Respiratory Physiology of *Neomyxine biniplicata*, the Slender Hagfish

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Abstract

Neomyxine biniplicata is one of the 79 currently described species of Myxinidae and one of only six species endemic to the waters about the east coast of New Zealand. Whilst perhaps not everyone's favourite animal species, hagfish are of great benefit to the ocean ecology and recycle decaying matter and return it to the ocean floor as nutrients. Also, hagfish form part of the diet of fish and ocean dwelling mammals that are not deterred by the slimy emissions hagfish use, to such great effect, to defend themselves. There are two sub-families of Myxinidae; the Eptatretinae (eptatretids) and the Myxininae (myxinids), and each occupy different ecological niches. Species of eptatretids are generally found on the seafloor whereas myxinids are specialist burrowers. Adaptation to burrowing can be seen in the external respiratory anatomy of the myxinids which exhibit two external apertures compared to the condition of the eptatretids where each gill is evacuated by a dedicated efferent duct. Although both eptatretids and myxinids experience conditions of hypoxia and anoxia, myxinids are considered better adapted to these conditions and, of the hagfish species examined to date, exhibit a lower critical pressure of oxygen (P_{crit}) or the point at which oxygen consumption ceases to be independent of dissolved environmental oxygen. When subjected to closed-system respirometry, specimens of *N. biniplicata* were confirmed to have a P_{crit} of 27mmHg although this was dependent upon environmental temperature and experimental method. The fish were not distressed by conditions of severe hypoxia, in contrast to *Eptatretus cirrhatus* which were examined in an earlier set of experiments and found to have a P_{crit} of 50mmHg at which point the fish became highly agitated. At an oxygen tension of 20mmHg, *N. biniplicata* exhibited no discomfort or desire to escape and merely lay quietly. However, when confronted with acute temperature alterations that were beyond the normal thermal range *N. biniplicata* reacted with agitation and escape behaviour. The ventilatory rate increased exponentially at temperatures above 12 °C and slowed in response to immersion in water cooler than 10 °C although the

thermal range differed in two seasonal populations examined as did the physical condition of each population. Observations of *N. biniplicata* in captivity revealed the species to be social in habit and sensitive to light, sound and vibration. The social habit of the fish influenced the results obtained during experiments and this must be considered when planning further experiments. *N. biniplicata* is a species adapted to a hypoxic environment but are very sensitive to temperature outside a narrow thermal range. Seasonal migration is a possibility.

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Thank you, Bill, for taking me into your physiology lab – full of kit and gubbins, all waiting to be fiddled with, and from which exciting experiments can be built. Thank you for bearing with my strange ideas and gently shoving me off in the right direction, for putting up with me wandering into your office waving spare bits of hagfish and for wading through the drafts. Thank you for your sage advice, without which this would be just a tottering and incoherent pile of words.

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Chapter One

Introduction

1.1 Family Myxinidae

Over 200 years has passed since western natural philosophers discovered, and were confounded by hagfish, which, to this day, continue to perplex and fascinate. The Atlantic hagfish was first examined by Kalm in 753, but it was Linnaeus (1754) who named the fish *Myxine glutinosa* for its uninhibitedly generous release of sticky slime, literally, "slime glue" from which derives the family name: Myxinidae.

Initially Linnaeus classified hagfish as a type of worm (*Vermes intesta*). In due course, the Swedish professor of natural history, Retzius and Abilgaard, the Danish zoologist recognised hagfish as a species of fish similar in appearance to the lamprey. Johannes Müller, the German anatomist, reclassified hagfish, alongside the lamprey, as both a vertebrate and cyclostome (round mouth). The two members of the cyclostome class were categorised by their mouthparts; the lamprey as Hyperoartia (whole palate) and the hagfish, which were described as Hypoartia or perforated palate. Cyclostomes are also classified as Agnatha – without jaws – as opposed to the gnathostomes that exhibit jaws derived from gill arches. Hagfish retain the notochord, which in gnathostomes is replaced by the vertebral column.

Myxinidae are basal vertebrates very distantly related to humans. The anatomy and physiology of hagfish is specialised and distinctive and quite removed from that of other vertebrates, and despite decades of research by dedicated hagfish enthusiasts, so much of the life history of hagfish remains unknown (Fänge, 1998; Martini, 1998a; Pough, Janis, & Heiser, 2013).

Neomyxine biniplicata is a member of the ancient family Myxinidae, the ancestors of which emerged in the Cambrian Period. Throughout the Cambrian, land masses

travelled about the earth, and the composition of the atmosphere and the climate vacillated. Hagfish adapted and evolved and now have existed, anatomically unchanged, within the oceans for 300 million years (Bardack, 1998; Fernholm, 1998; Cavalcanti & Gallo, 2008; Donoghue & Keating, 2014). Myxinidae are more ancient than the coelacanth and inhabited the oceans in their current physical form for some 100 million years before the arrival of the dinosaurs in the Triassic Period (Martini, 1998a; Knapp et al., 2011; Steyer, 2012; Langer, Ramezani & Da Rosa, 2018).

1.2 The Palaeozoic Era

The Palaeozoic Era, which lasted for most of the Phanerozoic Aeon, began 540 million years ago (mya) with the Cambrian and ended 252 mya with the Permian. Throughout the Palaeozoic, emerging species disappeared through periodic episodes of mass extinction caused by climatic and geological events. Tectonic plate movement caused land to form and dissociate again and the concentration of atmospheric carbon dioxide and oxygen rose and fell, while simultaneously, the climate warmed and cooled (Ilves & Randall, 2007; Pough et al., 2013). Atmospheric oxygen rose to its highest level during the Carboniferous while levels of carbon dioxide slowly decreased during the Cambrian, Ordovician and Silurian, rising again in the Cretaceous and Jurassic (Dudley, 1998; McCallum, 2015). Glaciation was followed by ice ages that occurred throughout the Devonian, Carboniferous and Permian Periods whilst incoming asteroids and volcanic activity in the Siberian marked the Permian and Triassic boundary. During the Cambrian, Ordovician and Devonian Periods, aquatic species multiplied and diversified only to be extinguished in episodic periods of mass extinctions. By end of the Permian 95% of marine species, and other forms of life, had been extinguished (Ilves & Randall, 2007; Pough et al., 2013). The mass extinction of life that occurred during the Permian allowed new species to evolve but throughout, hagfish endured. During the Silurian Era - 460 mya - the first vertebrates and the ancestors of modern hagfish and all other

vertebrates, the Ostracoderms arose (Forey & Janvier, 1994; Steyer, 2012; Guilbaud et al, 2018).

