-ATPase (NKA). Rh-glycoproteins are present in both invertebrates and vertebrates, and transport NH_3 and NH_4^+ across the basolateral and apical membranes of the cell. AMTs, found exclusively in invertebrates, transport NH_4^+ across the basolateral and apical membranes. Finally, with NKA, K^+ can be replaced by NH_4^+ since they have the same hydrated ionic radius, transporting NH_4^+ across the basolateral membrane and into the cell. Image adapted from C.H. Huang & Peng, 2005; Weihrauch & Allen, 2018; Wright & Wood, 2009, and created with BioRender.

1.4 Metabolic rate

Metabolic rate (MO_2) is the energetic cost of living, specifically, the amount of energy an organism must expend to maintain homeostasis (Auer et al., 2018; Hulbert & Else, 2000). MO_2 is linked to numerous key aspects of life, such as growth, survival, reproduction, and immunity (Pettersen et al., 2018). In short, measurement of oxygen consumption (MO_2) is used to assess animal health (Innes & Houlihan, 1985). The amount of oxygen consumed has various implications, such as indicating an animal's ability to grow in its environment and changes in its MO_2 to adapt to that environment (Costa, 1988; Innes & Houlihan, 1985; Jobling, 1981; Storey & Storey, 1990). Whole-animal MO_2 is influenced by several factors, including body size, activity level, temperature, physiological state, anabolism, and food intake (Chabot et al., 2016).

MO_2 can be quantified and assessed under different physiological and environmental conditions, resulting in different MO_2 measures, including basal metabolic rate (BMR), standard metabolic rate (SMR), mass-specific BMR, routine metabolic rate (RMR), maximum metabolic rate (MMR), stress-induced metabolic rate, and standard specific dynamic action (SDA). BMR and SMR refer to the minimum level of sustainable metabolism that occurs when an animal is not stressed, inactive, not digesting food, and non-reproductive (Norin & Metcalfe, 2019). BMR applies to endotherms (warm-blooded animals), and SMR applies to ectotherms (cold-blooded animals) (Norin & Metcalfe, 2019). So, BMR and SMR define the minimal cost of living that cannot be avoided. Mass-specific BMR or mass-specific MO_2 is the resting energy that is expended per unit of body mass; in other words, it is the rate at which an organism uses energy relative to its total body mass (Wang et al., 2011). Therefore, BMR varies with body mass (Hulbert & Else, 2004). RMR is defined as the oxygen consumption measured during minimal or moderate, uncontrolled motor activity (Brett, 1962; Chabot et al., 2016; Dall, 1986; Killen et al., 2021). On the other hand, MMR is the rate of oxygen uptake that an animal can achieve to create ATP aerobically and is consistent with the highest level of activity (Brett, 1962; Killen et al., 2021). Additionally, stress-induced MO_2 is another MO_2 measure that can be assessed. Studies have shown that stressors, including starvation stress and thermal fluctuations, can elicit changes in MO_2, such as suppression or increases of MO_2 (Lalouette et al., 2011; Shokri et al., 2026; D. W. Zhang et al., 2019). Finally, SDA is the metabolic response (MO_2) during meal digestion, which typically increases MO_2 (Secor, 2009).

MO_2 varies among individuals, and this variation can be caused by several factors, including growth, survival, and behaviour (Auer et al., 2018). It is important to note that in many animals, there is intraspecific variation in MO_2 (Metcalfe et al., 2016; Yoon et al., 2023). This

intraspecific variation in MO_2 results from genetics and environment (Metcalfe et al., 2016; Yoon et al., 2023). Therefore, it could be implied that animals of the same species from geographically distinct environments may potentially display differences in MO_2. Although MO_2 does not directly measure ammonia detoxification, it serves as a proxy for whole-organism energy expenditure, indicating metabolic demand or physiological stress, which could potentially influence nitrogen cycling and the organism's capacity to handle ammonia (A. Maas et al., 2012; Mégevand et al., 2022; Xiao et al., 2019). Given that MO_2 variation can be in response to the metabolic demand caused by physiological stress, the link between MO_2, survival, and enzymatic responses is crucial to establish to assess the ammonia tolerance of an organism.

1.5 Rationale and objectives

1.5.1 Rationale

Ammonia remains a prominent toxicant in aquatic environments and poses a threat, causing changes in communities, ecosystems, and biodiversity (Yun et al., 2026; T. X. Zhang et al., 2023). Despite playing a key role in nitrogen cycling, high ammonia levels can cause many damaging effects, including negative impacts on survival and growth, nitrogen metabolism, reproductive capacity, ion balance, intercellular mechanisms, and overall metabolism (Yun et al., 2026; T. X. Zhang et al., 2023). While ammonia tolerance and detoxification mechanisms have been well examined in traditional model organisms, such as vertebrates like fish and some invertebrates, cnidarian species, including the starlet sea anemone, have not been widely examined. As previously stated, aquatic invertebrates and terrestrial animals are vulnerable to ammonia and its toxic effects (Randall & Tsui, 2002; Weihrauch & Allen, 2018; T. X. Zhang et al., 2023). Currently, there is still limited understanding of the toxic effects of ammonia on aquatic invertebrates (Weihrauch & Allen, 2018; T. X. Zhang et al., 2023). Understanding the impact of

ammonia toxicity on aquatic invertebrates is crucial, as they are vital for global biodiversity and constitute the majority of organisms in aquatic ecosystems (Collier et al., 2016). The few studies that have focused on the effects of ammonia intoxication on aquatic invertebrates have found comparable results to those in vertebrates (T. X. Zhang et al., 2023). Some of these results include decreases in growth, increased susceptibility to pathogens, and abnormal osmoregulatory function (T. X. Zhang et al., 2023).

While certain cnidarian species rely on symbiotic relationships with photosynthetic algae (zooxanthellae) to detoxify and incorporate ammonia, the starlet sea anemone lacks this obligate zooxanthella symbiont that contributes to nitrogen assimilation (Glass & Barott, 2025; Goldstone, 2008; Porat & Chadwick-Furman, 2005; Starcevic et al., 2008). Despite its asymbiotic nature, preliminary evidence from our lab suggested that starlet sea anemones are extremely ammonia-tolerant. However, the mechanisms behind this tolerance are poorly understood. In this study, I aimed to determine the tolerance of the starlet sea anemone to high environmental ammonia (HEA) and explore ammonia-handling and metabolism strategies.

1.5.2 Objectives

In this study, starlet sea anemones collected from two different populations, New Hampshire and Nova Scotia, were used. Since ammonia tolerance is defined as an organism's ability to survive and maintain physiological functions in high ammonia, survival, physiological, and biochemical processes were examined (M. Liu et al., 2021; Lu et al., 2018; T. X. Zhang et al., 2023). The *first objective* of this study was to examine the survival and MO_2 of the starlet sea anemone in varying concentrations of ammonium chloride (NH_4Cl). The *second objective* of this study was to examine the enzyme activity of ammonia-detoxifying enzymes GS, GDH, ALT, and AST as a possible mechanism for ammonia tolerance. Finally, the *third objective* of this study was

to compare physiological responses to ammonia across populations, since geographically isolated populations may exhibit divergent physiological responses.

Chapter 2: Results and Discussion

2.1 INTRODUCTION

2.1.1 Ammonia and survival

As previously mentioned, ammonia has many detrimental effects on organisms, including impairing or decreasing survival. This decrease in survival results from ammonia causing decreases in growth rates and suppressing feeding and molting behaviour, resulting in abnormal development of organisms and their body shape (Zhang et al., 2023). Research on the Japanese sea perch (*Lateolabrax japonicus*) and mealworm larvae (*Tenebrio molitor*) demonstrated that exposure to ammonia resulted in reduced growth and increased mortality (Rybalka & Brygadyrenko, 2026; M. Zhang et al., 2022). Similarly, it was reported that exposing the South African abalone (*Haliotis midae*) and the greenlip abalone (*Haliotis laevigata*) significantly reduced the growth rate of these organisms (J. O. Harris et al., 1998; Reddy-Lopata et al., 2006). In a study conducted on juvenile Atlantic cod (*Gadus morhua*), it was suggested that ammonia may decrease food intake, which could be a potential reason for the decreases in growth and survival observed (Foss et al., 2004). This decrease in food intake means that the energy needed to maintain growth has to be reallocated to body maintenance (T. X. Zhang et al., 2023). Examining survival directly illustrates the toxicity of ammonia on an organism; however, it also provides insight into the organism's tolerance for ammonia.

2.1.2 Ammonia tolerance

Ammonia tolerance refers to an organism's ability to survive in high ammonia environments and maintain its normal physiological functions (M. Liu et al., 2021; Lu et al., 2018; T. X. Zhang et al., 2023). To evaluate ammonia tolerance, survival rates, growth and development, and physiological and biochemical parameters are analyzed under high ammonia stress (T. X. Zhang et al., 2023). Ammonia tolerance can be affected by many factors, such as age or life stage

and feeding (T. X. Zhang et al., 2023). For instance, in shrimp, it was discovered that the nauplius stage had higher ammonia tolerance compared to larval, juvenile, and adult stages (Ostrensky & Wasielesky, 1995). Feeding has also been shown to increase ammonia tolerance of the gastropod, *Bellamya aeruginosa* (M. Liu et al., 2022). Apart from the examples provided above, ammonia tolerance can be influenced by the environment or geographical location, where the initial habitat location impacts an organism's ability to tolerate ammonia (Gergs et al., 2013; Prenter et al., 2004). Given that the anemones used in this study are from geographically distinct locations, the ammonia tolerance of both populations may differ.

2.1.3 Enzymatic detoxification in cnidarians

Ammonia detoxification in cnidarians, including the sea anemone *Urticina felina* and the coral species *Alcyonium digitatum*, has been assessed, revealing that cnidarians release ammonium into the environment via direct excretion (Migné & Davoult, 1997). However, studies have also shown that apart from direct excretion, cnidarians also use ammonium assimilation to convert ammonia into organic compounds, such as amino acids, through glutamate- and glutamine-based pathways (Cui et al., 2023; Davy et al., 2012; Pernice et al., 2012; Roberts et al., 2001). Most cnidarians, such as some sea anemone species and reef-building corals, have symbiotic relationships with dinoflagellates, also known as zooxanthellae, that, together with their host (the cnidarian species), use enzymes such as GDH and GS to assimilate ammonium (Cui et al., 2023; Davy et al., 2012; Pernice et al., 2012; Roberts et al., 2001). Although the cnidarian and dinoflagellate partners are both capable of ammonium assimilation, it has been suggested that the dinoflagellate partner is the primary site of ammonium assimilation (Catmull et al., 1987; Dudler & Miller, 1988; Grover et al., 2002; Lipschultz & Cook, 2002).

As mentioned above, cnidarians, such as corals, have symbiotic relationships with photosynthetic algae, also known as zooxanthellae, that are part of the genus *Symbiodinium* (Rädecker et al., 2015). Due to this symbiotic relationship, nitrogen cycling looks different. Nitrogen is conserved through a recycling system that involves the coral host, its algal symbionts, and associated microbes (Rädecker et al., 2015). When the coral produces NH_4^+ as a waste product of metabolism, the NH_4^+ is taken up by the algal symbionts and incorporated into amino acids through nitrogen-assimilation pathways, such as those catalyzed by GS and GDH (Cui et al., 2023; Rädecker et al., 2015). This NH_4^+ is used by the algal partner for growth, photosynthesis, and production of nutrients that are transferred back to and used by the coral host (Cui et al., 2023; Rädecker et al., 2015). Based on the above, it is of interest how the asymbiotic starlet sea anemone cycles nitrogen and uses these enzymes to assimilate ammonia.

2.1.4 Metabolic rate and ammonia

Among the many physiological processes that ammonia has been reported to affect, MO_2 is one of them. Ammonia is known to act as a metabolic stressor, disrupting homeostasis and the energy required to maintain it (Barton, 2002; Sandblom et al., 2016). When ammonia disrupts homeostasis, organisms must reallocate energy from growth and reproduction to survival and maintenance (Barton, 2002; Sandblom et al., 2016). Many studies have shown that high ammonia exposure directly affects MO_2 of organisms, such as the giant freshwater prawn (*Macrobrachium rosenbergii*), *Daphnia magna*, marine medaka (*Oryzias melastigma*), and greenlip abalone (*Haliotis laevigata*) (Emadi et al., 2026; Gao et al., 2017; J. O. Harris et al., 1998; Nash et al., 2022).

In aquatic organisms, specifically invertebrates, ammonia toxicity can have numerous endpoints for MO_2. Previous literature has shown contrasting results of ammonia effects on MO_2,

such as some studies revealing reduced activity, behavioural impairment, reduced feeding, and metabolic suppression, while others show elevated metabolism and increased energetic costs (Gao et al., 2017; J. O. Harris et al., 1998; Y. Ma et al., 2024; Miranda-Filho et al., 2009; Nash et al., 2022). In other words, ammonia exposure does not always increase oxygen consumption or MO_2.

Despite studies showing that ammonia exposure is linked to an increase in MO_2 (oxygen consumption), it is important to note that increases in oxygen consumption could also be an indicator of detoxification, stress responses, ion regulation and/or osmoregulation, behavioural agitation (i.e., increased swimming, contractions, and/or increased movement in response to stress), tissue repair, and altered substrate utilization (i.e., promoting catabolism of lipids and amino acids) as these require substantial energetic investments (Das & Mukherjee, 2000; Fulton et al., 2013; Lemos et al., 2003; Mack & Bely, 2025; Maginniss & Wells, 1969; Miranda-Filho et al., 2009; Morgan & Iwama, 1998; Normant-Saremba et al., 2015; Racotta & Hernández-Herrera, 2000; Ren et al., 2022; Silvestre, 2020; Torreblanca et al., 1987; Vilas et al., 2006; Xiao et al., 2019). Being that the starlet sea anemone lacks a symbiotic relationship with the photosynthetic algae, zooxanthellae, and it has been suggested that the zooxanthellae partner is the primary site of ammonium assimilation, which includes the use of detoxifying enzymes, it is of interest how the MO_2 of the starlet sea anemone responds to ammonia exposure (Catmull et al., 1987; Dudler & Miller, 1988; Grover et al., 2002; Lipschultz & Cook, 2002).

2.1.5 Hypotheses

For this study, it was *hypothesized* that ammonia exposure would impair the survival of anemones from each population. This hypothesis is supported by previous studies that have shown that high ammonia decreases the survival of invertebrates and vertebrates, such as the South African abalone, greenlip abalone, the mealworm, Atlantic cod, and the Japanese sea perch (Foss

et al., 2004; J. O. Harris et al., 1998; Reddy-Lopata et al., 2006; Rybalka & Brygadyrenko, 2026; M. Zhang et al., 2022). It was *predicted* that fed individuals would have higher survival compared to fasted individuals, since feeding has been shown to increase ammonia tolerance and survival (M. Liu et al., 2022). Since anemones used in this study were collected from geographically isolated populations, it was *hypothesized* that the populations may exhibit divergent physiological responses, showing differences in ammonia tolerance. This is supported by previous studies that have demonstrated that starlet sea anemones locally adapt to their environment and have shown diverging responses to environmental stressors when from different populations (Friedman et al., 2018; Reitzel et al., 2013). Literature has shown that the water quality of salt marshes from Nova Scotia and New Hampshire differs, such as tidal cycles, which can change salinity and ammonia levels, further supporting that ammonia tolerance between populations may differ (Daly & Mathieson, 1981; Desplanque & Mossman, 2001).

Ammonia-detoxifying or -metabolizing enzymes are a primary route animals use to regulate ammonia levels in the body or cells. The activity of these ammonia-detoxifying enzymes has been examined in various organisms, illustrating their response to ammonia exposure. Therefore, it was *hypothesized* that the activities of GS, GDH, ALT, and AST would be impacted by ammonia exposure. Since studies have shown in whiteleg shrimp (*Litopenaeus vannamei*), Nile tilapia (*Oreochromis niloticus*), largemouth bass (*Micropterus salmoides*), freshwater mollusk (*Solenaia oleivora*), and the Antarctic pteropod mollusk (*Clione limacina antarctica*) that the activity levels of these enzymes have changed, it was *predicted* that enzyme activity would change in response to ammonia exposure (Hegazi et al., 2010; A. Maas et al., 2012; Qiu et al., 2018; T. Zhang et al., 2024; Zou et al., 2023).

Finally, it was *hypothesized* that ammonia would impact the MO_2 of anemones from each population. Based on evidence from studies conducted in prawns (*Macrobrachium rosenbergii* and *Penaeus chinensis*), marine medaka (*Oryzias melastigma*), greenlip abalone (*Haliotis laevigata*), and hybrid groupers (*Epinephelus fuscoguttatus* x *E. lanceolatus*) (Cao et al., 2024; Chen & Nan, 1993; Emadi et al., 2026; Gao et al., 2017; J. O. Harris et al., 1998), it was *predicted* that ammonia would increase the MO_2 of anemones, since ammonia is a metabolic stressor and disrupts homeostasis, requiring the anemones to divert their energy for survival and maintenance (Barton, 2002; Sandblom et al., 2016). It was also *hypothesized* that ammonia handling mechanisms or metabolic patterns between the two anemone populations would be similar (Chew et al., 2005).

2.2 MATERIALS AND METHODS

For this study, all chemical reagents were supplied by Sigma-Aldrich (Oakville, Ontario, Canada).

2.2.1 Anemone collection

Male and female *N. vectensis*, starlet sea anemones, were collected from separate estuarine (salt marsh pools) locations: Head of Chezzetcook, Nova Scotia, Canada (Latitude: 44°44’4.9308” N, Longitude: 63°15’21.5968” W), Rainbow Haven, Nova Scotia, Canada (Latitude: 44°39’04.2” N, Longitude: 63°25’00.5” W), and Rye, New Hampshire, USA, located behind Wallis Sands Beach (Latitude: 43°01’39.4” N, Latitude: 70°43’34.8” W) between June and August of 2024 and October 2025 for a recent population from the Chezzetcook Inlet, with salinity around 20 to 30 parts per thousand (p.p.t.). Within each location, anemones were collected from salt marsh pools near one another. The anemones were extracted from the sediment using a wide-bore transfer pipette and placed into containers with water from the salt marsh pool (20 to 30 p.p.t.) for transport

to York University. Anemones were housed in plastic containers with 15 to 20 p.p.t. of artificial seawater (ASW) (Instant Ocean, Blacksburg, Virginia, USA) in the Department of Biology at York University.