1.3 The Ostracoderms

The forebears of modern cyclostomes and gnathostomes were the Ostracoderms (Forey & Janvier, 1994; Donoghue & Keating, 2014; Janvier & Sansom, 2016). Ostracoderms, now long extinct, were small armour-plated fish and all specimens that have been unearthed to date were without jaws or paired fins. The mouth was round and contained strange moveable plates which have no analogue in any extant life form. The reason so many have been preserved is that the carapace is formed of dermal bone that has preserved the form of the soft body within (Pough et al.,2013). Many were of extraordinary design but still exhibited various vertebrate characteristics such as a calcified skeleton. Anaspid-like agnathans conform to the cyclostome pattern and some were more derived than modern cyclostomes (Forey & Janvier, 1993; Donoghue & Keating, 2014). The Ostracoderms form a paraphyletic collection of taxa and it is likely that the hagfishes and the lampreys descend from their own Ostracoderm ancestors (Janvier & Sansom, 2016).

1.4 Early vertebrate evolution and relationships

Two groups of vertebrates emerged from the Paleozoic Age: the cyclostomes and the gnathostomes (Forey & Janvier, 1994; Donoghue, Smith & Sansom, 2004). The cyclostomes or jawless Agatha comprise the Myxinidae and Petromyzontids, or the hagfish and the lampreys respectively (Donoghue & Keating, 2014). The gnathostomes developed jaws and this group includes the cartilaginous and bony fish and *Homo sapiens*. Myxinidae deviated from the main vertebrate line, the gnathostomes, over 500 million years ago and are the only surviving members of the class Pteraspidomorphi, the first vertebrates with a mineralised dermal skeleton (Forey & Janvier, 1994; Powell, Kavanaugh, & Sower, 2005; Bardack, 1998; Blair & Hedges, 2005; Near, 2009; Sills & Palermo, 2013; Donoghue & Keating, 2014;

Sugahara et al., 2016). The evolutionary relationship to later, evolved vertebrates is illustrated in Figure 1.1.

The relationship between the cyclostomes and gnathostomes has been investigated and intensely debated for 200 years (Janvier, 1996). It reduces to whether hagfish are more closely related to lampreys (the cyclostome monophyly) or whether gnathostomes descend more directly from either (the cyclostome paraphyly). The prevailing theory alters as new discoveries, both archaeological and genetic, emerge (Donoghue, 2014; Brazeau & Friedmann, 2015). The phylogenetic relationship between the cyclostomes is confused by the anatomical alterations that each family evolved to accommodate adaptation to their ecological niche (Ota & Kuratani, 2007). The difficulty constructing the phylogenetic relationship between cyclostomes and gnathostomes is also compounded, because living cyclostomes do not have a mineralised skeleton and preserved soft-bodied fossils are exceedingly rare (Janvier & Sansom, 2016).

Morphological cladistic analysis places lampreys and gnathostomes as closest relatives (Romer, 1966; Forey & Janvier, 1993, Janvier, 2007a; Near, 2009), whilst molecular phylogenetic investigations have positioned hagfish and lampreys together in a monophyly of cyclostomes (Kuratani & Ota, 2008; Heimberg, Cowper-Sal-lari, Sémon, Donoghue, & Peterson, 2010). Although molecular data supports a monophyly between the hagfish and lampreys, expression profiles of micro-RNA suggest the last common ancestor of cyclostomes and gnathostomes may have been more anatomically complex than modern cyclostomes. This was the premise put forward by Stensiö: that early cyclostomes had paired fins and a skeleton constructed of bone, as seen in the earliest jawed vertebrates (Stensiö, 1927; Janvier, 2010).

To understand the ancestral state and early evolutionary process of the vertebrates the RIKEN Evolutionary Morphology Laboratory has devised a novel method of procuring the embryos of the Japanese hagfish *E. burgeri* (Oisi, Kakitani, Kuratani, &

Ota, 2015). These embryos are used in comparative analysis of embryonic gene expression patterns.

Work on the hagfish embryos has added an additional layer of complexity to the debate over phylogeny between the cyclostomes and gnathostomes, because the embryonic hagfish brain has been interpreted as being closer in design to the gnathostome group than to that of the lamprey (Sugahara et al., 2016). The prevailing hypothesis for many years has been that lamprey and gnathostomes are more closely related (Forey & Janvier, 1994). In hagfish, as with all other living vertebrates, the neural crest cells delaminate and migrate, and embryological and gene expression analysis has shown that hagfish and gnathostomes share conserved developmental chondrogenesis of the vertebral elements (Holland & Chen, 2001; Ota & Kuratani, 2007; Ota, Fujimoto, Oisi, & Kuratani, 2013). Comparison of brain regionalisation in lamprey larvae and hagfish embryos indicate there was no convergent evolution between hagfish and gnathostomes (Sugahara et al., 2016).

Examination of pharyngula-stage hagfish embryos has revealed features that were present in a common ancestor of all the vertebrates (Ota, Kuraku, & Kuratani, 2007). Also, histological, and molecular investigations of *Epatatretus burgeri* embryos demonstrate the expression of regulatory genes, analogous to those synthesised in gnathostomes: *Pax*6, *Pax*3/7, *Sox*Ea and *Sox*9. Synthesis of these genes indicate the hagfish neural crest is programmed by molecular processes that are specific to vertebrates and indicate that divergence from the main vertebrate lineage occurred in the Cambrian Period (Ota, Kuraku & Kuratani, 2007). Comparison of the embryos of the Japanese inshore hagfish, *E. burgeri*; the Japanese lamprey, *L. japonicum;* and the adult catshark, *S. torzame* by molecular phylogenetic and synteny conservation analyses has established that the structure of the gnathostome embryonic brain is shared with hagfish but not the lamprey. In jawed vertebrates the medial ganglion eminence develops from the frontal section of the neural tube in an area called the

rhombic lip that expresses *Pax*6 and this region is clearly present in hagfish (Sugahara et al., 2016).

The anatomical divisions seen in brains of crown gnathostomes is not a modern development and dates to the last vertebrate ancestor before divergence of the cyclostomes and gnathostomes 500 mya (Sugahara et al., 2016). Morphological comparative analysis of the Middle Devonian Ostracoderm, *Palaeospondylus gunni*, with extant *E. burgeri* embryos has shown homologous skeletal parts and a vertebral column. The craniofacial development does not follow gnathostome patterning and *P. gunni* is probably a stem hagfish from the Middle Devonian (393–383 Ma). (Hirasawa, Oisi, & Kuratani, 2016).

In the meantime, and until further fossilised evidence is unearthed, the phylogenetic relationship between the cyclostomes and the gnathostomes remains unresolved (Brazeau & Friedmann, 2015).

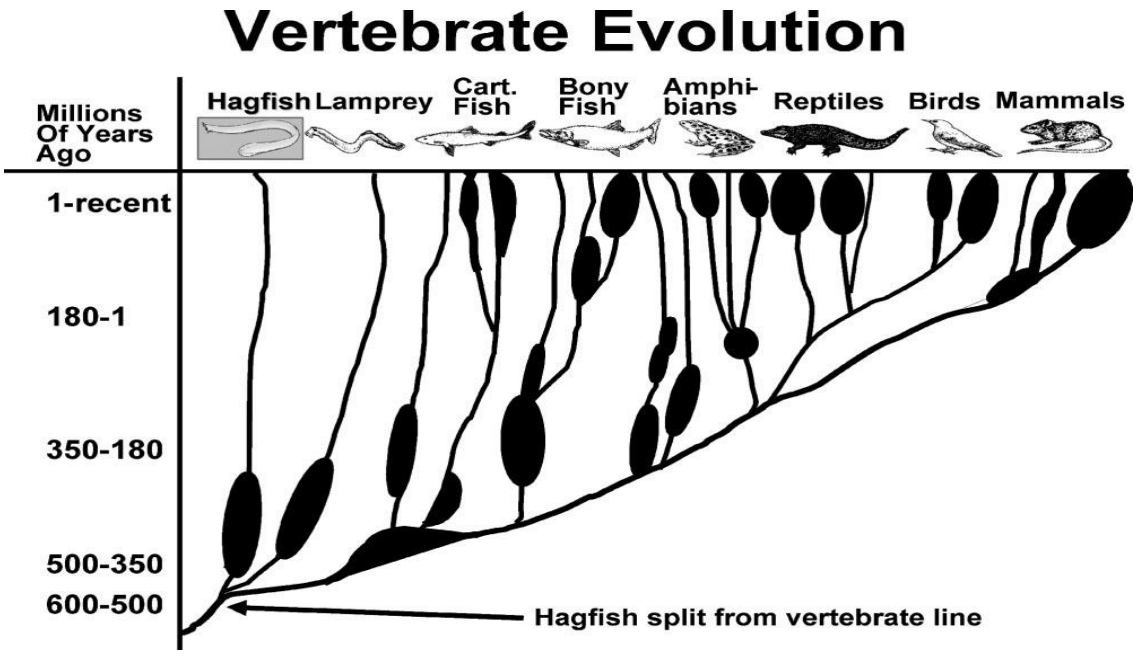

Fig. 1.1. Schematic diagram of evolutionary relationship of hagfish to later evolved vertebrates. From: Powell, Kavanaugh, & Sower (2005).

1.5 Sub-families of Myxinidae

There are two sub-families of Myxinidae, Eptatretinae (eptatretids) and Myxininae (myxinids), and representatives of both occupy the waters about New Zealand (Roberts, Stewart, & Struthers, 2015). The anatomical characteristic that separates the Myxinidae into the sub-families are the efferent branchial ducts that evacuate water from the gills. The inter-familial difference reflects the ecological niche each occupies (Adam & Strahan, 1963; Fernholm, 1998). The non-burrowing Eptatretinae inhabit substrates of gravel and rock and may be seen curled up on ridges, whereas species of Myxininae burrow beneath the soft benthic ooze where they may lie without ventilating for hours at a time (Strahan, 1958; Honma, 1998; Currie & Edwards, 2010). In eptatretids each gill is evacuated by its own efferent duct whereas in myxinids the gills empty into a common duct which is evacuated by two bilateral external apertures. The gill anatomy of the eptatretids is considered to be the less evolved state (Cole, 1913; Fernholm, 1998; Martini, 1998b; Zintzen et al., 2015).

1.6 Myxinidae of New Zealand

Currently there are six genera of Myxinidae worldwide. Of the 79 described species six, from both sub-families, are endemic to waters around New Zealand. *Eptatratus cirrhatus* is found in the south east of Australian waters (Knapp et al., 2011; Martini & Beulig, 2013; Roberts et al., 2015). *Neomyxine biniplicata* is a species of Myxininae (Richardson, 1953; Zintzen et al., 2015). The genus *Neomyxine* was first described in 1951 and *Neomyxine biniplicata* was discovered in 1953 (Richardson & Jowett, 1951; Richardson, 1953; Zintzen et al., 2015). A specimen of *N. biniplicata* in captivity is illustrated in Figure 1.2.

Fig. 1.2. Specimen of *Neomyxine biniplicata* in captivity.

1.7 Myxinidae: ecology, commerce and conservation

All hagfish described so far are marine species that inhabit the seabeds of temperate oceans where they are important constituents of benthic communities and in commercially unfished regions are vast in number (Cole, 1913; Fernholm, 1998; Martini, 1998b; Møller & Jones, 2007). As well as being included in the diet of marine mammals and invertebrates that are undeterred by the slime, hagfish are essential members of the contingent of housekeepers that keep the seabed clear of rotting detritus (Martini, 1998b). Hagfish consume dead and decaying matter including tonnes of by-catch discarded by commercial trawlers (Dayton & Hessler, 1972; Lesser, Martini, & Heiser, 1996; Zintzen, et al., 2011). *Eptatretus hexatrema*, a species of hagfish that lives in the Southern Atlantic Ocean, is found to be especially numerous in areas close to fish-processing plants (Kench, 1989).

Hagfish are important to the ecology of the ocean, recycling nutrients from decaying matter and returning these to the sea floor through burrowing (Martini 1998b; St

Martin, 2001). Decreases in commercial flounder catches have been reported after hagfishing began on an industrial scale in the north-western Atlantic Ocean. It may be that the benthic ecosystem can no longer support some fish populations if deprived of hagfish and their beneficial environmental services (Martini 1998a; St Martin, 2001; Gorbmann, Kobayashi, Honma, & Matsuyama, 1990; NEFSC 2003; Ellis, Rowe, & Lotze, 2015).

Hagfish have become a commercially valuable species and are sought for their skin, meat, and slime, which is used as a cleaning agent (Knapp et al., 2011). Since the 1980s global catches of finned fish have declined and interest has turned to lower trophic level species such as herring and sardine (*Clupeidae*) and hagfishes (Anderson, Flemming, Watson, Lotze, 2011). The earliest commercial fishing of hagfish began in Japan during WWII and by the 1980s, through uncontrolled fishing, the number of hagfishes caught was much reduced, and this situation was repeated in Korean waters during the 1990s (Gorbmann et al., 1990).