2.2.2 Maintenance of anemones

We maintained anemones in individual containers (~50-90 anemones per container), each containing 15 to 20 p.p.t. of ASW (~ pH 7.52-7.8), with individuals sourced from the same location and clone line (Figure 5). The containers were kept on a laboratory bench at room temperature (21°C) in normal lighting (12h:12h; light:dark cycle). Background ammonia levels were checked every month using a testing kit [API Ammonia (NH_3/NH_4^+) Test Kit, API Fishcare] and were maintained around 0 parts per million (p.p.m.) to 0.50 p.p.m. Weekly water changes were conducted by decanting the water in their containers and replacing it with fresh ASW. The anemones were fed freshly hatched brine shrimp every two days. To culture the *Artemia*, approximately 2 g of *Artemia* eggs (Brine Shrimp Direct, Brine Shrimp Eggs, Utah, U.S.A) was added to 500 mL of 28 to 30 p.p.t. ASW in a separatory funnel. The funnel was then incubated at 30°C for 24 to 48 hours, with aeration. After 24 to 48 hours, the funnel was removed from the incubator and placed in its holder. To ensure that only hatched shrimp larvae were fed to the anemones, the funnel was placed under a lamp to ensure that unhatched larvae went to the bottom and were discarded. Once the unhatched larvae were removed, the hatched larvae that were actively swimming and orange in colour were collected in a small beaker and fed to the anemones using a transfer pipette. The amount of *Artemia* given to each container varied depending on the number of anemones present in the container. For instance, a container containing over 50 anemones received 2.5 mL of *Artemia*.

2.2.3 Development of general populations

To create a general population to use in this study, one individual from multiple clone lines (Nova Scotia or New Hampshire; Chezzetcook and Rainbow Haven, together, made the Nova Scotia general population) was placed into a container, ensuring each genotype from the population was represented (Figure 5). To create the clone lines used to create the general population in this study, anemones collected directly from the salt marshes were bisected. Once the anemones were bisected, they were placed into separate containers. If wild-caught populations were used, we would not know whether the anemones were all clones of each other. Using separate clone lines rather than wild-caught animals allowed us to control for overrepresentation of single genotypes, minimizing line-specific bias. Furthermore, this experimental setup for the general populations permitted equal representation from each line to ensure the general populations reflected the genetic diversity across the clone lines.

Anemone bisection. Using a wide-bore transfer pipette, adult anemones were extracted from their containers and placed into Petri dishes filled with 15 to 20 p.p.t. ASW. After the anemones were transferred to Petri dishes, they were left undisturbed for 30 minutes before bisections occurred. This 30-minute wait time allowed the anemones to relax and adjust their posture. After 30 minutes, the anemones were bisected using a scalpel (anemones were cut in the middle of their body column horizontally). This method allowed the anemone to regenerate a fully functioning head or foot.

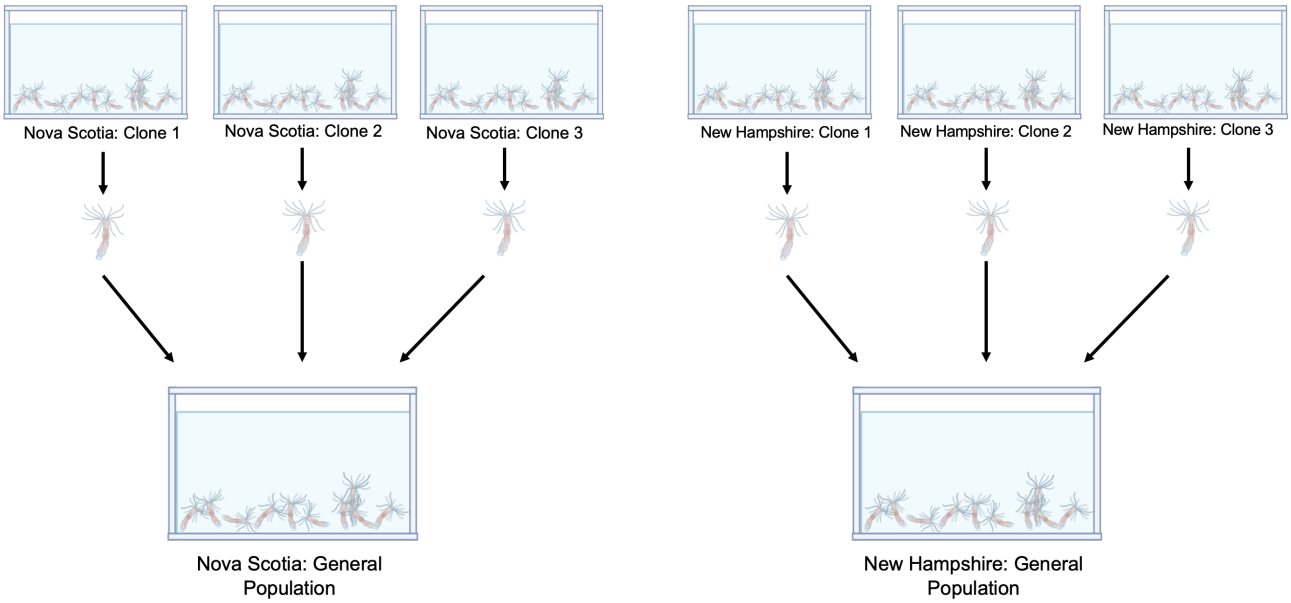

Figure 5. Maintenance of anemones and generation of general populations. Anemones were maintained in containers with 15 to 20 p.p.t. ASW. Clone lines were used to make general populations for Nova Scotia and New Hampshire anemones. One individual from each clone line of the respective population was added to ensure all genotypes were represented in the general populations used. Image created using BioRender.

2.2.4 Ammonia treatments

To analyze the effects of ammonia on anemone survival, anemones from both general populations were used and split into two groups: fed and fasted. Fed individuals were fed to satiation daily with *Artemia* (approximately 40-50 *Artemia* per dish). After the anemones were fed, unconsumed *Artemia* were removed daily. Individuals in the fasting group remained fasted for the entire duration of the experiment. The anemones were exposed to various concentrations of ammonium chloride (NH_4Cl) for 11 days (Figure 6). The tested concentrations were: 0 $mmol^{-1}$ (mM) (control), 0.125 mM, 0.15 mM, 0.25 mM, and 0.5 mM. To ensure there was no effect of pH on the results obtained, the pH of all ammonia concentrations and 18 p.p.t. ASW were measured (Hanna HI2020 edge multiparameter pH meter, Hanna Instruments Canada Inc, Laval, Quebec,

Canada). The pH for all solutions ranged from 7.35 to 7.5. Ammonia solutions were made in 18 p.p.t. ASW. The tested concentrations were selected based on environmental relevance and preliminary survival experiments that revealed concentrations above 0.5 mM caused 100% mortality within 24 hours of exposure (see Figure A2 in Appendix A).

For each concentration, five to six individuals from each population were placed in a Petri dish. Each group underwent chronic exposure for 11 days. At the end of the 11-day exposure period, the number of surviving individuals in each concentration group was counted. The ammonia exposure in this study was static (ammonia solutions in the Petri dishes were not changed during the 11-day exposure). Survival experiments were conducted in duplicates (two Petri dishes per concentration) to avoid pseudo-replication.

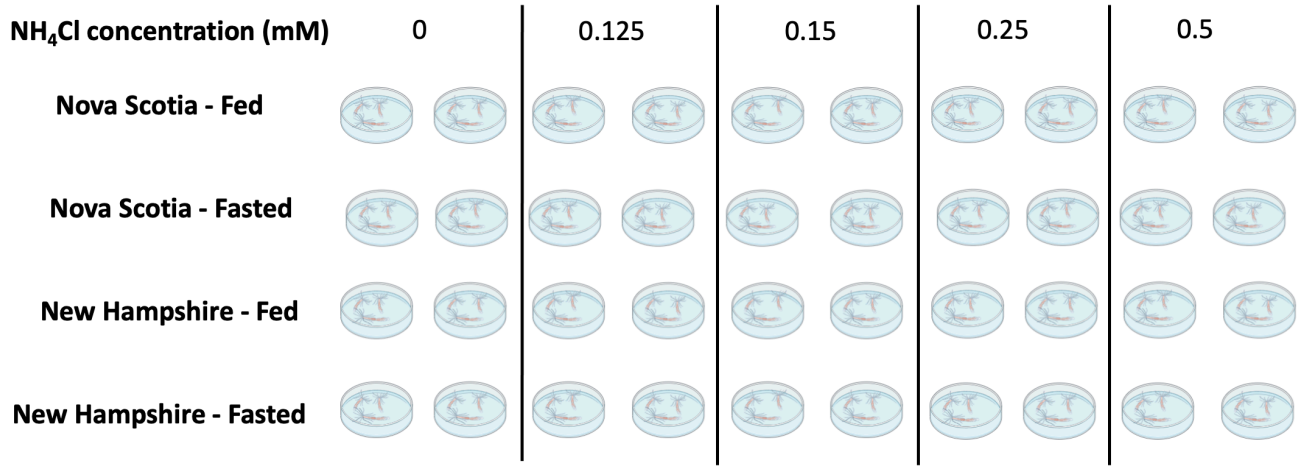

Figure 6. Experimental setup. This illustration depicts the experimental setup used to conduct the 11-day survival, respirometry, and enzyme experiments. Survival experiments were conducted in duplicate. Image created using BioRender.

2.2.5 Enzyme assays

To examine the activity of the ammonia-detoxifying enzymes: glutamine synthetase (GS), glutamate dehydrogenase (GDH), alanine aminotransferase (ALT), and aspartate aminotransferase

(AST), enzyme assays were used. Enzyme assays were performed on surviving anemones (whole-body) from the survival and respirometry experiments. Approximately three to five anemones (~0.200 grams) were homogenized on ice in a homogenization buffer containing 20 mmol L^{-1} 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (pH 7.5), 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), and 0.1% Triton-X. After homogenization, the samples were centrifuged at 10,000 ***g*** for 5 minutes at 4°C to allow for a pellet of debris to form (Thermo Fisher Scientific, Mississauga, ON, Canada). Following centrifugation, the supernatants were then collected, placed on ice, and used to measure enzyme activity and protein concentration. The plates for each assay were read at 26°C using a microplate reader (BioTek Instruments, Winooski, Vermont, USA).

For each enzyme assay, a positive control tissue sample (*Carassius auratus*, goldfish, gill tissue) was run on the plate to verify the presence of enzyme activity and ensure that the running buffers and cocktails were working.

Glutamine synthetase (GS) was measured by the glutamyl transferase assay. The reaction mixture contained a running buffer containing 50 mmol L^{-1} HEPES (pH 6.7), 60 mmol L^{-1} glutamine, 15 mmol L^{-1} hydroxylamine, 0.4 mmol L^{-1} adenosine 5′-diphosphate sodium salt (ADP), 20 mmol L^{-1} sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \bullet 7H_2O$), 0.3 mol L^{-1} manganese (II) chloride ($MnCl_2$), and 10 µL of the supernatant. In a 1.5 mL tube, 187 µL of the running buffer, 3 µL of $MnCl_2$, and 10 µL of the supernatant were added. The reaction of the blanks (negative control) was stopped immediately (time = 0) by adding 60 µL of a ferric chloride ($FeCl_3$) stopping reagent [50% hydrochloric acid (HCl): 24% trichloroacetic acid (TCA): 10% $FeCl_3$ in 0.2 N HCl]. The tubes were mixed by vortexing (Analog Vortex Mixer, Pennsylvania, U.S.A.) and placed on the lab bench to incubate. The experimental tubes were incubated for 60

minutes at room temperature. Following incubation, the experimental reaction was stopped by adding 60 µL of the stopping reagent, and all tubes were centrifuged at 13,000 ***g*** for 1 minute. 200 µL from each tube was pipetted into a 96-well enzyme assay microplate and read at 540 nm.

The assays for GDH, ALT, and AST were all measured in the microplate reader using a continuous measurement of change in absorbance. All the following assays measure the decrease in absorbance at 340 nm as NADH is converted to NAD^+.

Glutamate dehydrogenase (GDH) was measured using a reaction mixture containing 0.12 mmol L^{-1} NADH, 250 mmol L^{-1} ammonium acetate, 1 mmol L^{-1} ADP, and 14 mmol L^{-1} α-ketoglutarate in 50 mmol L^{-1} HEPES (pH 7.5). For the experimental samples, 140 µL of the running buffer, 50 µL of α-ketoglutarate, and 10 µL of the supernatant were pipetted into the 96-well plate. However, for the control, the 10 µL of supernatant and 50 µL of α-ketoglutarate were substituted for 60 µL of homogenization buffer. The microplate was read at 340 nm. This GDH assay measured the amination pathway (α-ketoglutarate + NH_4^+ + NADH → glutamate).

Alanine aminotransferase (ALT) was measured using a reaction mixture containing 0.12 mmol L^{-1} NADH, 200 mmol L^{-1} alanine, 0.025 mmol L^{-1} pyridoxal 5' phosphate (PLP), and 10.5 mmol L^{-1} α-ketoglutarate in 50 mmol L^{-1} HEPES (pH 7.5). For the experimental samples, 137 µL of the running buffer, 40 µL of α-ketoglutarate, 13 µL of lactate dehydrogenase (LDH), and 10 µL of the supernatant were pipetted into the 96-well plate. For the control, the 10 µL of supernatant and 40 µL of α-ketoglutarate were substituted for 50 µL of homogenization buffer. The microplate was read at 340 nm.

Aspartate aminotransferase (AST) was measured using a reaction mixture containing 0.12 mmol L^{-1} NADH, 40 mmol L^{-1} L-aspartic acid sodium salt monohydrate, 0.025 mmol L^{-1} PLP, and 7 mmol L^{-1} α-ketoglutarate in 50 mmol L^{-1} HEPES (pH 7.5). For the experimental samples, 161

µL of the running buffer, 25 µL of α-ketoglutarate, 4 µL of malate dehydrogenase (MDH), and 10 µL of the supernatant were pipetted into the 96-well plate. For the control, the 10 µL of supernatant and 25 µL of α-ketoglutarate were substituted for 35 µL of homogenization buffer. The microplate was read at 340 nm.

2.2.6 Bicinchoninic acid (BCA) assay

A BCA assay was used to measure the protein concentration of the anemone samples used in the enzyme assays and to normalize enzyme activity to protein. To perform the BCA assay, bicinchoninic acid solution (Reagent A) and copper (II) sulfate solution (Reagent B) were mixed in a 50:1 dilution. The bovine serum albumin (BSA) standards (1, 0.75, 0.5, and 0.25 mg/mL) (Bio-Rad Laboratories, Inc, U.S.A) were used to determine the concentrations of the unknown samples. The anemone samples (the unknowns) were diluted 1:10. In a 96-well microplate, 200 µL of the Reagent A + Reagent B solution was added to each well. To each well, 20 µL of the BSA standards, anemone samples, and homogenization buffer (negative control) were added in their respective wells (completed in duplicates). To observe the reaction and colour change, the plate was incubated in the microplate reader for 15 minutes at 37°C. After 15 minutes, the plate was read at 562 nm. The average absorbance of the standards was plotted to make a standard curve and to calculate a linear regression ($y = mx + b$), which was used to calculate the protein concentrations of the anemone samples.

2.2.7 Microplate respirometry

To examine the effect of ammonia on metabolic rate (MO_2), a microplate respirometer (SensorDish Reader system, Loligo Systems, Viborg, Denmark) was used. After 11 days of exposure during the survival experiment, the surviving anemones were used to measure oxygen consumption and ultimately calculate MO_2 (Figure 6). Fed anemones were fed with *Artemia* one

hour before respirometry was conducted. This timing was chosen to ensure standardized feeding across the anemones and allowed the anemones to finish feeding and minimize the influence of SDA, which is known to elevate oxygen consumption post-feeding. Fasted individuals remained fasted.

Anemones were placed into individual wells of the glass microplate respirometer to measure respiration (oxygen consumption) (Figure 7). Each NH_4Cl concentration was measured on a separate plate to avoid mixing solutions. Each well was filled with the appropriate solution (18 p.p.t. ASW, 0.125, 0.15, 0.25, or 0.5 mM NH_4Cl). Negative controls (blanks = no anemone in well) were run on each plate in the last column, where the wells were filled with the appropriate solution to account for non-biological oxygen consumption. Finally, the loaded plate was covered with an adhesive plastic seal and monitored for 6 to 24 hours to allow the percent air saturation to reach 10% or below. All plates were measured at the temperature of the lab (25°C), 965 mBar air pressure, and 18 p.p.t. salinity.