In the Northern Atlantic there are eight hagfishing regions supplying the Asian market landing up to 10 million kg of Atlantic hagfish annually, which are harvested off the coast of Maine and Massachusetts (Gorbmann et al, 1990; Powell et al., 2005). Hagfish catches in California, British Columbia, Maine and Massachusetts have peaked with very low catches or none landed at all (St Martin, 2001). Since 2001 hagfishing enterprises have developed in New Zealand (Martin & Beulig, 2013), and large increases in numbers caught have been reported with the result that hagfishing enterprises are now expanding southwards, although catches have declined since 2010 (Ellis et al, 2015). In New Zealand waters no distinction between species of hagfish is made by the Ministry for Primary Industries (MPI), and unlimited catches are allowed under a general commercial fishing permit (Zintzen et al., 2015; MPI). *N. biniplicata* has been listed as a by-catch of commercial trawling and is probably vulnerable in both shallow and deep waters (Mincarone, 2013). Hagfish that are brought to the surface as by-catch do not survive because of the rapid alteration in

salinity and temperature (Lesser et al., 1996), and trawling degrades their habitat, threatening hagfish populations (Knapp et al., 2011).

To manage and conserve the hagfish, information on their life cycle, feeding habits and ecology will be critical to the regulation and development of sustainable fishing practice (Anderson et al., 2011; Knapp et al.,2011). Research into hagfish reproduction suggests that hagfish growth is slow, about 2 cm annually, and possibly a period of up to 13 years is required for an Atlantic hagfish to reach sexual maturity which happens at a length of 36 cm (van der Meer & Kooijman, 2014).

1.8 Population trend of Neomyxine biniplicata

The population trend is unknown but *Neomyxine biniplicata* is an endemic species with a restricted range. Data of population numbers does not exist and there are no conservation measures in place (Mincarone, 2013). Zintzen et al., (2015) records that only six specimens of *N. biniplicata* were caught in Kaikoura and none at sites around White Island during preparations for their paper on morphometrics of New Zealand hagfish.

For this research 6 fishing expeditions were required to procure 20 specimens and no more than 5 fish were caught at any one time. Whether the fish escaped the traps, had moved from areas in which they had previously been caught, or population numbers are low is not known. Lyttleton Harbour is currently being dredged and deposits are being discarded in the region off Pigeon Bay Heads where *N. biniplicata* were seen previously in large numbers (R. Bishop, personal communication, 2018). The dumping of the sediments may have obscured and polluted the sea bed in that area.

1.9 General characteristics of Myxinidae anatomy

In works devoted to the anatomy and physiology of Myxinidae words such as peculiar and unique are often used as adjectives.

Hagfish, often mistaken for eels, are long, thin, soft-skinned marine vertebrates (Hardisty, 1979). All hagfish have one funnel-shaped nostril or nasopharyngeal opening. The nostril and mouth are surrounded by three (Richardson, 1951; Zintzen et al., 2015) or four pairs of sensory barbels (tentacles) (Dawson, 1963; Mok, 2001). The dental apparatus of hagfish, whilst very efficient, is exceptionally odd. Hagfish have one internal palatine tooth and two scythe-shaped keratin dental plates that support two rows of lingual teeth. The dental plates rest concealed within the oral cavity and protract during feeding (Dawson, 1963). The hagfish bite force is equal to that of a turtle (Zintzen et al., 2011).

In eptatretids vestigial optical tissue is situated beneath a layer of skin, whilst in myxinids it is further concealed under a layer of muscle. (Strahan, 1963a; Locket & Jørgensen, 1998). Interpretations on the state of the eyes of Myxinidae differ. Hagfish eyes may demonstrate an earlier stage of vertebrate evolution (Collin & Lamb, 2016) or are examples of the evolutionary trend that eliminates the eyes of cave-dwelling or burrowing animals, and optical degeneration is an adaptation to life in deep water (Ross, 1963; Hardisty, 1979; Locket & Jørgensen, 1998). Heimberg et al. (2010) regard the eyes of the Myxinidae as being degenerate and regressed.

Even so, hagfish eyes remain sensitive to light, which may be important in maintaining circadian and diurnal rhythms. Photoreceptors are located within the skin of hagfish, particularly about the nose and around the cloaca (Newth & Ross, 1954). Species of eptatretids and myxinids held in aquaria have shown nocturnal activity (Gustafsen, 1935; Fernholm, 1974; Kabasawa, Ooka-Souda, & Takashima, 1993; Collin & Lamb, 2016). In the present study *N. biniplicata* were active between 9 pm and 4 am.

The inner ear of the hagfish is simple, consisting of single and bilateral semicircular canals containing three sensory epithelia and hair cells. The orientation of the hair cells senses rotational movements of the body and the surrounding environment (Jørgensen, 1998; McVean, 1998). Ross (1963) thought it likely that hagfish possessed

very poor auditory capacities, although it became obvious in the present study, during both ventilatory and hypoxia experiments, that *N. biniplicata* hear very well and react, even to a quietly spoken human voice, with an instantaneous but unsustained burst of hyperventilation.

The olfactory system in the hagfish is well developed, and hagfish possess a very acute sense of smell (Jensen, 1966). The olfactory organ, large in hagfish, is directly exposed to water entering the nasopharyngeal duct (Døving, 1998). Schreiner organs are sensory receptors located in hagfish skin and are particularly concentrated on the tentacles, nose and head but also present along the body and tail. It is likely that Schreiner organs may also sense chemical stimuli but do not seem to be involved in electro-receptivity (Braun & Northcutt, 1998; Collin, 2007). The tentacles of Myxinidae are exquisitely sensitive to physical contact (Greene, 1925).

Slime is the primary weapon of the hagfish. Different species possess varying numbers of slime pores, which are situated along both sides of the body between the head and tail (Spitzer & Koch, 1998; Zintzen et al., 2015). The exudate fibres combine with seawater to form a fibrous slime that is unlikely to be toxic but incapacitates gill-breathing fish, thereby decreasing competition for food and deterring other predators (Zintzen et al., 2011; Herr, Clifford, Goss, & Fudge, 2014). Slime glands can be deployed at will to repel and disable potential predators in a rapidly ejected deluge (Strahan, 1963a; Martini, 1998b). It may be that slime is important in hagfish innate immunity and protects against colonisation by pathogens and parasites (Zintzen et al., 2011).

Distinctive amongst most other marine vertebrates, modern hagfish maintain the invertebrate trait of conforming to their environment both osmotically and ionically (Robertson, 1963). The blood plasma of hagfish is very similar to that of marine invertebrates and may represent the evolutionary transition between invertebrates and vertebrates (Holland & Chen, 2001; Clifford, Zimmer, Wood, & Goss, 2015). The osmotic concentration of seawater (mOsm) is between 1000 mOsm and 1150 mOsm

and the blood plasma concentration of Myxinidae is 1062 mOsm (Fänge, 1998). Unlike most marine fish, which are hypo-osmotic to seawater and must regulate both osmotically and ionically to maintain homeostasis, hagfish need not expend energy osmo-regulating, and this contributes to the very low basal metabolic rates maintained by the animals (Munz & Morris, 1965; Steffensen, Johansen, Sindberg, Sørensen, & Møller, 1984; Forster, 1990). Myxinidae have been shown to be unable to tolerate conditions of low salinity and if placed into water below 25‰ will exude a deluge of slime, become comatose and die (Gustafsen, 1935; Johansen, 1963).

Myxinidae do not exhibit sexual dimorphism nor any apparent primary sex organs (Strahan & Honma, 1960; Hardisty, 1979; Bardack, 1998; Patzner, 1998), although Jensen (1966) reported that female *E. stoutii* were larger than the males of the species.

Over the course of millennia, the anatomy of Myxinidae has evolved as the animals adapted to benthic conditions and hagfish progressed to a form that had no further use of external eyes, vertebrae, and the lateral line, although embryological hagfish retain a vestigial lateral line (Wicht & Tusch, 1998; Ota & Kuratani, 2007; Janvier, 2007a; Kuraku, Meyer, & Kuratani, 2008; Kuratani & Ota, 2008; Ota et al., 2013).

1.10 Oxygen in the marine environment

Free unbonded oxygen dissolved in water is used by Myxinidae and other marine organisms that respire aerobically to power metabolism (Fry, 1947; Nelson, 2016). Oxygen enters seawater via the air or as a photosynthetic by-product of aquatic plants, algae and phytoplankton.

In marine environments, where the water is open and unstratified, oxygen saturation will be 100% of air saturation, where the water contains dissolved gas molecules in equilibrium. The percentage of each gas in the water is equal to the percentage in the atmosphere or the partial pressure (vanLoon & Duffy, 2007). For several reasons, oxygen saturation is often reduced in the marine environment. Oxygen minimum zones (OMZ) often occur on continental shelves that extend

around land masses. Here, through the natural contours of the seabed, oxygen saturation is often reduced at depths between 400 m and 1000 m. In unmixed water and flocculant matter floating above benthic sediments and through microbial respiration, hypoxic and anoxic conditions will exist (Childress & Seibel, 1998; Seibel, Schneider, Kaartvedt, Wishner, & Daly, 2016). The natural layering of water that comes about through thermoclines, haloclines and pycnoclines are causes of lower oxygen saturation (Breitburg, Hondorp, Daias, & Diaz, 2009). Oxygen minimum zones occur in the mesopelagic zone at between 200 m and 1000 m in depth and in aphotic zones below 1000 m where light does not penetrate, and photosynthesis cannot occur. The activities of humans are also a cause of OMZ, and both anthropogenic and natural OMZs are expanding (Seibel et al., 2016).

The oxygen concentration of sea water varies according to the temperature of the water (Claireaux & Chabot, 2016). The partial pressure of oxygen (PO_2) in seawater is inversely correlated with temperature and is affected by salinity. In seawater with a salinity of 35‰, oxygen concentration at 0 °C is 11mg kg^{-1}, 9 mg kg^{-1} at 10 °C reducing to 7 mg kg^{-1} at 20 °C (Fry, 1947; Fry, 1971; vanLoon & Duffy, 2007; Farrell & Richards, 2009).

Hypoxia is the low partial pressure of oxygen and it has no precisely defined reference point. For aquatic ectotherms, severely hypoxic conditions lie between a PO_2 of between 10 mmHg and 30 mmHg with anoxia being the complete absence of oxygen (Malte & Lomhalt, 1979; Seibel et al., 2016). The critical PO_2 (P_{crit}) defines the point at which an organism's oxygen consumption is no longer independent of oxygen tension and anaerobic metabolism intervenes (Seibel et al., 2016). A low P_{crit} predicts a greater tolerance to hypoxia as it indicates improved oxygen uptake and transport to the tissues in low oxygen conditions (Speers-Roesch, Mandic, Groom, & Richards, 2013).

1.11 Adaptation to hypoxia and anoxia

Myxinids and eptatretids occupy different substrates within the benthic landscape with eptatretids resting on the substrate and myxinids burrowing within (Strahan, 1958; Strahan, 1963b; Fernholm, 1998). Through their burrowing habit, species of myxinid are better adapted to hypoxia and anoxic conditions. Burrowing or not, low concentrations of dissolved oxygen are encountered by all benthic organisms, and hagfish submerged beneath the turbid substrate or within decaying corpses will be subsumed within an environment that may be completely devoid of oxygen (Baker, Sardella, Rummer, Sackville, & Brauner, 2015).

Myxinids respond differently to hypoxia and in the species examined so far have a lower P_{crit} than eptatretids, which may have a more aerobic lifestyle (Forster, 1989; Forster, 1998). *Eptatretus cirrhatus* has a P_{crit} of between 45 mmHg and 50 mmHg and struggle and attempt to escape confinement when PO_2 is reduced to that level (Forster,1989; Axelsson, Farrell, & Nilsson, 1990; Coxon & Davison, 2010). Unlike *E. hexatrema* that became limp at a P_{crit} below 50 mmHg (Kench, 1989), *E. stoutii* made urgent and determined attempts to escape at 22 mmHg. This compares to specimens of *M. glutinosa* subjected to severe hypoxia and found to have a P_{crit} of 11–22 mmHg with no attempt to escape (Perry, Fritsche, & Thomas, 1993).

Myxinidae have adapted and survived because they have specialised in hypoxia tolerance (Ilves & Randall, 2007). Between the central circulatory system and subcutaneous sinuses, Myxinidae hold a large volume of blood which both facilitates the storage of oxygen and the capacity to dilute metabolic wastes including high concentrations of ammonia and HCO_3^-, which disrupt the acid-base balance (Davison, Baldwin, Davie, Forster, & Satchell, 1990; Forster, Russell, Hambleton, & Olsen, 2001; Cox, Sandblom, Richards, & Farrell, 2011; Gillis et al., 2015; Clifford, Weinrauch, & Goss, 2018). The hagfish heart has high myoglobin and glycogen content to support cardiac function (Davison et al., 1990; Forster, Davie, Davison, Satchell, & Well, 1991), a very low heart rate and the lowest blood pressure recorded

in a vertebrate, which enable hagfish to function and live at depth in an environment that varies between low concentrations of oxygen and complete anoxia (Johansen & Strahan, 1963; Forster, Davie, Davison, Satchell, & Well, 1988; Lesser et al., 1996; Martini, 1998b).