2.2.7.1 Anemone weight

To determine the wet mass of the anemones, anemones were removed from the respirometer and measured immediately (Figure 8). Empty 1.5 mL tubes were filled with the respective solution for each anemone used in the respirometry and weighed using an analytical balance scale (Mettler Toledo, Mississauga, ON, Canada). Anemones were added to their respective tube and weighed. To determine the final wet mass (in grams) of each anemone, the difference between the filled tube with the anemone and the tube filled without the anemone was used:

$$Anemone\ wet\ mass\ (g) = (Filled\ tube\ with\ Anemone) - (Filled\ tube\ without\ anemone)$$

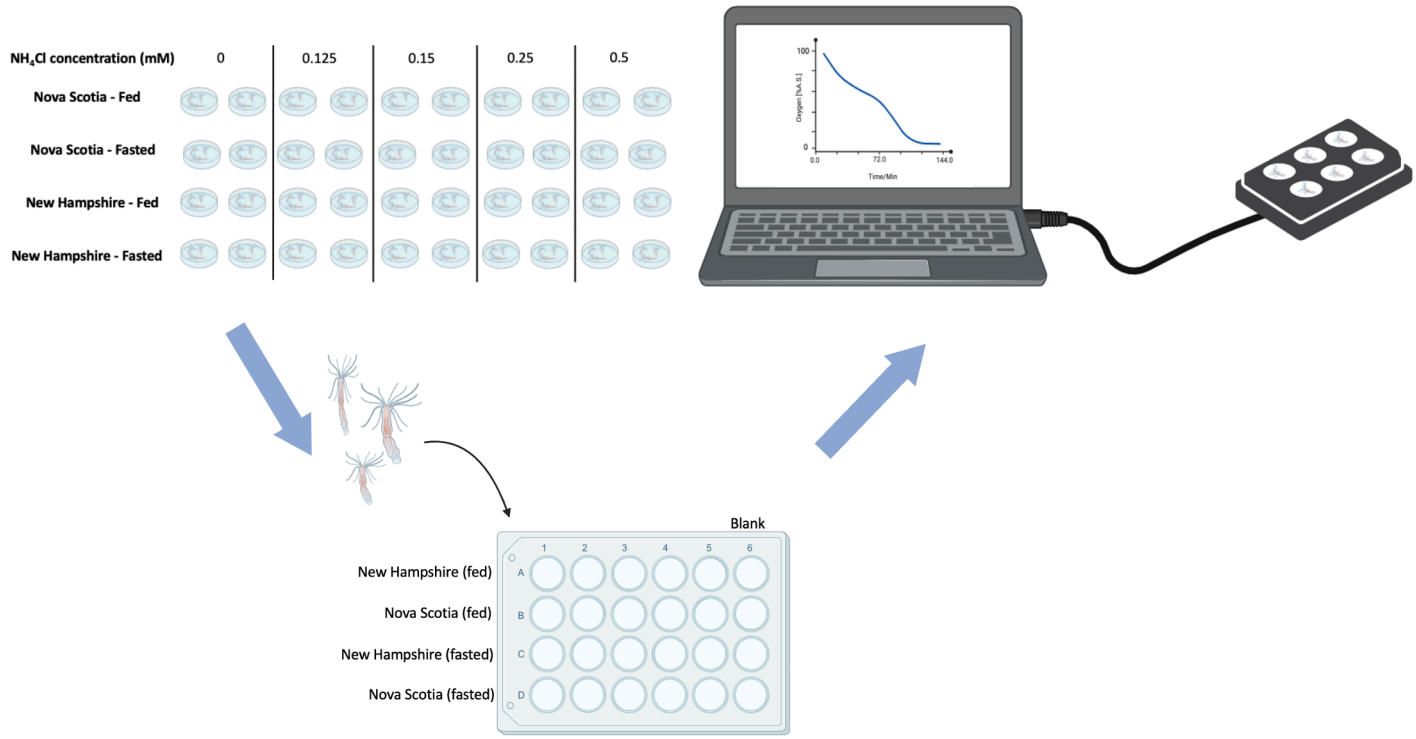

Figure 7. Respirometer microplate setup. To measure respiration (oxygen consumption) and metabolic rate (MO_2), a microplate respirometer was used. Anemones from the ammonia treatments were placed into the wells with their respective solution: 0 mM (18 p.p.t. ASW), 0.125 mM, 0.15 mM, 0.25 mM, or 0.5 mM NH_4Cl. Each ammonia concentration was allocated its own plate (i.e., one plate for each concentration) to avoid mixing of solutions. Image created using BioRender.

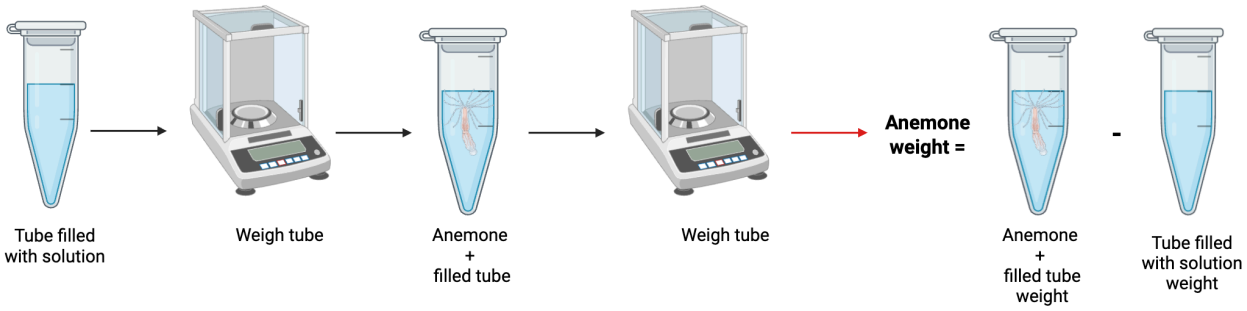

Figure 8. Weighing wet mass of anemones. To determine the wet mass of the anemones used in all experiments (survival, enzymes, and microplate respirometry), filled 1.5 mL tubes were weighed. Afterward, anemones were added to the tubes and weighed again. To determine the final anemone wet mass (weight), the difference between the filled tube with the anemone and the filled tube without the anemone was used. Image created using BioRender.

2.2.8 Statistical analyses

All graphs and statistical analyses were carried out using R version 4.6.0.

Enzyme activity data were analyzed using a three-way ANOVA to test the effects of population, feeding state (fed vs. fasted), and ammonia concentration on enzyme activity. Assumptions of normality and homogeneity of variance were assessed using the log-transformed enzyme activity. Post hoc comparisons were performed using estimated marginal means (emmeans) to examine differences among feeding states within the same population, ammonia concentrations within feeding states, population groups, and overall population differences.

Linear mixed-effects models were fit in R using the lme4 package to assess the effects of population, diet, ammonia concentration, and their interactions on MO_2 as fixed effects. Analyses were performed separately for each air saturation category: high, medium, and low. Estimated marginal means (emmeans) and pairwise comparisons were obtained using Kenward–Roger approximated degrees of freedom. Tukey-adjusted post hoc comparisons between concentration groups generated compact letter displays for figure annotation.

2.3 RESULTS

2.3.1 Survival experiments

To examine the effects of ammonia on survival, anemones from Nova Scotia and New Hampshire were exposed to various concentrations of NH_4Cl for 11 days. The results from the initial survival experiments (February 2025) showed that in both populations, fed individuals experienced significant decreases in survival (Figures 9 and 10). Interestingly, in both populations, a 100% survival rate was observed for fasted individuals, except for individuals from the 0.5 mM group, where they had 0% survival after 6 days. However, the survival rates of fed individuals showed a different trend, displaying reduced survival at higher ammonia concentrations. Nova Scotia fed individuals experienced a decrease in survival at all concentrations except for the 0 mM (control) and 0.0625 mM groups (Figure 9). Fed individuals from New Hampshire also had similar survival, with a decrease in survival at all concentrations except for 0 mM (Figure 10). The lethal concentrations (LC_{10} = 10% mortality; LC_{25} = 25% mortality; LC_{50} = 50% mortality; LC_{75} = 75% mortality) of both populations for fasted anemones were similar, revealing that the sensitivity of fasted anemones from both population to ammonia were similar (Nova Scotia: LC_{10} = 0.335 mM; LC_{25} = 0.344 mM; LC_{50} = 0.354 mM; LC_{75} = 0.363 mM, and New Hampshire: LC_{10} = 0.333 mM; LC_{25} = 0.343 mM; LC_{50} = 0.354 mM; LC_{75} = 0.364 mM, see Figure A1 in Appendix A for LC graphs and calculations, LCs reported as proportion mortality). However, the LCs for the fed individuals from both populations differed. The LCs for the Nova Scotia fed group was: LC_{10} = 0.113 mM; LC_{25} = 0.152 mM; LC_{50} = 0.203 mM; LC_{75} = 0.271 mM, and New Hampshire was: LC_{10} = 0.059 mM; LC_{25} = 0.091 mM; LC_{50} = 0.141 mM; LC_{75} = 0.22 mM. Based on these calculated LC values, fed individuals from New Hampshire were found to have higher sensitivity

to ammonia exposure, due to having lower LC values. Overall, given the LC values, fed individuals from both populations showed higher sensitivity to ammonia than fasted individuals.

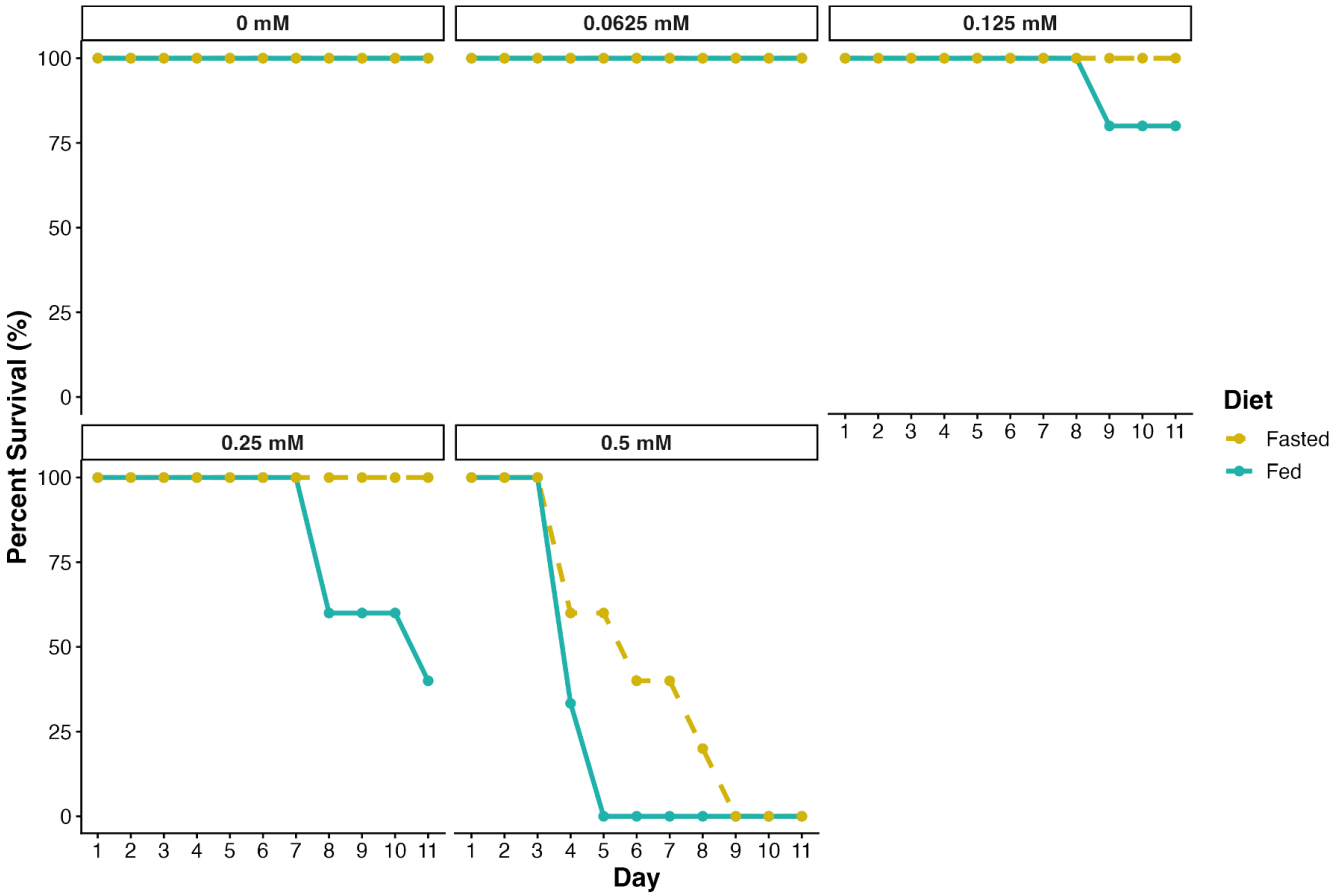

Figure 9. Initial survival of the Nova Scotia general population in ammonia. Fasted (gold, dashed line) and fed (turquoise, solid line) anemones from the Nova Scotia general population were exposed to varying concentrations of ammonia (0.0625 mM, 0.125 mM, 0.25 mM, and 0.5 mM NH_4Cl) for 11 days. Fasted individuals had 100% survival in all concentrations, except in 0.5 mM, where 100% mortality occurred after 8 days. Fed individuals experienced a reduction in survival in 0.125 mM and 0.25 mM after 6 days. Fed individuals displayed a steep decrease in survival in 0.5 mM after 4 days, resulting in 100% mortality on Day 5. The lethal concentrations (LCs, proportion mortality) are: LC_{10} = 0.335 mM; LC_{25} = 0.344 mM; LC_{50} = 0.354 mM; LC_{75} = 0.363 mM (fasted) and LC_{10} = 0.113 mM; LC_{25} = 0.152 mM; LC_{50} = 0.203 mM; LC_{75} = 0.271 mM (fed) (LC_{10} = 10% mortality; LC_{25} = 25% mortality; LC_{50} = 50% mortality; LC_{75} = 75% mortality) (n = 5 for fasted and fed individuals).

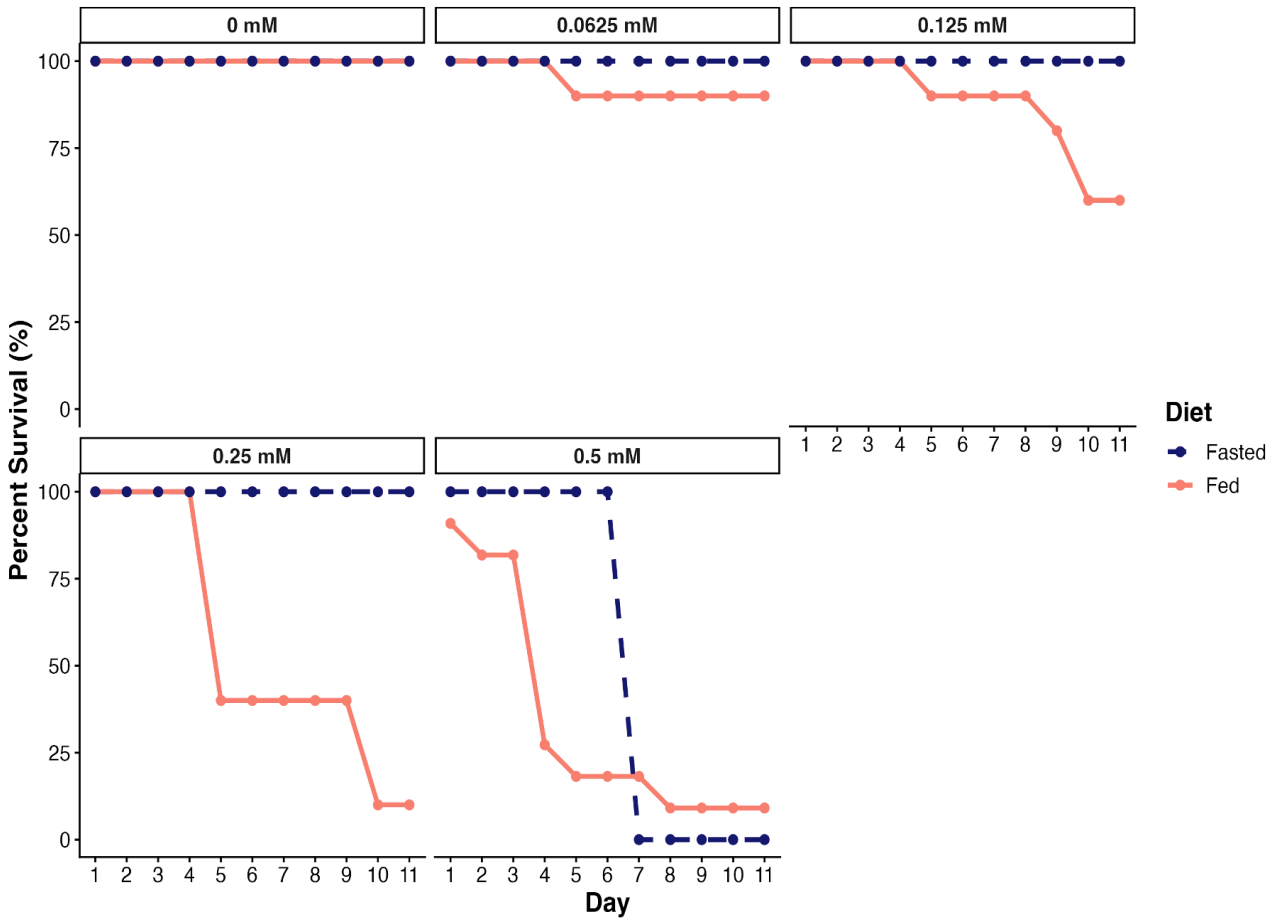

Figure 10. Initial survival of New Hampshire general population in ammonia. Fasted (purple, dashed line) and fed (salmon, solid line) anemones from the New Hampshire general population were exposed to varying concentrations of ammonia (0.0625 mM, 0.125 mM, 0.25 mM, and 0.5 mM NH_4Cl) for 11 days. Fasted individuals had 100% survival in all concentrations, except in 0.5 mM, where 100% mortality occurred after 6 days. Fed individuals experienced a reduction in survival in 0.0625 mM, 0.125 mM, and 0.25 mM after 4 days. Fed individuals displayed an immediate decrease in survival in 0.5 mM during Day 1 and continued to decrease. The lethal concentrations (LCs, proportion mortality) are: LC_{10} = 0.333 mM; LC_{25} = 0.343 mM; LC_{50} = 0.354 mM; LC_{75} = 0.364 mM (fasted) and LC_{10} = 0.059 mM; LC_{25} = 0.091 mM; LC_{50} = 0.141 mM; LC_{75} = 0.22 mM (fed) (LC_{10} = 10% mortality; LC_{25} = 25% mortality; LC_{50} = 50% mortality; LC_{75} = 75% mortality) (n = 5 for fasted and fed individuals).

When survival experiments were repeated (November 2025 and January 2026), surprisingly, fed individuals from both populations showed 100% survival in all concentrations (Figures 11 and 12). However, fasted individuals showed a reduction in survival, but only at the highest concentration of 0.5 mM. Fasted individuals from Nova Scotia had a 70% survival (Figure 11), and New Hampshire individuals had a 90% survival (Figure 12) in 0.5 mM of ammonia. Due to minimal reductions in survival observed in this survival trial, LCs could not be calculated. Compared to the results from the first set of survival experiments (Figures 9 and 10), these results showed that fasted individuals displayed a decrease in survival, instead of fed individuals exhibiting decreased survival, as observed in the initial survival experiments.