1.12 The cardiovascular system

Hagfish are well supplied with hearts. The cardiovascular system is driven by two hearts formed of cardiac tissue: the branchial and portal hearts. The branchial heart has three in-line chambers – the *sinus venosus*, atrium and a ventricle – and is without innervation (Forster, 1998; Farrell, 2007). The portal heart, a feature particular to hagfish, moves blood exiting the gut to the branchial heart via the liver (Fänge, Bloom, & Östlund, 1963; Forster, 1998). Peripheral circulation is assisted by two bilateral organs in the head and tail; the cardinal and caudal hearts. The caudal heart has innervation and is driven by skeletal muscle (Forster et al., 1991). Amongst vertebrates, hagfishes have the largest volume of blood, of which some 30% is contained within a network of subcutaneous sinuses (Forster et al., 1991; Lomholt & Franko-Dossar, 1998; Davison, 2016).

1.13 Respiration and ventilation

In hagfish gills, ventilation is powered by the movement of the velum, which draws water into the velar chamber and propels it onwards through afferent gill ducts and across the gills. The velum is a cartilaginous process that moves vertically within the velar chamber (Strahan, 1958; Johansen & Strahan, 1963). Oxygen and blood come into contact at the gill lamellae contained within the gill sacs. Gill numbers range between 5 and 14 pairs in species of Eptatretinae and each gill has its own efferent duct, whereas in Myxininae, gill numbers are between 5 and 7 pairs, which are evacuated by a single pair of efferent ducts (Johansen & Strahan, 1963; Bartels, 1998).

1.14 Marine environment of New Zealand

New Zealand is directly in the path of the Antarctic Circumpolar Current, which travels through, and connects, the world's oceans. Cold water that originated in the Atlantic Deep-Water zone flows eastwards around the islands of New Zealand (Gordon, 2004; Davis, 1991). Salinity of the seawater about the coast of the South Island is 34–35% rising to 35–36% towards the top of the North Island. The oxygen content of open seawater is around 6 mL per litre. The temperature of surface seawater to the east of the coast of New Zealand varies seasonally between 10 °C and 17.5 °C (Willmer, Stone, & Johnston, 2005; World Sea Temperature, n.d.). Whilst the water temperatures at the surface alters seasonally, seawater at depth remains within a narrow range (Nelson, 2006). The estimate for the temperature of the sea floor is between 8 °C and 13 °C (Levitus, n.d.). Most marine animals that inhabit this region are ectothermic and, apart from seasonal migrations where the animal may encounter temperatures that vary within 1–2 °C, remain within a preferred temperature zone depending upon the depth occupied (Willmer et al., 2005).

1.15 Environmental temperature

Modern Myxinidae are specialists with 300 million years of benthic experience. As with most marine animals, Myxinidae are ectothermic and do not regulate their body temperature, which remains very close to that of their environment and sudden alterations in temperature have lethal effects (Adam & Strahan, 1963; Martini, 1998b). Marine ectotherms may be able to escape unfavourable water temperatures by moving away (Fry, 1947; Fry, 1971). This is seen in *E. burgerii* when the species makes an annual migration to deeper and cooler water during the warmer months of the Northern hemisphere (Fernholm, 1974; Ota & Kuratani, 2006).

Many species of Myxinidae inhabit oceans at depths greater than 30 m in water temperatures of 5–13 °C and are unable to survive water temperatures above 22 °C (Adam & Strahan, 1963; Steffensen et al, 1984; Martini, 1998b). Against the

background of climate change, global warming and the expansion of oxygen minimum zones within the oceans, respirometry and oxygen consumption research is relevant (Childress & Seibel, 1998; Farrell, Eliason, Sandblom, & Clark, 2009; Holt & Jørgensen, 2018).

1.16 Range and environment of Neomyxine biniplicata

Neomyxine biniplicata, the slender hagfish, is endemic to the waters around the eastern coastline of New Zealand. The geographical range of this species extends along the eastern coast of both islands of New Zealand, between the northern Bay of Plenty and Pegasus Bay in the south, encompassing Cook Strait and the Western Chatham Rise (Figure 1.4). (Mincarone, 2013; Zintzen et al., 2015). *Neomyxine biniplicata* inhabit the ocean floor at depths between 35 m and 396 m (Roberts et al., 2015), although the specimens for this research were extracted from seabed at around 20 m. Of the sea-floor temperature at the depths occupied by *Neomyxine biniplicata* no data exists, but best estimates are between 8 °C and 13 °C (Levitus, n.d.; Willmer et al., 2005). *Neomyxine biniplicata* is a member of the Myxininae, known for their burrowing habit (Martini, 1998b). A specimen of the substrate into which they burrow was retrieved from the sea floor in Pegasus Bay and found to be a fine, soft, greenish-coloured silt typical of the substrate that is found in areas of stable benthos. The Wentworth cladistic scale of sediment grain for this type of substrate is 0.06–7.8 μm in particle size (Nelson, 2006).

1.17 Neomyxine biniplicata

Neomyxine biniplicata is based on *Myxine biniplicata* as a new genus in Myxinidae (Richardson, 1953). Another hagfish endemic to New Zealand waters is *Neomyxine caesiovitta*. This species occupies the same genus and is distinguishable from *N. biniplicata* by a blue line along both flanks and a greater number of slime pores, 161–199 along the trunk compared with 131–180 in *N. biniplicata*. The slime pores of *N. caesiovitta* may be defined by a darkened rim (Roberts et al., 2015). Coloration in

Neomyxine biniplicata ranges between pale pink and grey with soft, naked skin (Figures, 1.3 1.5). Some fish exhibit dark spots of pigmentation whilst scar tissue is white, (Figure 1.2).

Morphometrics record this fish as growing to 425 mm in length although some of the specimens caught for this research have been longer; the longest being 550 mm. The body is like that of an eel with a single fin fold extending from the dorsal tail tip to the ventrally situated gill apertures. The tail is rounded. This species has three pairs of barbels (tentacles), two pairs nasal and one pair about the oral cavity. There are no eye spots. Slime pores are white and situated ventro-laterally on both sides of the body and run the entire length of the fish. Slime pores vary in both number between fish, and in some instances, laterally within a fish. There are seven pairs of internal gills which are evacuated by one pair of ventral gill apertures (Richardson, 1953; Spitzer & Koch, 1998; Zintzen et al., 2015).