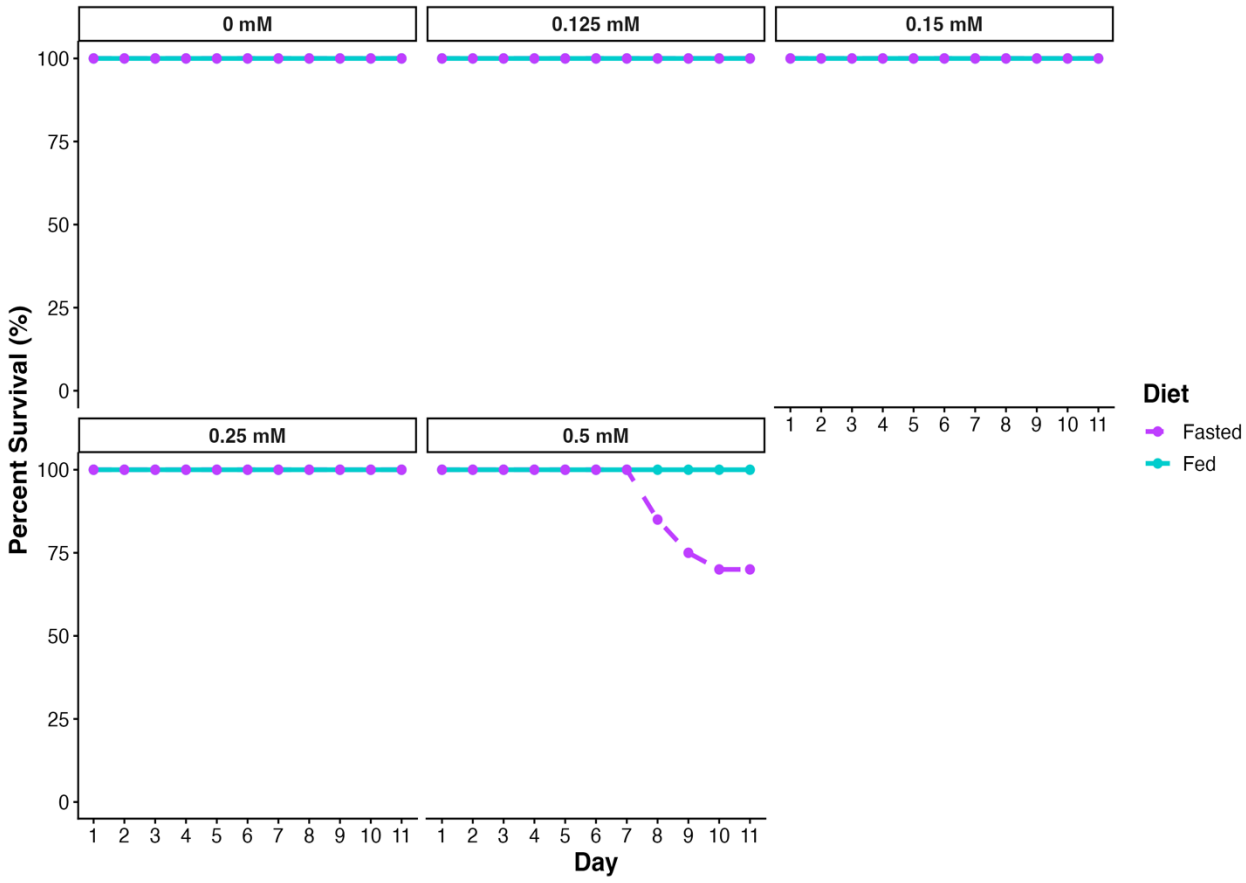

Figure 11. Nova Scotia general population ammonia survival. Fasted (purple, dashed line) and fed (blue, solid line) anemones from the Nova Scotia general population were exposed to varying concentrations of ammonia (0.125 mM, 0.15 mM, 0.25 mM, and 0.5 mM NH_4Cl) for 11 days. Fed individuals had 100% survival in all concentrations. Fasted individuals had 100% survival in 0 mM, 0.125 mM, 0.15 mM, and 0.25 mM. However, fasted individuals in 0.5 mM NH_4Cl had a survival percentage of 70% (n = 20 for fasted and fed individuals).

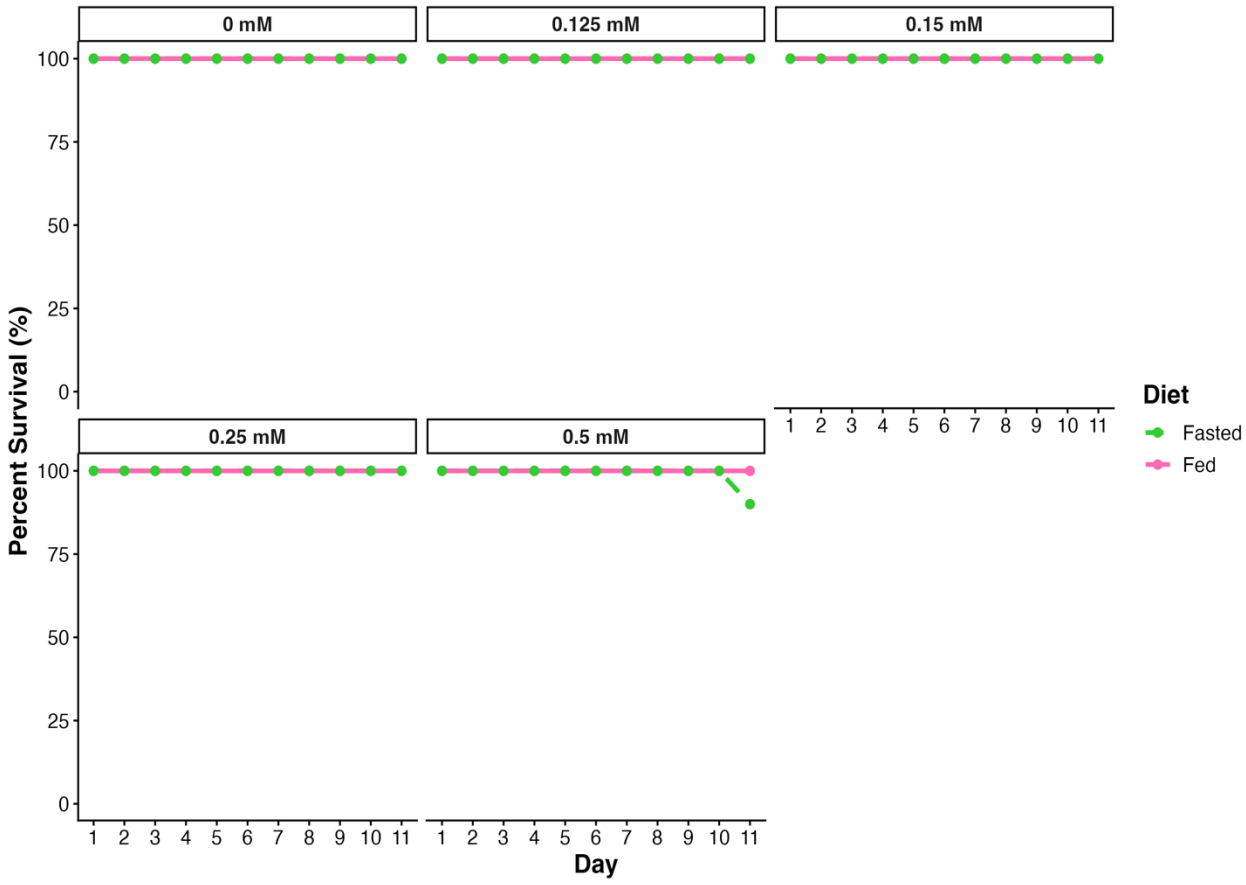

Figure 12. New Hampshire general population ammonia survival. Fasted (green, dashed line) and fed (pink, solid line) anemones from the New Hampshire general population were exposed to varying concentrations of ammonia (0.125 mM, 0.15 mM, 0.25 mM, and 0.5 mM NH_4Cl) for 11 days. Fed individuals had 100% survival in all concentrations. Fasted individuals had 100% survival in 0 mM, 0.125 mM, 0.15 mM, and 0.25 mM. However, fasted individuals in 0.5 mM NH_4Cl had a survival percentage of 90% (n = 10 for fasted and fed individuals).

2.3.2 The effect of ammonia exposure on GDH activity

To examine the effect of ammonia on GDH activity, fed and fasted individuals from each population were used (Figure 13). The results from the three-way ANOVA showed that GDH activity was not significantly affected by population, diet, or ammonia concentration as main effects. However, a significant Population x Diet interaction ($p = 0.0221$) indicated that the effects of diet differed between the two populations. The results from the post-hoc analysis used to interpret and explain the Population x Diet interaction detected by the ANOVA revealed that Nova Scotia fasted anemones had significantly higher GDH activity than New Hampshire fasted anemones ($p = 0.046$). Interestingly, there were no population differences in fed individuals.

Furthermore, there was a significant effect of ammonia concentration on GDH activity ($p = 0.0202$). The post-hoc test used to explain this ammonia effect suggested an increase in GDH activity in higher ammonia concentrations (0.25 mM and 0.5 mM; $p = 0.0373$ and $p = 0.0213$, respectively) in fasted individuals from Nova Scotia. Apart from this ammonia effect, no other ammonia-related pairwise comparisons (i.e., differences between ammonia concentrations within each Population x Diet combination) or three-way interactions (Population x Diet x Ammonia) were significant.

2.3.3 The effect of ammonia exposure on GS

Along with GDH, GS activity was examined in response to ammonia exposure (Figure 14). Similar to the GDH results, the ANOVA for GS indicated no significant main effect of population, diet, or ammonia concentration on GS activity. Two interactions had significant effects on GS activity: Population x Diet ($p = 0.040$) and Diet x Ammonia ($p = 0.000041$). These interactions revealed that GS activity depended on feeding state (diet) and ammonia concentration. The post-hoc analysis used to explain the Population x Diet effect showed that Nova Scotia fasted anemones

had higher GS activity compared to fasted anemones from New Hampshire ($p = 0.0132$), further revealing that GS activity in fasted individuals differed between populations. The post-hoc tests also revealed that GS activity was significantly higher in fed New Hampshire individuals than fasted New Hampshire individuals ($p = 0.0163$). Therefore, diet effects only existed in the New Hampshire population. For GS activity, effects of ammonia concentration were only observed in the New Hampshire population. This ammonia-related difference in GS activity was population- and diet-dependent. In fed individuals from New Hampshire, GS activity differed between 0.125 mM and 0.5 mM ammonia ($p = 0.0214$) and between 0.25 mM and 0.5 mM ammonia ($p = 0.0319$), where 0.125 mM and 0.25 mM anemones had higher GS activity compared to 0.5 mM anemones, explaining the Diet x Ammonia interaction. In fasted New Hampshire anemones, GS activity was significantly higher in 0.25 mM compared to 0.15 mM anemones ($p = 0.0487$), which further explained the Diet x Ammonia interaction in New Hampshire anemones. No significant ammonia-related differences in Nova Scotia anemones were observed. Additionally, no Population x Ammonia or three-way interactions were observed.

Overall, the most outstanding results from the enzyme assays are that enzyme activity for both GS and GDH was not completely and consistently dependent on ammonia concentration.

2.3.4 The effect of ammonia exposure on ALT and AST

Although ALT and AST activity were examined in both populations of anemones (fed and fasted), no detection of activity for these enzymes was made in the assays used in this study.

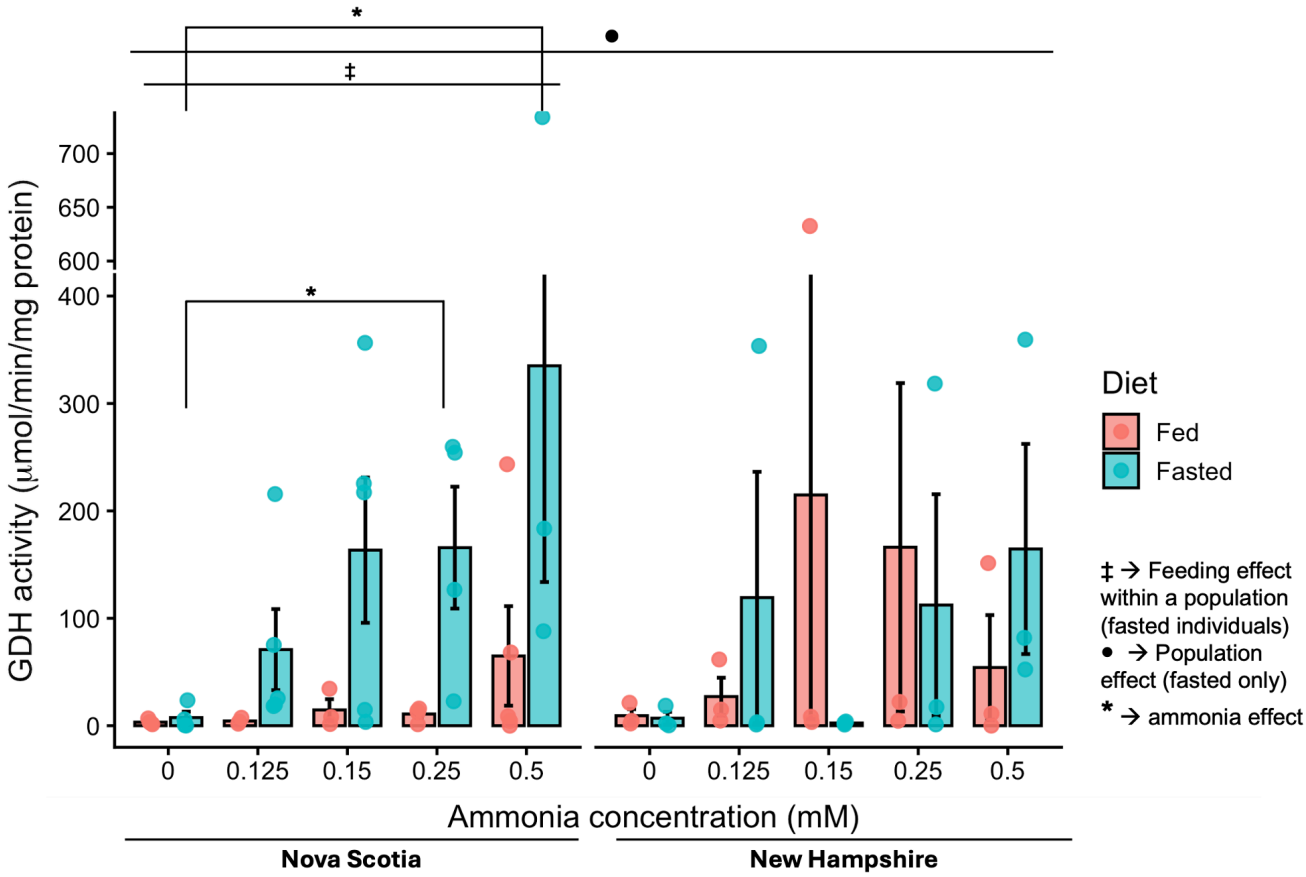

Figure 13. The effect of ammonia on GDH activity in Nova Scotia and New Hampshire. The graph illustrates whole-body GDH activity normalized to protein concentration in fasted (blue) and fed (salmon) anemones from both populations in ammonia (0, 0.125, 0.15, 0.25, and 0.5 mM). The data show a significant difference in GDH activity across populations in the fasted group (•, $p < 0.05$; $p = 0.046$), where Nova Scotia fasted individuals displayed a significant increase in GDH activity compared to New Hampshire fasted individuals. It was also observed that in Nova Scotia, there was a diet effect; fasted individuals had significantly higher GDH activity than fed individuals (‡, $p < 0.01$; $p = 0.0025$). However, no diet effect was shown in the New Hampshire population. Finally, an ammonia effect was only observed in Nova Scotia fasted individuals [0 mM vs. 0.25 mM ($p = 0.0373$) and 0 mM vs. 0.5 mM ($p = 0.0213$)], where 0.25 mM and 0.5 mM fasted individuals had significantly higher activity than 0 mM individuals (*, $p < 0.05$, Tukey-adjusted). [n = 3-5 for Nova Scotia (fed and fasted) and New Hampshire (fed and fasted)]. Annotations (symbols) denote significant pairwise comparisons identified by estimated marginal means (emmeans) analyses. Graph shows mean ± SEM.