The trophic levels of feeding in *Neomyxine biniplicata* were investigated using the stable isotopes carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in specimens caught at a depth of 50 m. Stomach content analysis revealed the remains of food obtained from higher trophic levels including carrion and invertebrates dredged from beneath the silt. Hagfish store lipids in their muscles, which may be used in times of fasting (Flood, 1998; Zintzen et al., 2011). The lipid fraction of the muscle of this species was not high in comparison with *Eptatretus cirrhatus*, which may indicate that *Neomyxine biniplicata* feeds regularly. Hagfish are known to be scavengers and adaptable feeders and have also been filmed actively hunting prey. (Zintzen et al., 2011; Zinzten, Rogers, Roberts, Stewart, & Anderson, 2013).

To date information about *Neomyxine biniplicata* is limited to morphometric data obtained from deceased specimens, the regions and depth of the ocean the species inhabits, and diet (Currie & Edwards, 2010; Zintzen et al., 2013; Zintzen et al., 2015). The IUCN lists this species as data deficient (IUCN, 2013).

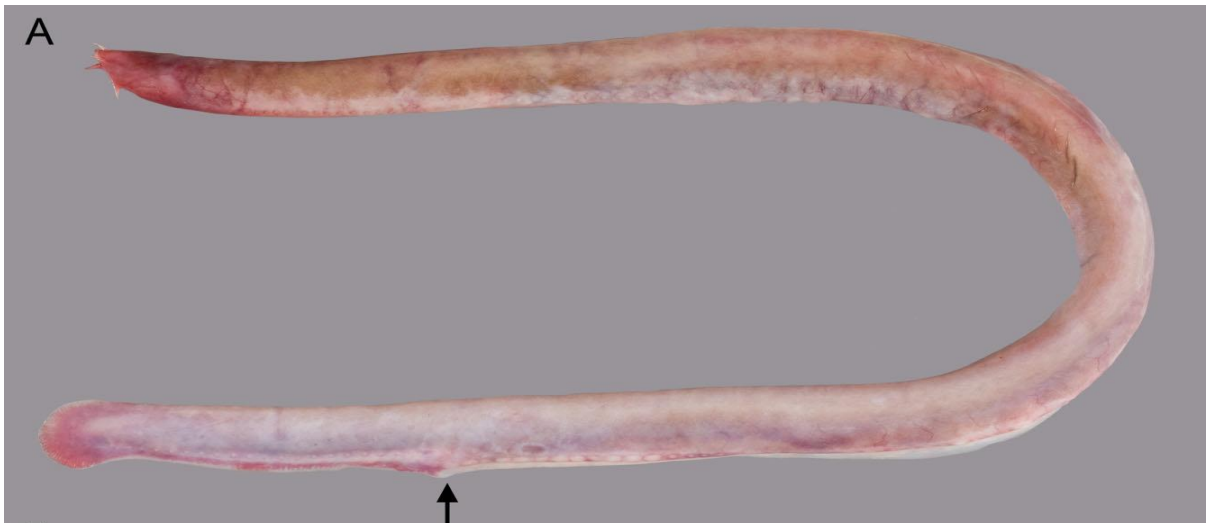

Fig: 1.3. Fresh colour images of *Neomyxine biniplicata*. From: Roberts et al., 2015

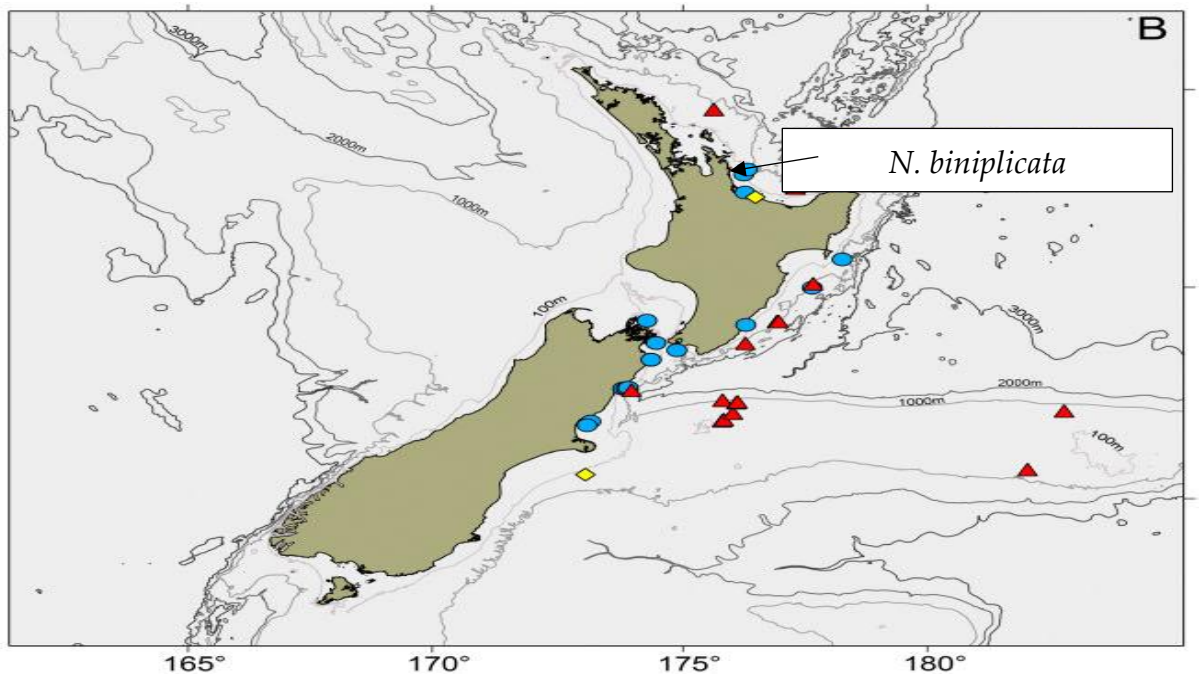

Fig.1.4. Distribution map of *Neomyxine biniplicata* (blue dot). Adapted from: Zintzen et al., 2015

Fig. 1.5. *Neomyxine biniplicata* in captivity. The substrate was lifted from the seabed in the area where the fish were captured. The smaller hagfish on the right has burrowed and the nose is protruding close to the head of the second hagfish.

Thesis objectives

This is the first study of the respiratory physiology of *Neomyxine biniplicata* and although some specimens have been successfully held in captivity in the aquarium of the University of Canterbury, this may be the first instance of the species being more closely observed. The main objective of this thesis was to test and understand the respiratory physiology of *N. biniplicata*. In addition, observing the habits, behaviour and interaction between individuals and their surroundings will increase our knowledge of this ancient species.