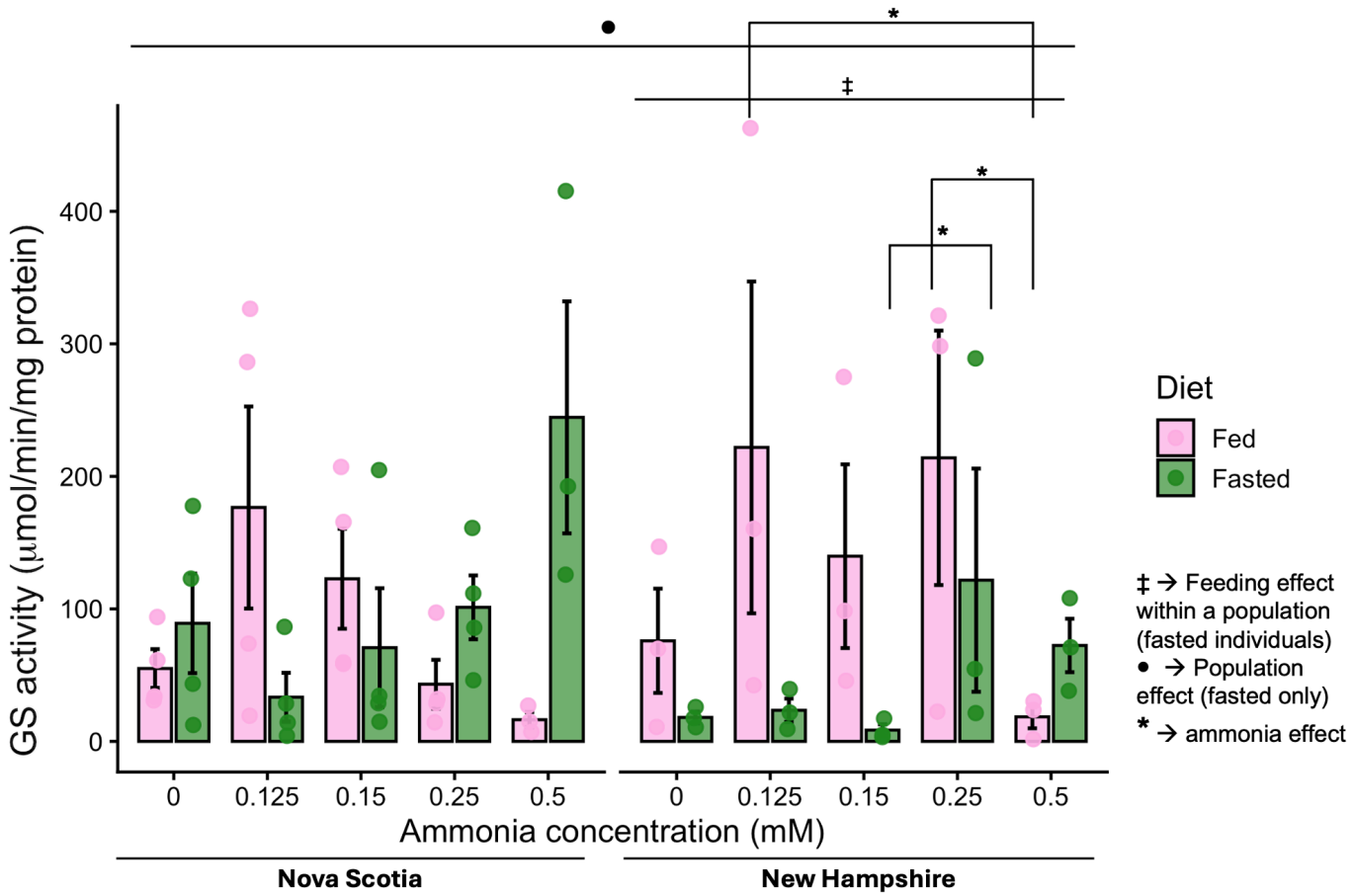

Figure 14. The effect of ammonia on GS activity in Nova Scotia and New Hampshire. The graph illustrates whole-body GS activity normalized to protein concentration in fasted (green) and fed (pink) anemones from both populations in ammonia (0, 0.125, 0.15, 0.25, and 0.5 mM). The data show a significant difference in GS activity between populations; fasted anemones from Nova Scotia had significantly higher GS activity than New Hampshire fasted individuals (•, $p < 0.05$; $p = 0.0132$). The data also revealed a feeding effect in New Hampshire only (‡, $p < 0.05$; $p = 0.0163$); fed individuals generally had higher GS activity. Finally, an ammonia effect was only observed in New Hampshire for fed and fasted individuals [0.125 mM vs. 0.5 mM (fed), 0.25 mM vs. 0.5 mM (fed), and 0.15 mM vs. 0.25 mM (fasted)] (*, $p < 0.05$, Tukey-adjusted). For fed individuals from New Hampshire, individuals in 0.125 mM and 0.25 mM had significantly higher GS activity than individuals in 0.5 mM ($p = 0.0214$ and $p = 0.0319$, respectively). For fasted individuals from New Hampshire, anemones in 0.15 mM had significantly lower GS activity than those in 0.25 mM ($p = 0.0487$). [n = 3-5 for Nova Scotia (fed and fasted) and New Hampshire (fed and fasted)]. Annotations (symbols) denote significant pairwise comparisons identified by estimated marginal means (emmeans) analyses. Graph shows mean ± SEM.

2.3.5 MO_2 and oxygen consumption

To examine the effect of ammonia on MO_2, the oxygen consumption of anemones from both populations was observed (Figure 15). To analyze MO_2, the data are presented as mass-specific MO_2, calculated at different percent air saturation, which is equivalent to the oxygen available in the microplate well at the time of the measurements. The high percent air saturation bin (Figure 15A), which represents air saturation above 60%, indicated a population difference for fasted individuals at 0.125 mM. At higher air saturation, there were some significant differences in MO_2 between fed and fasted individuals in the Nova Scotia population. However, in the New Hampshire population, fed individuals had significantly higher MO_2 than fasted individuals at higher ammonia concentrations, 0.25 mM and 0.5 mM. At higher air saturation, the ammonia effect was apparent in increasing ammonia concentrations. At medium air saturation (31% to 59%) (Figure 15B), both populations showed that fed individuals had significantly higher MO_2 compared to fasted individuals. In Nova Scotia, no effect of ammonia concentration on MO_2 was observed. Interestingly, within the medium bin for New Hampshire, ammonia appeared to have a significant effect on MO_2, where fed individuals had higher MO_2 at higher ammonia concentrations. Finally, in the low air saturation bin (<30%) (Figure 15C), the effect of diet was only present in the Nova Scotia population at 0.15 mM and 0.25 mM, where fed individuals had higher MO_2. It is important to note that based on pairwise comparisons, there was a strong influence of a Diet x Ammonia interaction in the high and medium air saturation bins, meaning that the effect of ammonia concentration on MO_2 was dependent on whether the anemones were fed or fasted, showing that at higher ammonia concentrations, fed individuals had higher MO_2.

2.3.6 Effect of ammonia on MO_2 (oxygen consumption rate) patterns

To understand the ammonia handling strategies and physiological responses to ammonia of the two populations, regarding MO_2, the metabolic patterns were examined (Figure 16). The results showed that at 0 mM, there was a significant difference between fed and fasted anemones within each population, with fed individuals having higher MO_2. However, there was no significant difference between fed and fasted anemones when ammonia was present (0.5 mM). Overall, both populations exhibited the same pattern, meaning their responses were the same.

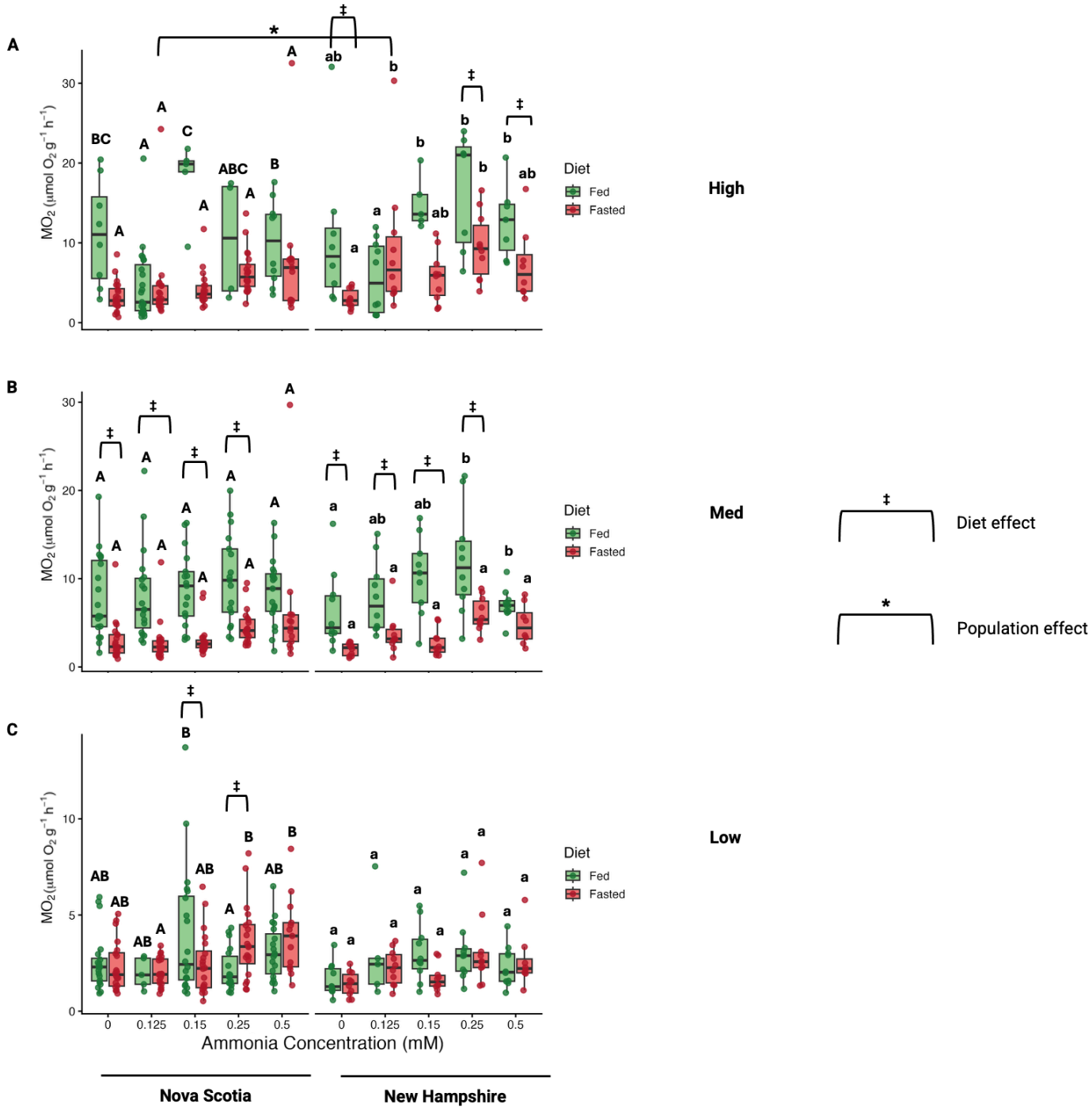

Figure 15. The effects of ammonia on mass-specific MO_2. The graph shows the mass-specific MO_2 of Nova Scotia and New Hampshire populations, based on individual anemone mass. The MO_2 is shown for the three bins of percent air saturation: **(A)** high (> 60%); **(B)** medium (31% to 59%); **(C)** low (< 30%). The data present population and feeding effects (diet). Letters indicate the effect of ammonia concentration (uppercase = Nova Scotia, lowercase = New Hampshire). The data reveal that fed individuals generally had higher MO_2 compared to fasted individuals. ‡, $p < 0.05$ [n = 20 for Nova Scotia (fed and fasted), n = 10 for New Hampshire (fed and fasted)].

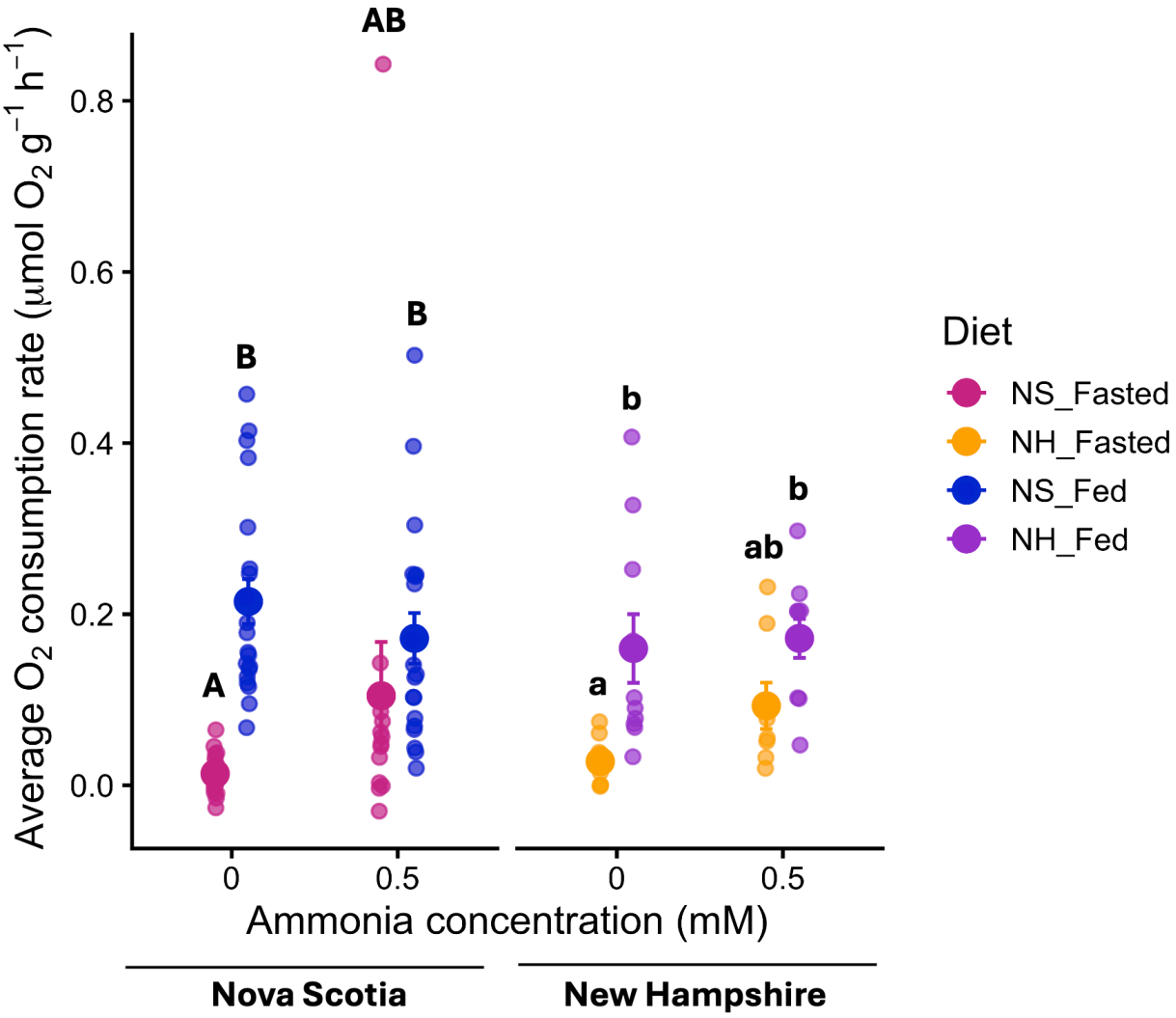

Figure 16. MO_2 patterns of Nova Scotia and New Hampshire anemones in high ammonia. The graph shows MO_2 patterns of the Nova Scotia and New Hampshire populations in 0 mM and 0.5 mM ammonia (NH_4Cl). The data reveal that at 0 mM ammonia, there was a significant difference between fed and fasted anemones in both populations, where fed anemones had significantly higher MO_2 than fasted anemones. The data also show that at a high ammonia concentration (0.5 mM), there was no significant difference between fed and fasted anemones from both populations. Overall, the results showed that both populations had the same MO_2 patterns. Different letters indicate significant differences (Tukey HSD) [uppercase letters denote Nova Scotia (NS); lowercase letters denote New Hampshire (NH)]. Nova Scotia: n = 20 (for fed and fasted); New Hampshire: n =10 (for fed and fasted). Values are presented as mean ± SEM.

2.4 DISCUSSION

2.4.1 The effect of ammonia on the survival rate of anemones from Nova Scotia and New Hampshire

It is known that ammonia impacts many organisms, including invertebrates (T. X. Zhang et al., 2023). Previous literature has shown that high ammonia levels cause a decrease in survival of various organisms, such as the greenlip abalone and cinnamon river shrimp (J. O. Harris et al., 1998; Miranda et al., 2025). Although the survival results showed different patterns of ammonia survival over time, a reduction in survival was still observed; the literature supports these results. However, when diet is considered, the literature shows feeding improves survival in high ammonia compared to fasting. The results collected from the first survival experiments (Figures 9 and 10) show that fed individuals had higher sensitivity to ammonia. Despite the expectation that fasted individuals would have higher sensitivity to high ammonia, the results showed the opposite. This unexpected result can be rationalized by various explanations shown in previous literature. A possible explanation for this result is that an increase in ammonia production and excretion was elicited by feeding of *Artemia*, a high-protein and amino acid-rich food (Herawati et al., 2014). A study conducted in the freshwater leech (*Nephelopsis obscura*) showed that feeding of a high-protein diet caused a significant increase in ammonia excretion (Quijada-Rodriguez et al., 2015). The suggestion that feeding can increase endogenous ammonia production and excretion caused by elevated nitrogen metabolism post-feeding is strongly supported (Brett & Zala, 1975; Bucking, 2017; Bucking & Wood, 2008; Wicks & Randall, 2002a). Given this information, if feeding increased internal ammonia production and ultimately ammonia excretion, this could have acted as an added stressor, causing further accumulation of ammonia in the external environment, leading to increased mortality of fed individuals.

In the results obtained from this study, fasted individuals showed lower sensitivity to ammonia in the first trial of survival experiments, but were the only group to experience reduced survival in ammonia in the second trial of survival experiments (Figures 11 and 12). Compared to feeding, literature shows stronger support for fasted individuals having higher susceptibility to ammonia, causing a decrease in survival, and proposing that feeding increases ammonia tolerance in high environmental ammonia (Uddin et al., 2024; Wicks & Randall, 2002a). It was found in juvenile rainbow trout (*Oncorhynchus mykiss*) that fed fish had higher LC_{50} levels in ammonia compared to fasted fish, showing that fed trout were less sensitive to ammonia toxicity than fasted trout, revealing that feeding increased ammonia tolerance (Wicks & Randall, 2002a). When the effects of ammonia were examined in common carp (*Cyprinus carpio*), it was also found that feeding protected the carp against ammonia's toxic effects (Diricx et al., 2013). Similar results were also discovered in *Carassius auratus gibelio*, the gibel carp, where it was found that fed carp had higher survival rates in ammonia compared to unfed carp (Uddin et al., 2024). Uddin et al. (2024) also found that fed carp showed improvements in ammonia detoxification, since they exhibited higher concentrations of GS, GDH, and glutamine in their liver, suggesting that fed fish had more energy for ammonia detoxification. This mechanism suggests that fed animals have greater metabolic resources; therefore, enhancing the capacity to detoxify ammonia through metabolic pathways, such as glutamine synthesis, which was also shown in another study in rainbow trout, where although fed trout had significantly higher plasma ammonia, they had higher GS activity (Wicks & Randall, 2002b). When exposed to high ammonia, organisms tend to adjust their nitrogen metabolism to rid the ammonia in their cells, which is energy-consuming (Cooper & Plum, 1987). Therefore, it is possible that feeding could be the source of energy aquatic invertebrates, such as the starlet sea anemone, require to detoxify ammonia, and ultimately grow

and survive under high and even lethal concentrations of ammonia (T. X. Zhang et al., 2023). According to the literature, it is likely that similar responses may occur in the starlet sea anemone. In this case, fed and fasted individuals might utilize ammonia-detoxifying enzymes, such as GS, GDH, ALT, and AST, at different rates to detoxify ammonia.

As previously mentioned, the dominant form of ammonia (NH_4^+ or NH_3) in solution depends on temperature, salinity, and pH. Given that the ammonia solutions were made in 18 p.p.t. ASW, with pH around 7.5, and survival experiments were conducted in the laboratory around 25°C, the NH_3 fraction compared to NH_4^+ remains low, and NH_4^+ still dominates, meaning that subtle decreases in survival would be expected compared to if NH_3 was dominant (Thurston et al., 1981; Yan et al., 2019). If pH, temperature, and salinity were to increase during the survival experiments, NH_3 would dominate, meaning the toxicity to the anemones would increase since NH_3 could diffuse more easily and rapidly through biological membranes than NH_4^+ (Ip & Chew, 2010; Randall & Tsui, 2002; Thurston et al., 1981; Wilkie, 2002; Yan et al., 2019).

2.4.2 Ammonia tolerance across populations

Although the results revealed that New Hampshire fed individuals had higher sensitivity because they had lower LC values in the first trial of survival experiments, with small sample sizes and no statistical comparisons and/or confidence intervals around the LC estimates, we cannot claim a true population difference between anemones. Given this lack of statistics, we could only suggest a possible population difference. These results on fed individuals from the first survival trial could not be replicated; therefore, the feeding effect was not reproducible. Since statistics were not conducted on survival data and LC estimates could not be made in the second trial of survival experiments, it cannot be said that one population was more tolerant than the other. Even though it was expected that the two populations would differ in ammonia tolerance in their

survival, it cannot be definitively stated that this was observed; rather, we could suggest, based on this study's results, a possibility of population differences. The claim that could be made for this study is that the observed contrasting results are consistent with acclimation; however, there are other explanations that could be made.

The literature shows that different populations of the starlet sea anemone exhibit local adaptation to environmental stress, suggesting that populations would tolerate stress differently (Friedman et al., 2018). Based on these contrasting results, it is suggested that long-term laboratory rearing and maintenance may have influenced ammonia sensitivity. Previously, it has been demonstrated that stress tolerance in organisms can change in the laboratory due to acclimation, a physiological process, or adaptation, a genetic process (Sun et al., 2018). Based on this acclimation or adaptation, stress tolerance can increase or decrease in the laboratory setting (Sun et al., 2018). To show the possibility of lab acclimation, preacclimation and acclimation studies have shown their impacts on survival results. It was shown in *Daphnia magna* that pre-exposure to environmental pollutants, such as copper, lead, and zinc, resulted in resistance to these metals, demonstrated by sustained survival under exposure (LeBlanc, 1982). Multigeneration acclimation to copper was also shown in marine copepods (*Tigriopus japonicus* and *Tigriopus californicus*) (Kwok et al., 2009; Sun et al., 2014). Reef-building corals (*Acropora nana*) exposed to high temperatures for 11 days showed rapid acclimation and higher heat resilience within 7 days, meaning their heat tolerance increased (Bay & Palumbi, 2015). Furthermore, continuous exposure of *Daphnia pulex* and *Simocephalus vetulus* to cyanobacteria showed that over time, they evolved higher tolerance (Jiang et al., 2016). Since the anemones were maintained in containers with multiple other anemones, and possibly uneaten *Artemia*, it is plausible that over the months, ammonia in the containers increased above levels in the wild, inducing ammonia tolerance, since

decomposition and nitrogenous waste excretion from other animals are main sources for ammonia accumulation in aquatic environments (Edwards et al., 2024). It has been shown that ammonium levels in salt marsh sediment range from 100 μM to 1.5 mM; therefore, if ammonia in the containers was above this range for a long period of time, the anemones could have acclimated to these ammonia levels over time (Carmona et al., 2021). In general, the populations of anemones showing different patterns of ammonia toxicity in the laboratory over time are likely, since toxicity varies between wild and laboratory-raised animals (Yun et al., 2026). The literature has indicated that ammonia toxicity in natural water systems (i.e., lakes, streams and rivers) may be less harmful than in laboratory conditions, since natural water systems have factors that can reduce the harmful effects of ammonia (X. Liu et al., 2024). Some of these factors include dissolved organic matter, suspended particles, and microbial communities (X. Liu et al., 2024). Several other alternative explanations for the observed differences arise, including seasonal and age differences. A study on freshwater crustacean invertebrates showed that season did impact ammonia toxicity and overall survival rate, specifically showing a difference in ammonia tolerance across seasons between species (Dehedin et al., 2013). In other words, LC_{50} at different ammonia concentrations varied across seasons and temperature (Dehedin et al., 2013). Therefore, it is possible that season and temperature influenced the survival results of this study. Variable differences in ammonia tolerance or survival of aquatic invertebrates have been observed based on life stage and age (Ostrensky & Wasielesky, 1995; T. X. Zhang et al., 2023; Zhao et al., 1997). For instance, crustacean larvae have a weaker tolerance and resistance to ammonia due to their metabolic capacity associated with growth and their physiological structure (T. X. Zhang et al., 2023; Zhao et al., 1997). However, in shrimp, the nauplius stage showed higher tolerance to ammonia compared to the juvenile and adult stages, consistent with reduced ammonia tolerance as they age

and develop, further suggesting accelerated energy metabolism in the nauplius stage that supplies the energy demand to alleviate the toxic effects of ammonia (Ostrensky & Wasielesky, 1995). It is possible that anemones from an earlier or later developmental stage were chosen, causing inconsistencies in the data. Selecting individuals from similar developmental stages would potentially minimize the effect of age or developmental stage. With this evidence in mind, the results from this study are surprising, since an initial dramatic decrease in survival was observed; however, over time, the survival rate increased. Even with evidence from studies that have shown that anemones can locally adapt to their environment, the experiments conducted in this study cannot support this, since genetic evidence is required to conclude whether the populations differ genetically, which could be done using amplified fragment length polymorphism (AFLP) fingerprinting, allowing for more inference about the genotype of our two anemone populations (Reitzel et al., 2008). The data from this study align more with the suggestion of differences being attributed to local environmental conditions, such as the laboratory.

The tolerance of the starlet sea anemone has been demonstrated in many studies, including those focusing on thermal tolerance, oxidative and heat-related stress, ultraviolet (UV), and salinity (Friedman et al., 2018; Reitzel et al., 2013; Sachkova et al., 2020). Collectively, these studies showed that populations exhibit a broad range of environmental tolerance, with geographic variation as a strong driver of physiological performance, consistent with local adaptation. The study that examined thermal tolerance in the anemone showed that thermal stress affected growth, development, and regeneration, revealing population differences in optimal temperatures, consistent with divergence in thermal physiology (Reitzel et al., 2013). Similarly, population differences were also observed under salinity stress, where tolerance of salinity was dependent on the native environment of the anemone (Sachkova et al., 2020). Furthermore, a study that looked

at oxidative stress in clonal lines revealed the effects of genetics on stress-response (Friedman et al., 2018). These findings support the starlet sea anemone being physiologically plastic, with local adaptation as a strong driver in responses and tolerance to stress (Glon et al., 2020; Sachkova et al., 2020).

Overall, this study found that ammonia does impact the survival of the starlet sea anemone. The results showed that fed and fasted individuals experienced reductions in survival; however, contrasting results were observed after 9 to 11 months. Based on the different results from the two trials of survival experiments, it was observed that survival rate increased over time, leaving room for the suggestion that laboratory maintenance may have led to the anemones acclimating to their environments and increasing survival in ammonia. The results were also consistent with possible population differences in survival rate when exposed to ammonia, in the first trial of survival experiments; however, this population difference was not reproducible and was lost over time. With these results as evidence and despite the inconsistency, the expectation that ammonia exposure impairs survival was observed.

2.4.3 The effect of ammonia on GDH and GS activity

As previously stated, GS and GDH are key enzymes for ammonia detoxification, making the examination of their activity important for the aim of this study. The overall results from the enzyme assays for GDH and GS (Figures 11 and 12) showed that both anemone populations exhibited distinct enzyme activity in response to diet (fed and fasted) under ammonia exposure. These results were also observed with consistent population differences under fasted conditions for GDH and GS, suggesting a possible population-specific regulation of ammonia and nitrogen metabolism during energy limitation. Both populations showed plastic and stable enzyme activity. For instance, for GDH, Nova Scotia displayed a strong response to energy availability, with fasted

individuals showing higher GDH activity, suggesting activity plasticity. However, for GS activity, Nova Scotia showed stable responses, with diet not strongly influencing GS activity. On the other hand, New Hampshire anemones showed a stable response for GDH activity, with no influence of diet on enzyme activity. But for GS activity, New Hampshire demonstrated plasticity in their responses, which were influenced by diet, where fed individuals had higher enzyme activity than fasted individuals.

The most important result obtained from the enzyme assays revealed that GDH and GS activity was not dependent on ammonia concentration. Most vertebrate and invertebrate studies have shown that increasing ammonia elicits stronger enzyme responses, making the results from this study stand out (Hegazi et al., 2010; Qiu et al., 2018; T. Zhang et al., 2024; Zou et al., 2023). Since the results showed no clear ammonia-dependent pattern or trend, it cannot be said that ammonia itself is directly regulating GDH and GS activity or that changes in enzyme activity suggest a response to ammonia exposure. Given these results, it leads to the exploration of alternative explanations. Some alternative explanations or factors that could explain enzyme variation include feeding status, energetic state, acclimation history, and individual variation. In terms of feeding or nutritional status affecting enzyme activity, invertebrate studies have shown that enzyme activity and nitrogen metabolism depend on the organisms' feeding and nutritional status (Brito et al., 2000; Cui et al., 2023; James Bond et al., 2025). In shrimp (*Litopenaeus setiferus*), it was revealed that feeding altered digestive enzyme activity, such as protease, demonstrating that nutritional status alone can influence enzyme activity (Brito et al., 2000). Similar results were found in mussels (*Mytilus californianus*), where the activity of the digestive enzyme amylase increased and decreased based on diet and feeding regime (James Bond et al., 2025). Another cnidarian-focused study showed that enzyme activity in corals (*Stylophora*

pistillata and *Acropora hemprichii*) and sea anemones (*Exaiptasia diaphana*) depended on the nutritional and carbon status of the cnidarian host and not just environmental ammonium, meaning enzyme activity may have represented differences in nutritional state and carbon availability from the host, rather than a direct response to ammonia concentration, which also relates to energetic state (Cui et al., 2023). Studies in coral demonstrated that metabolic processes are dynamically regulated across diel cycles (shifts in environmental conditions and biological activity) and that physiological shifts can occur without observing changes in enzyme activity, further highlighting the importance of the metabolic regulation that is mediated by symbiotic relationships (Levy et al., 2006; Linsmayer et al., 2020). With this evidence, it can be suggested that enzyme activity may reflect the metabolic state and energetic availability of the cnidarian holobiont (the host organism and its associated microorganisms) (Levy et al., 2006; Linsmayer et al., 2020). Lastly, acclimation history and individual variation are possible alternatives for enzyme variation. A study on coral adaptation and acclimation demonstrated that over time, reef communities and populations' responses to environmental change appear, and that their physiological traits could differ depending on their prior exposure history (Buddemeier & Smith, 1999). This provides further support for differences in enzyme activity across anemone populations; these differing enzyme responses may reflect environmental history or long-term acclimation, rather than direct ammonia regulation. Individuals within each anemone population could have different genotypes that could influence their enzyme activity and the nitrogen assimilation pathway favoured (Darling et al., 2004). Altogether, metabolic activity may be highly variable, as shown in rocky intertidal invertebrates (mussels, barnacles, whelks, and sea stars), where metabolic indicators and physiological responses were influenced by multiple environmental factors simultaneously, rather than driven by a single factor (Dahlhoff et al., 2002).

Since this study focused on a cnidarian species, it is possible that variation in enzyme activity may reflect enzyme regulation at the level of the holobiont (host + symbionts + microbiome), rather than a direct response of the cnidarian host (the starlet sea anemone) to high environmental ammonia. Studies have shown that in cnidarians, the holobiont regulates nitrogen assimilation cycling and particularly the glutamine synthetase/glutamate synthase (GS-GOGAT)-mediated nitrogen assimilation cycle; this may reflect the reason enzyme activity did not show an ammonia concentration-dependent response, which was initially anticipated (Cui et al., 2023; Rädecker et al., 2015). Overall, the microbial community of cnidarians and the cnidarian holobiont are important for the health of the cnidarian host by aiding in metabolism, making it a possible explanation for the physiological changes observed (Stabili et al., 2018). Furthermore, while the starlet sea anemone lacks a symbiotic relationship with zooxanthellae, it does have symbiotic relationships with bacteria associated with its microbiome, meaning the holobiont of the starlet sea anemone, specifically the microbes, may possibly play a role in ammonia detoxification (Glass & Barott, 2025; Har et al., 2015). However, a study that examined the starlet sea anemone and its ammonia transporters suggested that the main excretory mode for the starlet sea anemone is diffusion (Andrikou et al., 2019). With the evidence from these studies, it provides support for the explanation that the microbial community along with other modes of excretion, such as diffusion, may be alternative mechanisms in which the starlet sea anemone mitigates ammonia detoxification, assimilation, and handling.

2.4.4 The influence of diet, ammonia, and population on GDH and GS activity

One of the surprising results obtained in this study was high GDH activity in fasted individuals from Nova Scotia (Figure 11). It was expected that fed individuals would have higher enzyme activity since previous literature has shown increases in GDH and GS activity when

organisms are fed; however, the results for GDH showed that Nova Scotia fasted individuals had higher activity (Luczkowska et al., 2024). In a study conducted on *Carassius auratus gibelio*, the gibel carp, it was shown that GS and GDH activity increased in fed individuals when exposed to ammonia, showing higher detoxification metabolism, which helped fed fish survive longer in high ammonia (Uddin et al., 2024). In contrast, fed individuals from New Hampshire showed significantly higher GS activity, supporting the prediction made for this study. Previous studies in mice have shown that GDH activity increased when they were continuously fed or fed high-protein diets (Luczkowska et al., 2024). It is worth noting that the *Artemia* given to the fed group is known to have high levels of proteins and amino acids, further supporting the initial prediction that fed individuals would exhibit higher activity of GDH and GS in high environmental ammonia (Herawati et al., 2014). Studies also indicate that fed animals with higher levels of GS and GDH may have more energy available for ammonia detoxification compared to fasted individuals (Uddin et al., 2024).

The measured GDH activity in the amination direction was higher in fasted individuals compared to fed individuals. Due to GDH having a central role in amino acid, energy, and nitrogen metabolism, and catalyzing a reversible reaction (deamination and amination), the obtained results must be addressed cautiously. Although a possible explanation for higher GDH activity in fasted individuals could be provided, this change in enzyme activity alone cannot determine the direction of nitrogen flux. Without additional measurements of glutamate, α-ketoglutarate, and glutamine, we cannot infer whether GDH was consuming or producing ammonia. Studies have shown that measuring glutamine, glutamate, and α-ketoglutarate is important when trying to understand the direction of nitrogen metabolism under ammonia exposure (Sanderson et al., 2010). It has been shown that prolonged fasting or starvation can cause an accumulation of amino acids that are

metabolized (Felig et al., 1969). When there is an addition of endogenous ammonia produced by the breakdown of amino acids, and a high ammonia external environment, this would cause significant increases in GDH and GS activity, as both enzymes catalyze reactions that convert ammonium (NH_4^+) to glutamate or glutamine (Castegna & Menga, 2018; Plaitakis et al., 2017). In the study by Uddin et al. (2024), it was also found that high ammonia resulted in decreased food consumption in the gibel carp, which subsequently led to a reduction in GDH and GS activity. It was also found in this study that higher detoxification was observed at lower ammonia concentrations, while excessive exposure to high ammonia concentrations inhibited feeding and damaged detoxification organs, resulting in reduced detoxification activity in the fish, including GDH and GS (Uddin et al., 2024). Based on this evidence, another possible explanation for these results is that chronic exposure to ammonia for 11 days reduced food consumption among the fed anemones. This decrease in the consumption of *Artemia* would ultimately lead to a reduction in the activities of GDH and GS.

Additionally, the results did not show a clear or gradual increase in enzyme activity as ammonia concentration increased, despite the expectation that higher ammonia concentrations would significantly increase the activity of GS and GDH. In the gibel carp study, it was shown that GDH and GS activity did not show a clear increase with increasing ammonia concentrations; instead, it showed lower enzyme activity at higher ammonia concentrations, and significantly higher enzyme activity at lower ammonia concentrations (Uddin et al., 2024). A possible reason for not observing the anticipated trend is that the anemones are using another pathway or mechanism for ammonia detoxification, previously shown in fish (Yu & Liu, 2026). A study using the euryhaline four-eyed sleeper (*Bostrychus sinensis*) showed that GDH and GS activity were non-linear and tissue-specific, meaning activity did not always increase with ammonia

concentration and tissues exhibited distinct activity responses (Peh et al., 2010). Using this evidence, it could be strongly suggested that using whole-body samples rather than distinct tissues may have influenced enzyme activity. It is possible that because enzyme activity for GDH or GS increased in one tissue and decreased in another, the signal may have disappeared, since enzyme activity was averaged across the whole animal. If specific tissues, such as the mesenteries of the anemone, were used to conduct enzyme assays, there might have been a difference in enzyme activity; perhaps higher enzyme activity may have been observed, since these structures are the primary digestive organs that secrete many enzymes (Figure A3; Appendix C) (Babonis et al., 2016). Another reason as to why enzyme activity did not increase with increasing ammonia is the timing of sampling. In a study conducted in shrimp (*Litopenaeus vannamei*), it was shown that when enzyme activity for GDH, GS, ALT, and AST was sampled every two hours for 8 hours after exposure to ammonia, it was shown that generally, enzyme activity would peak early on and then decrease once the 8-hour mark approached; however, other enzymes showed peaks closer to 8 hours (Sui et al., 2023). This study supports the suggestion that peak enzyme activity is variable; therefore, if samples were sampled after the peak, the expected response would not be observed. In the case of the anemones, it is possible that optimal and peak enzyme activity were missed when analysis took place 11 days after exposure, prompting a future step of measuring enzyme activity at different time points after and/or during ammonia exposure. With this extra information, it could provide a clear assessment of enzyme activity. Without measuring ammonia (i.e., ammonia excretion), glutamine, or glutamate concentration in specific tissues of the anemone, it becomes difficult to determine whether the enzyme activity observed is biologically meaningful.

Finally, another result observed during the enzyme assays was that ALT and AST activities were not detectable in the anemones. However, this result does not necessarily indicate the absence

of these enzymes in the starlet sea anemone. Rather, their activities may have been below assay detection limit or indistinguishable from the background signal under the experimental conditions used. Goldfish tissue samples were used as a positive control to ensure that enzyme activity was detected with the running buffer. The results from the goldfish sample showed high ALT and AST activity, further supporting that the activity for these enzymes was not distinguishable in the anemone samples. The enzyme protocols used in this study were adapted from vertebrate studies, which could mean that the protocol may need to be revised for invertebrate models (Bucking et al., 2013; Pelster et al., 2015). As stated previously, ALT and AST are transaminases, meaning they convert amino acids, alanine and aspartate, to glutamate, showing that they do not directly participate in ammonia assimilation like GS and GDH. Given the reactions catalyzed by ALT and AST, it could be said that their activity levels were rather low due to being responsible for nitrogen redistribution (Ellinger et al., 2011; X. J. Huang et al., 2006; Sookoian & Pirola, 2012; Toney, 2014).

Overall, the enzyme assay results revealed that both populations displayed varying GDH and GS activity across ammonia concentrations and between diets. Based on the enzyme results between both populations, they did not show similar physiological responses to ammonia, leaving the hypothesis regarding the populations having similar physiological responses to ammonia as partially rejected. Given that the anemones used are of the same species, it is possible that their local adaptation to their wild environments (Nova Scotia and New Hampshire) persisted, even after being maintained in the lab for three years. This suggestion is supported by literature that has shown that, despite the environment being controlled, population differences can persist due to countergradient variation, where genetic traits and environment act in opposite directions, causing overall phenotypic traits (physical or behavioural traits) to remain constant across environments

(Conover & Schultz, 1995). Furthermore, studies have also shown that despite having the same environmental conditions, local adaptation is a strong influence, with different populations showing differences in various parameters, such as physiology, life-history traits, growth, and survival (Fraser et al., 2011). Due to the distribution of the starlet sea anemone and population isolation, there is a lack of genetic similarity, further supporting that mechanisms used between the two populations may be different (Fraune et al., 2016). The results also partially disprove the prediction that ammonia would increase enzyme activity. Although some increased activity at higher concentrations was observed, it was not conserved and consistent across the two populations and enzymes.

2.4.5 The effect of ammonia on MO_2 and oxygen consumption

The results from this study revealed that generally, fed anemones had higher MO_2 than fasted anemones in each population (Figures 15A and 15B), supporting the initial prediction that fed individuals would have higher MO_2. The findings from the high and medium percent air saturation bins (Figures 15A and 15B) align with previous literature, which indicates that feeding increases oxygen consumption and, ultimately, MO_2 (Jobling, 1981). Meal size significantly affects metabolic oxygen consumption (MO_2). Research indicates that larger meals can considerably increase the rate of oxygen consumption and MO_2 due to the time required for digestion (Jobling & Davies, 1980). Studies have shown that food intake increases protein catabolism, leading to elevated oxygen consumption and ammonia excretion, further supporting the results from the respirometry experiments (Chew et al., 2005; Zakęś et al., 2006). In contrast, it was revealed in previous studies that fasting reduces MO_2, supporting the results that fasted individuals inherently have lower MO_2 (S. W. Y. Ma & Foster, 1986).

The MO_2 data demonstrated that ammonia exposure sometimes increased oxygen consumption, but this was not consistent. Based on these results and without measurements of ammonia excretion, glutamine or glutamate production, and ATP utilization, we cannot fully conclude or claim increased ammonia detoxification or increased costs of detoxification. However, we could suggest and possibly conclude that ammonia exposure increased energetic demands, but not ammonia detoxification increasing these energetic demands. These results lead to alternative explanations, such as stress, cell repair, and behaviour, which have been shown to influence MO_2 (Glazier & Gjoni, 2024). Stress has been shown to affect MO_2, especially when organisms are subjected to acute, chronic, or repeated stressors, causing MO_2 to rise (Kuti et al., 2022; Sokolova, 2021). For instance, a study conducted in benthic octopods (Cephalopoda) revealed that limited oxygen in an environment (hypoxia, a stressor) caused increased MO_2 (Seibel & Childress, 2000). There is a link connecting stress to cell repair, which is another factor known to influence MO_2 (Alotiby, 2024). When insects were exposed to low-temperature thermal fluctuations, oxidative stress and damage followed; this caused increases in MO_2 that were a result of the energy demand to repair cold-induced injuries in the insects (Lalouette et al., 2011). Studies conducted in the starlet sea anemone have shown that locomotor activity follows a rhythmic cycle that requires energy, increasing MO_2, and further demonstrating that MO_2 may fluctuate with endogenous rhythms, which could act independently of environmental ammonia exposure (Hendricks et al., 2012; A. E. Maas et al., 2016; Oren et al., 2015). Altogether, these listed factors all provide reasons for increases in MO_2.

Although ammonia concentration was shown to have some effect on MO_2 in both populations, this effect was diet-dependent, where fed individuals had higher MO_2 at increasing ammonia concentrations. Previous literature supports these results, showing that high ammonia

can increase MO_2 in other invertebrates and vertebrates, including prawns (*Penaeus chinensis*), greenlip abalone (*Haliotis laevigata*), and rainbow trout (*Salmo gairdneri*) (Chen et al., 1991; J. O. Harris et al., 1998; Smart, 1978). It is also known that fasting decreases oxygen consumption and therefore MO_2 (Fu et al., 2005). The lower MO_2 in fasted individuals could be explained by a lack of metabolic demand, compared to fed individuals who need to increase metabolic demand to handle protein synthesis and the required ATP, caused by amino acid turnover elicited by feeding (Fuery et al., 1998). Furthermore, feeding increases the demand for ATP since digestion and nutrient processing are metabolically expensive, elevating MO_2 in fed individuals (Cant et al., 1996). The factors discussed alongside fed individuals reflect the energetic costs associated with SDA; however, with fasted individuals, the results are consistent with the absence of SDA-related costs (Secor, 2009). As previously mentioned, MO_2 is also influenced by body size or body mass. Metabolic scaling principles such as Kleiber's law establish that increased body mass increases total metabolic rate, suggesting that larger organisms use more total energy than smaller organisms (Thommen et al., 2019). However, when we focus on mass-specific metabolic rate, this law implies that mass-specific MO_2 decreases as body mass increases, so metabolic rate per gram decreases (Thommen et al., 2019). Although this may pose as a possible explanation for the MO_2 data, the results disprove this law, since fed individuals, whose body mass was higher than fasted individuals, displayed higher MO_2 per gram anemone mass, further supporting the suggestion that the increased MO_2 observed was reflective of SDA.

Even though this study could not conclude an increase in ammonia detoxification, ammonia exposure is known to increase energetic demand (i.e., ATP demand) to maintain homeostasis disrupted by ammonia. In addition to increased ammonia, escalating the demand for ammonia detoxification, such as ammonia elimination, is also known to disrupt ion regulation and

acid-base regulation (pH), and increase cellular respiration stress (Armstrong et al., 1978; Hakvoort et al., 2017; R. Harris et al., 2001; Lin et al., 1993; Martinelle & Häggström, 1993; Sinha et al., 2012; Wright, 1995; T. X. Zhang et al., 2023; Zhou et al., 2020). Overall, feeding elevates metabolic activity in organisms due to the energetic cost associated with physiological processes, such as digestion, nutrient absorption, and protein synthesis (Cant et al., 1996; Fuery et al., 1998). These important processes require the acquisition of ATP; therefore, feeding status is closely linked to MO_2 and excretion of ammonia.

2.4.6 Respirometry strategies of anemones from Nova Scotia and New Hampshire

When oxygen consumption rate (MO_2) was examined more closely, Nova Scotia and New Hampshire populations showed the same metabolic patterns and responses (Figure 16). Although there was no significant difference between MO_2 at 0 mM and 0.5 mM ammonia, across both populations, average MO_2 tended to be higher in our 0.5 mM treatment, supporting the expectation that with no ammonia exposure, MO_2 would be lower, and that fasted individuals would demonstrate lower MO_2. These results are supported by literature that has shown that increases in ammonia cause an increase in MO_2 to maintain homeostasis (Chen et al., 1991; J. O. Harris et al., 1998; Smart, 1978). Due to a low sample size, we cannot say that Nova Scotia relies on GDH and New Hampshire relies on GS. To confirm whether the populations rely heavily on one enzyme over the other, it would require the assessment of other factors, such as metabolite and pathway flux measurements (i.e., nitrogen flux), gene expression, and inhibition experiments.

Together, the results from the enzyme assays and respirometry experiments allowed for a key finding to be discovered. Despite the populations displaying similar metabolic responses, enzyme responses between populations were different, showing that they use different biochemical strategies. To recall, Nova Scotia anemones showed changes in GDH activity, but stable GS

activity, while New Hampshire anemones showed stable GDH activity, but changes in GS activity. Physiological redundancy is a possible explanation, which is when redundant mechanisms overlap or compensate for other mechanisms or pathways to help maintain normal activity and physiological performance in organisms (Joyner, 2013). Compensatory actions and mechanisms are also another possible reason for differences in enzyme regulation, so redundancy can also allow for compensation (Joyner, 2013). The contrasting enzyme responses may also reflect degeneracy, where distinct physiological mechanisms, such as different enzyme reactions, perform similar functions and contribute to similar functional outcomes (Tononi et al., 1999). A paper focused on the effects of temperature on aerobic metabolism suggested that aerobic metabolism is controlled by multiple interacting pathways and systems, meaning that whole-organism oxygen consumption is influenced by factors such as enzymes, oxygen transport, and mitochondria (Schulte, 2015). Due to the many factors and systems that contribute to metabolic responses, it is likely there is no one-to-one relationship between enzyme activity and MO_2. Since metabolic pathways are complex, form networks, and could be the underlying effect of whole-organism performance including MO_2, it further supports the observation of different enzyme responses, but similar metabolic responses (Chandel, 2021; Seebacher & Beaman, 2022). In a coral energy metabolism study, it was found that significant enzyme activity differences occurred with corresponding changes in whole-organism metabolism, further suggesting that enzyme activity and metabolic responses may not always be reflective of each other (Linsmayer et al., 2020). Instead, it was found that energy metabolism was regulated through adjustments of metabolite fluxes, rather than enzyme abundance regulating energy metabolism (Linsmayer et al., 2020).

The results from the respirometry experiments showed that ammonia did have some influence on MO_2, especially at higher concentrations of ammonia, supporting the hypothesis that

ammonia would affect MO_2. However, it cannot be concluded that the increased oxygen consumption caused by ammonia exposure was indicative of increased ammonia detoxification. These results do not provide direct evidence for the observed MO_2 changes being used to regulate and handle ammonia and its impacts. Based on the results, it was discovered that the two anemone populations have similar MO_2 response patterns, suggesting similar handling strategies across populations. With these results, the hypothesis of similar handling strategies across populations can be partially accepted; however, we cannot confirm that metabolic responses are being used as an ammonia handling strategy.

2.4.7 An integrated model of ammonia handling in *Nematostella vectensis*

Based on the present findings and previous studies, we propose a hypothetical model of ammonia handling in *N. vectensis*. If we look at all the results together, we see the data support the assumption that the starlet sea anemone is ammonia-tolerant. More specifically, it was observed that the anemones from both populations could tolerate moderate ammonia. The data showed that survival decreased at higher ammonia concentrations, with fed and fasted individuals showing decreased survival. It was also observed that GDH and GS responses differed between populations, where Nova Scotia anemones showed plastic GDH activity and stable GS activity, but New Hampshire showed stable GDH activity and plastic GS activity. Despite differences in enzyme responses, both populations showed increases in MO_2, especially in fed individuals when no ammonia was present; however, with increased ammonia, this feeding effect was no longer apparent. Therefore, the two populations of anemones showed different biochemical responses, but similar energetic responses. Since we observed similar whole-animal energetic responses despite differing enzyme responses, it suggests that multiple physiological pathways may support ammonia tolerance in the starlet sea anemone, making this a notable result from this study. The

differences may indicate that Nova Scotia predominately adjusts GDH, while New Hampshire predominately adjusts GS. In other words, Nova Scotia could be using GDH as a primary enzyme, especially in fasted individuals, while fed individuals from New Hampshire use GS, suggesting that multiple routes or pathways might be used to accomplish a similar physiological goal.

Chapter 3: Conclusion, Limitations, and Future Directions

3.1 CONCLUSION

This study examined ammonia tolerance and handling strategies in the starlet sea anemone. We investigated the influence of population, diet, and ammonia concentration as individual and combined effects. The findings from this study provide insight into the ammonia tolerance of the starlet sea anemone, as research on this in aquatic invertebrates, especially cnidarians, is poorly understood compared to the studies conducted on aquatic vertebrates, such as fish. Despite being a natural byproduct, ammonia remains a prominent toxicant in aquatic environments, as it poses threats to communities, ecosystems, and biodiversity, which provides a basis for the importance of research that focuses on how these aquatic organisms maintain normal functioning and homeostasis in high ammonia environments (Yun et al., 2026; T. X. Zhang et al., 2023). Since the starlet sea anemone resides in estuarine environments that face fluctuating ammonia levels, they pose as an ideal model organism to assess this main objective (Klein et al., 2021; Layden et al., 2016; Stefanik et al., 2013).

This study discovered that the starlet sea anemone is relatively ammonia-tolerant; ammonia exposure impacted survival, there were differences in physiological and biochemical responses between geographically isolated populations, and ammonia exposure increased energetic costs. Despite the examination of known ammonia-detoxifying enzymes, this study did not discover detoxification mechanisms, pathways, or mechanisms of ammonia tolerance. However, the findings from the present study could contribute to understanding specific mechanisms of tolerance and detoxification.

More specifically, this study provides evidence for population-specific physiological responses to ammonia exposure. Our results showed that survival is affected by ammonia, initially revealing dramatic decreases in survival for both populations, with the fed New Hampshire

population showing increased ammonia sensitivity. Unexpectedly, survival experiments conducted several months later showed results consistent with increased ammonia tolerance, indicated by a higher survival rate. With these recent survival results, differences in population sensitivity were no longer apparent. Despite this notable result, we can still accept our hypothesis, since we did see impairments in survival in both survival trials.

The results obtained in the study also revealed a foundational understanding of how ammonia affects the physiology and biochemical processes of the starlet sea anemone. It was also observed that enzyme activity differed across populations. For GDH activity, diet influenced GDH responses; fasted individuals from Nova Scotia displayed higher activity than New Hampshire. It was also observed that in high ammonia concentrations, Nova Scotia fasted individuals exhibited higher GDH activity. However, New Hampshire fed individuals had higher GS activity compared to Nova Scotia fed individuals. Instead of seeing an increase in GS activity as ammonia concentration increased, a decrease was displayed. Collectively, the enzyme results indicated Population x Diet effects and ammonia effects. It was also discovered that there was no ALT or AST activity detected. Interestingly, ammonia did not show to cause a consistent increase in enzyme activity. Enzyme activity was not concentration-dependent, contradicting the prediction that ammonia exposure would increase enzyme activity. However, the hypothesis that ammonia would affect enzyme activity can be accepted, since ammonia did influence enzyme activity, just not in the expected direction and magnitude.

Finally, the MO_2 data did show ammonia effects between diets within the same population. The MO_2 results also showed that fed individuals had higher MO_2, as expected; however, with higher ammonia concentrations, this pattern was no longer apparent. Both populations showed the same metabolic pattern: similar MO_2 between fed and fasted anemones with increased ammonia

concentration. With these MO_2 results, the hypothesis that ammonia would affect MO_2 can be accepted, but the metabolic results do not confirm ammonia detoxification.

Based on the overall results, we can partially accept the hypothesis that physiological responses to ammonia across populations would be similar, but ammonia tolerance would differ. The initial survival results showed differences in ammonia toxicity; however, the effect of ammonia on survival in both populations appeared to be more similar over time. Since LC estimate comparisons could not be conducted statistically, we cannot directly address ammonia tolerance and population differences, but we can suggest possible population differences in ammonia toxicity. Regarding the physiological responses to ammonia, the enzyme analysis showed that the populations differed in their enzyme responses under ammonia exposure, leading to a partial rejection of the hypothesis. But the metabolic patterns showed that the populations had similar metabolic strategies, leading to partial acceptance of this hypothesis. This was a key result in this study, since enzyme activity was consistent with the suggestion that the populations are likely using different biochemical strategies, but exhibiting similar physiological outcomes at the MO_2 level. Since ammonia excretion, glutamate and glutamine concentration, and ATP utilization were not measured, we cannot conclude that the results from the respirometry experiments or enzyme assays showed increased ammonia detoxification, as these parameters could have been influenced by various factors. Therefore, we cannot classify these results as official ammonia handling strategies, but as metabolism strategies.

A recurring theme observed in this study is that elevated responses were attributed to adaptation and/or acclimation. However, these responses could also indicate stress. This leads to a final question of whether the starlet sea anemone is coping efficiently or struggling. It has been shown in aquatic invertebrates, and similarly in this study, that under ammonia stress, the body

undergoes metabolic readjustment to meet the increasing metabolic demands caused by ammonia exposure, which ultimately increases MO_2 and oxygen consumption, which could potentially lead to reduced fitness (T. X. Zhang et al., 2023). This demonstrates that ammonia stress is energetically costly, and even though there were subtle increases in enzyme activity, for instance, it does not necessarily mean that the anemone is effectively coping, because enzyme activity is also an energy cost that may not be sustainable for the animal. To truly understand whether the anemone is coping or struggling, more research focused on longer-term ammonia exposure and even reproductive success is needed.

Overall, the results show an effect of ammonia on survival, enzyme activity, and MO_2; however, ammonia detoxification mechanisms remain inconclusive, as further experiments and parameters need to be addressed. This study also emphasized the effect of diet on these physical, physiological, and biochemical parameters. Nonetheless, this study provides valuable insight into how cnidarian and aquatic invertebrate species handle ammonia and their implications for physiological and biochemical processes.

3.2 LIMITATIONS

While this study highlighted foundational understanding of the effects of ammonia on the starlet sea anemone, there were some limitations. For instance, a limitation of this study is that the water chemistry of the collection sites for both anemone populations was not measured. Having measurements of pH, salinity, ambient ammonia concentrations, temperature, and dissolved oxygen would help interpret the differences in ammonia tolerance between the two populations. Further, these measurements would help determine whether the observed tolerance and survival differences are a result of local adaptation, acclimatization, or other environmental factors. Ammonia concentrations used in this study were nominal and were not re-measured during the 11-

day exposure. Since ammonia levels in the Petri dishes could have changed over time due to feeding, microbial activity, uptake, or water chemistry, it could have affected the results observed in this study. It is possible that the tested ammonia concentrations increased during the duration of the experiment. Additionally, no metabolites, such as glutamine, glutamate, and α-ketoglutarate, were measured, which means we could not truly assess whether the observed enzyme activity was acting in ammonia detoxification. Measuring the concentration of these metabolites will help verify the direction of GDH measured and provide more insight into the role of these enzymes in the starlet sea anemone, since they are multifunctional. This study primarily focused on enzyme activity and did not explore ammonia transporter expression (Rh-like glycoproteins, AMTs, or NKA); therefore, it is possible that some ammonia excretion or detoxification took place at the transport level. Since we did not measure nitrogen flux or excretion, we could not distinguish ammonia excretion, detoxification, or storage. These would have further implications for what specifically may be going on physiologically. Also, this study demonstrated whole-animal enzyme activity, not tissue-specific activity. Collecting information on tissue-specific enzyme activity would have helped us determine if one or more tissues were responsible for enzyme activity. Since it was averaged across the whole body, it is possible that significant enzyme responses for particular tissues were muted or suppressed. During this study, an attempt to isolate individual tissues, such as the mesenteries, for enzyme assays was made (Figure A3; Appendix C). Since the mesenteries are the primary digestive organs of the starlet sea anemone, it is possible enzyme activity would be higher in these tissues (Babonis et al., 2016). For the analysis of enzyme activity, there was low statistical power, since the sample sizes were small. Increasing sample size could alter the trends and patterns observed in this study. Based on the design of the enzyme experiments,

it is possible that ALT and AST are present in the starlet sea anemone, but based on the threshold for our assays, we could not detect very low levels of these enzymes.

3.3 FUTURE DIRECTIONS

Future directions for this study include, conducting the same experiments on wild-caught anemones (conduct experiments immediately after collection), examining mRNA expression of ammonia transporters, such as Rh-glycoproteins and AMTs, measuring ammonia excretion and metabolites, assessing MO_2 changes in response to detoxification, stress, ion regulation and/or osmoregulation, behavioural agitation, tissue repair, and altered substrate utilization, inhibiting the microbiome with antibiotics, assessing the microbiome contribution to nitrogen assimilation, using clone lines, and examining ammonia toxicity across varying salinities. To truly establish ammonia tolerance of the starlet sea anemone and advance our understanding of ammonia toxicity in aquatic environments, laboratory and field data should be compared to help with the understanding of the reactions that occur in their natural water systems (Stroud & Ratcliff, 2025; Yun et al., 2026). The threshold for ammonia tolerance may differ between anemones in a controlled environment versus the wild. The present study focused on enzymatic responses, but did not assess ammonia transport pathways, despite evidence that Rh-like glycoproteins and AMTs may contribute substantially to ammonia handling in the starlet sea anemone (Andrikou et al., 2019). This would be beneficial to examine as a possible mechanism for ammonia detoxification. Alongside measuring mRNA expression of ammonia transporters using quantitative polymerase chain reaction (qPCR), ammonia excretion after ammonia exposure can also be measured to verify the excretion of ammonia and possibly validate the results from the enzyme assays or qPCRs, while providing more insight into the energy requirements, aligning with the purpose of measuring MO_2 (Lemos et al., 2003). Measuring metabolites will also help validate the role of GDH and GS and determine

whether ammonia detoxification took place. As previously mentioned, detoxification, stress, ion regulation and/or osmoregulation, behavioural agitation, tissue repair, and altered substrate utilization are alternative explanations for increases in MO_2; therefore, it will be of benefit to examine MO_2 responses to these factors, by exposing them to different stressors that would impact detoxification, ion and water regulation, locomotion or tentacle movement, and substrates associated with repair pathways. Due to the importance of symbiotic relationships and the microbial community in cnidarians, a comparative study between cnidarians with zooxanthellae and the starlet sea anemone can be conducted to assess the role of these microbes. Also, inhibiting the anemone's microbiome using antibiotics and then reassessing the parameters in this study will help establish whether the physiological responses to ammonia were driven directly by the cnidarian or by the microbial partner. Assessing the influence of the microbiome is integral to understanding how a cnidarian species tolerates ammonia and manages nitrogen assimilation, since the microbial community of cnidarians is essential for maintaining their health and survival in their environments. Using clone lines from each population would help control for genetic variation in each population to see if genotype drives ammonia tolerance and physiological responses to ammonia. A final proposed future direction is investigating ammonia toxicity across salinity levels. Since ammonia toxicity depends on pH, salinity, and temperature, it would be beneficial to analyze ammonia toxicity under different ranges of these parameters. More importantly, a recent study in the giant freshwater prawn (*Macrobrachium rosenbergii*) has shown that increasing salinity and ammonia causes significant increases in MO_2 (Emadi et al., 2026). Since this study kept salinity constant (18 p.p.t.), it would be interesting to see if results obtained in this study would differ based on varying salinity.

In conclusion, this study examined nitrogen metabolism in an asymbiotic cnidarian species, creating a foundation that would help us understand ammonia toxicity in aquatic invertebrates and will have further implications for how ammonia levels are assessed and managed in our water systems. This study establishes *Nematostella vectensis* as a promising model for investigating ammonia tolerance in asymbiotic cnidarians and sets the stage for a range of future experiments.

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Appendix

Appendix A: Supplementary Survival Figures

To calculate the lethal concentrations (LCs). The survival data were imported into R to compute mortality proportions for each ammonia concentration. Data from Day 11 of the survival experiment were analyzed with a two-parameter log-logistic dose-response model, using the package *drc*. The model used a binomial error distribution and the total number of individuals per treatment. Effective dose (ED) values corresponding to 10%, 25%, 50%, and 75% mortality (LC_{10}, LC_{25}, LC_{50}, and LC_{75}, respectively) were estimated using the fitted model. The predicted mortality probabilities were generated across the concentration range and plotted with the observed survival data from this study.

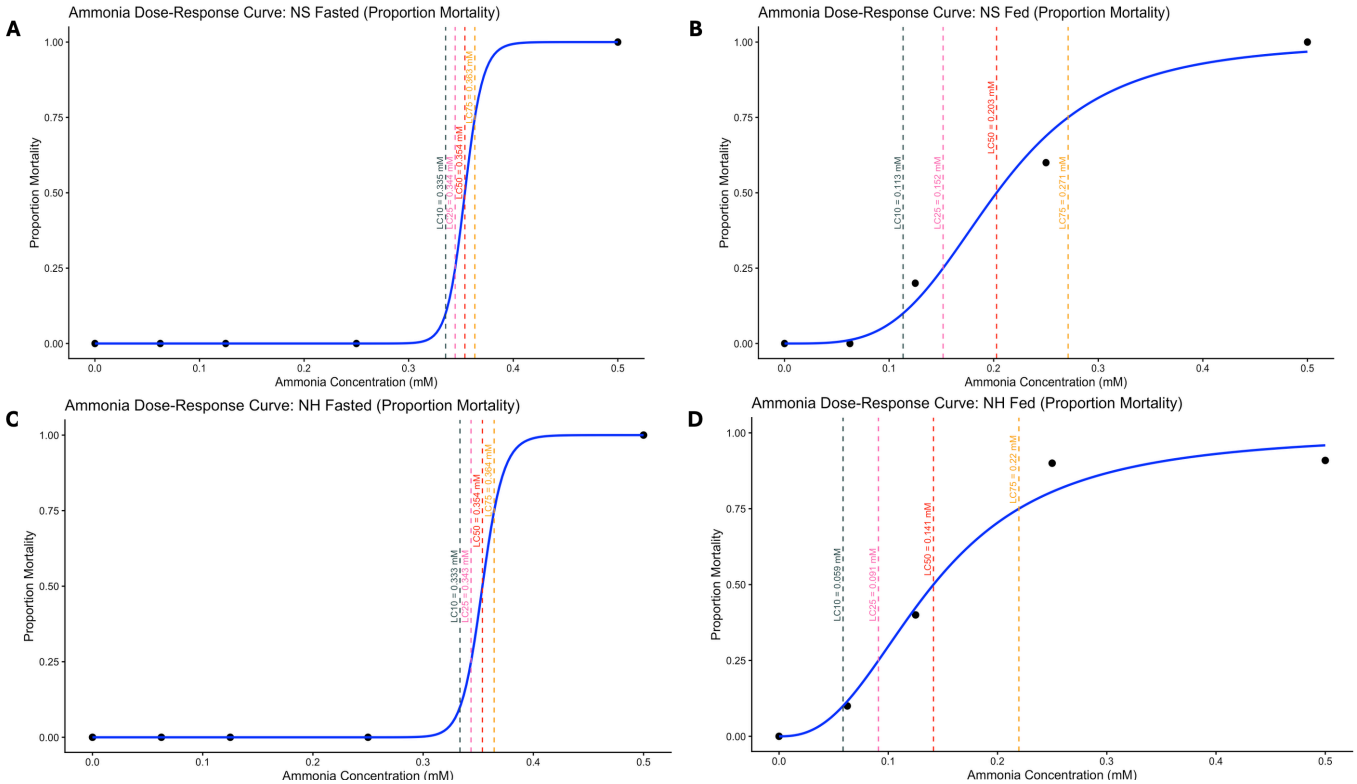

Figure A1. Ammonia dose-response curves (proportion mortality). To calculate the lethal concentration (LC) values, a dose-response relationship between ammonia concentration and proportion mortality on Day 11 for fasted and fed individuals of both populations [Nova Scotia (NS, **A** and **B**) and New Hampshire (NH, **C** and **D**)] was calculated. Points represent the observed mortality proportions, and the solid blue line represents the log-logistic model. The dashed vertical lines indicate estimated LC_{10}, LC_{25}, LC_{50}, and LC_{75} (10%, 25%, 50%, and 75% mortality, respectively).

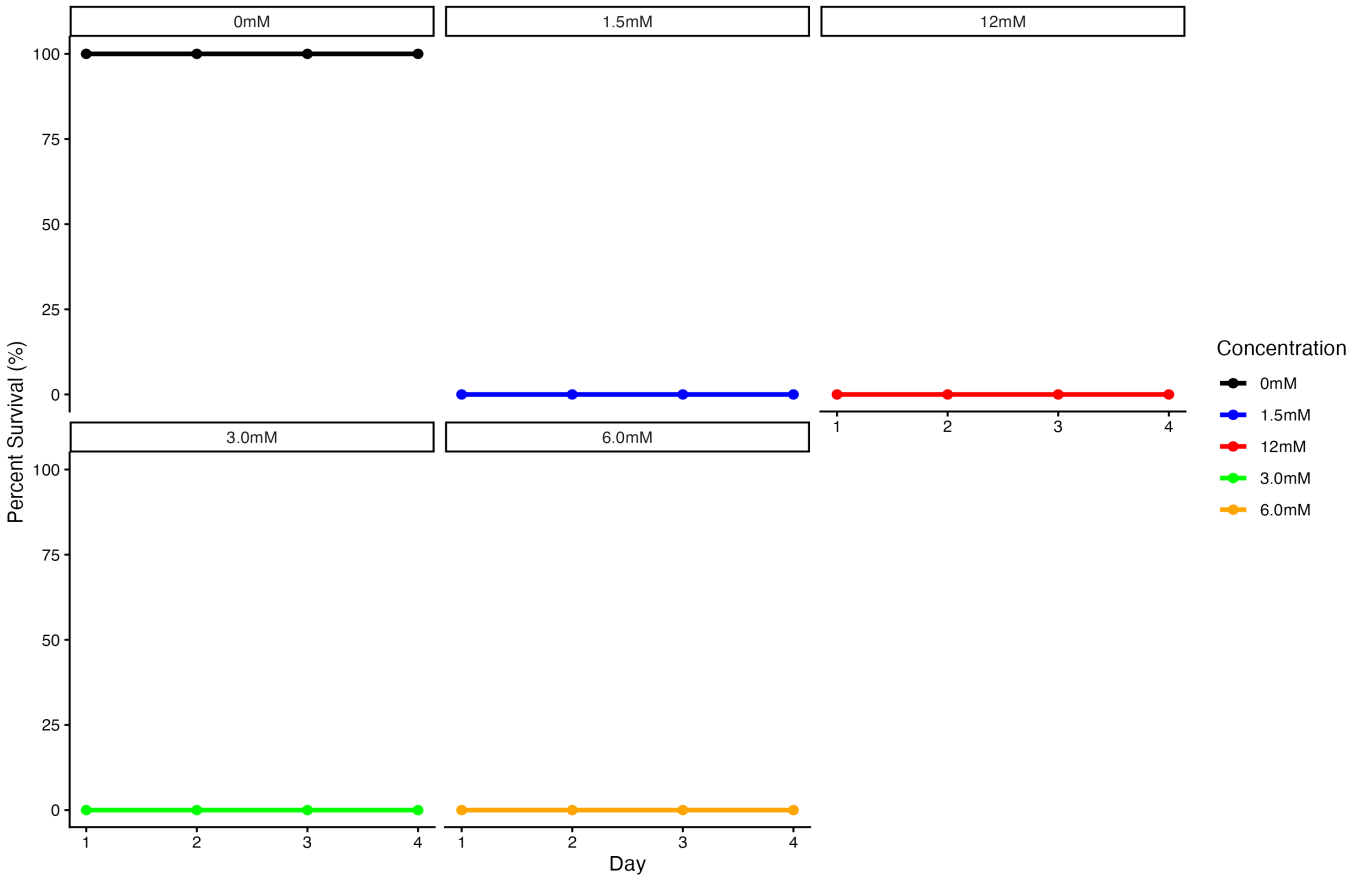

Figure A2. Anemone survival above 0.5 mM NH_4Cl. Since preliminary research from our lab suggested the anemones were tolerant of high ammonia levels (~3 mM), pilot survival experiments were conducted for this study. Ammonia concentrations of 1.5, 3.0, 6.0, and 12 mM were used to assess anemone survival within 96 hours (4 days). It was observed that 100% mortality occurred within 24 hours of exposure. 0 mM = control (18 p.pt. ASW) and n = 5 anemones per concentration.

Appendix B: Metabolic Rate Calculations

R code for respirometry calculations of MO_2:

To calculate MO_2 from the data provided from the Loligo respirometry software, a respirometry package in R was used:

R Code:

```
library(respirometry)

well_names <- colnames(O2_data_0_unfed)[-1]  #exclude Time column

bin_width <- 5

o2_unit <- "percent_a.s."

vol <- 0.0017

temp <- 25

sal <- 18

atm_pres <- 965

duration <- as.numeric(O2_data_0_unfed$Time)
```

Birk, M. A. (2025). *respirometry: Tools for conducting and analyzing respirometry experiments* (R package version 2.0.2). Comprehensive R Archive Network. https://CRAN.R-project.org/package=respirometry

Equations for MO_2 calculations:

Mass-specific MO_2:

$$\textit{Mass-specific } MO_2 = MO_2 \textit{ of anemone} / \textit{Mass of anemone (wet mass)}$$

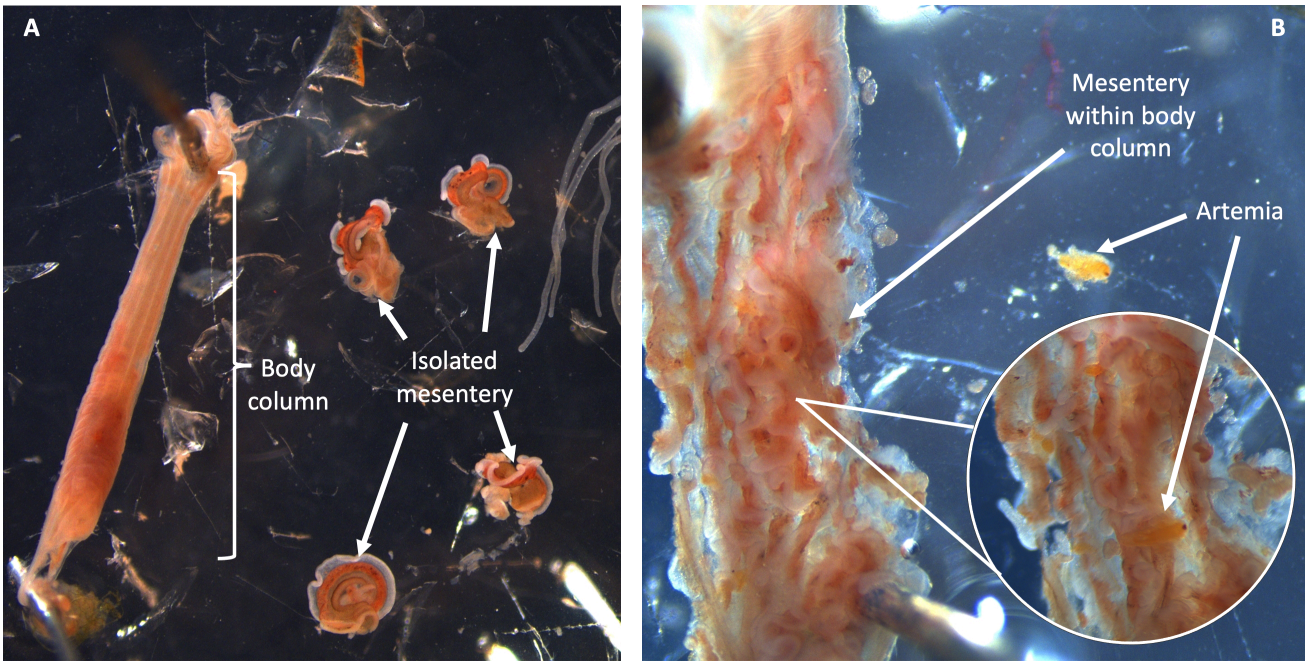

Figure A3. Isolation of *N. vectensis* mesenteries. As an attempt to conduct tissue-specific enzyme assays, the mesenteries, the primary digestive organs, of the anemone were dissected. Individual mesenteries were isolated from the body column after the removal of the physa and tentacles **(A)**. To confirm the site of digestion, the body column was cut vertically to expose the mesenteries, where *Artemia* was almost fully digested **(B)**.