Chapter 2: General experimental methods, animal husbandry and ethical consent.

Chapter 3: Ventilatory response to acute changes in temperature. Two seasonal populations were exposed to acute variations in water temperature. This was to establish the resting ventilatory rate, ventilation rate over a range of temperatures, the Q_{10} effect and to determine if seasonal acclimation occurs. Also, the results of this set of experiments will inform us of the range of temperatures suitable for the hypoxia experiments.

Chapter 4: Routine metabolic rate and critical oxygen tension in *N. biniplicata:* two experimental methods. Closed-system respirometry was used for these experiments. The specimens naturally reduce oxygen tension until the animals are compelled to conform with the ambient oxygen tension. These experiments were run at both acclimated temperature and a narrow range of water temperatures. Results will deliver information on the tolerance of specimens of *N. biniplicata* to hypoxia and allow for direct comparison with the Atlantic hagfish, *Myxine glutinosa* and *E. cirrhatus*, another hagfish endemic to this region and the subject of previous studies by this laboratory. The metabolic rate of oxygen consumption across a narrow range of temperatures will be calculated and compared with that of other species of hagfish. Data obtained will provide information on the species-specific P_{crit} or the level of dissolved oxygen at which *Neomyxine biniplicata* ceases to regulate oxygen consumption and will indicate how well the animals are adapted to a hypoxic environment.

Chapter 5: Observations of behaviour, habits, interactions between the animals and food preferences of *N. biniplicata* within the aquarium will be recorded and may be of use in future studies.

Chapter 6: Conclusions, limitations of the research and further research.

Chapter Two

General experimental methods

2.1 Ethics and consent

The Animal Ethics Committee (AEC) of the University of Canterbury approved the use of specimens of *Neomyxine biniplicata*, the slender hagfish, for this research: Approval Ref. 2017/26R. Care and husbandry of the animals was strictly in accordance with the AEC's Code of Ethical Conduct.

2.2 Capture of experimental animals

The slender hagfish were caught in Pegasus Bay, between 3 km and 4 km off the east coast of the South Island of New Zealand, and directly east of Pigeon Bay Heads and Taylors Mistake. A map of the area in which the hagfish were caught is illustrated in Figure 2.1. Gee Minnow traps (Figure 2.2), were used to catch the hagfish. The traps were 228 mm by 444 mm in dimension and constructed of 6.3 mm galvanised steel wire mesh with a 25.4 mm opening at each end.

The traps were baited with salmon (*Oncorhynchus tshawytscha*) and lowered into Pegasus Bay, and specimens of *Neomyxine biniplicata* were captured at depths between 20 m and 22 m. Following capture, the fish were inserted into a large plastic container filled with seawater and transported over land to the aquarium at the University of Canterbury. The fish proved to be unexpectedly elusive and were caught over six fishing expeditions between November 2017 and June 2018.

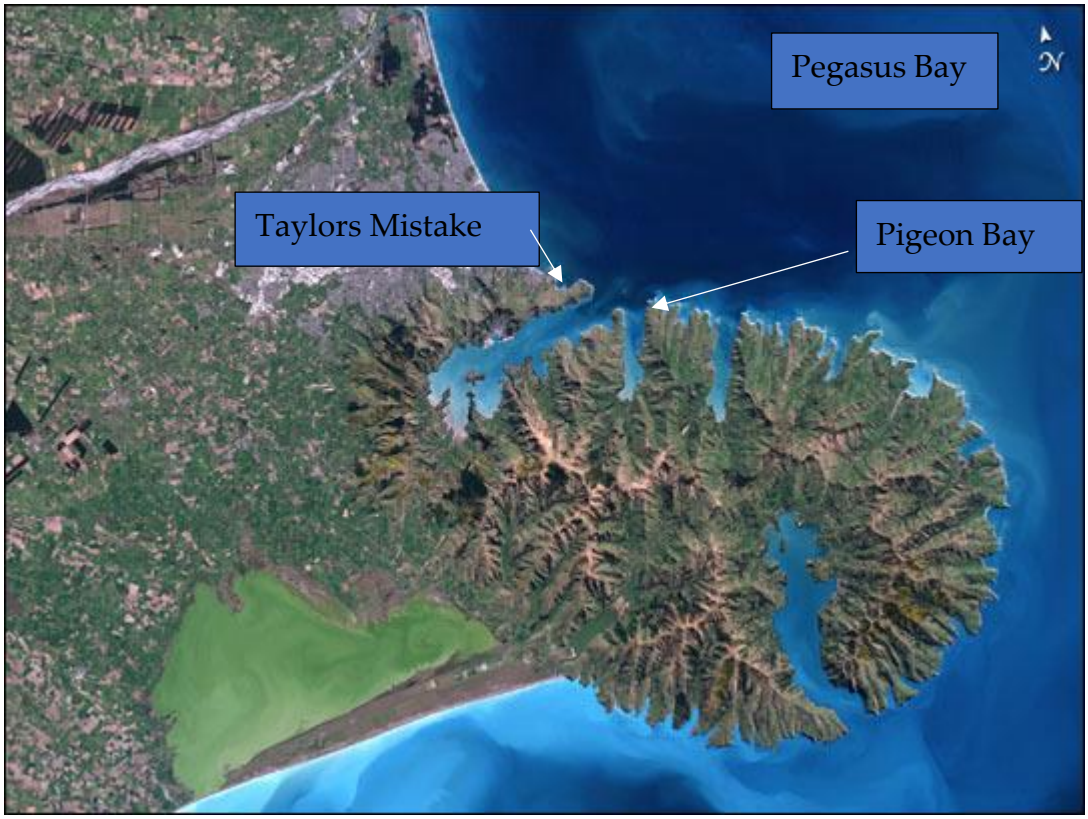

Fig. 2.1. Map of Banks Peninsula and Pegasus Bay, adapted from Earth Observatory Images, NASA: sites of capture of *N. biniplicata* (https://earthobservatory.nasa.gov/images/3217/christchurch-new-zealand)

Fig. 2.2. Gee minnow trap

2.3 Aquarium

The hagfish were housed inside the aquarium at the university where they were installed into a separate acrylic tank, covered to exclude most light, containing 300 L

of fresh seawater of 34–35‰ and held at 12.5 ± 0.3 °C. The tank was connected to the recirculating system and a protein skimmer. Salinity and water temperature were tested twice weekly and approximately one third of the water in the tank was changed every week. The fish were exposed to a photoperiod of 11.50L:11.50D hours with 10 minutes of dim light to simulate dawn and dusk. All fish survived transportation and a period of ten days acclimation was allocated to let fish caught between November and February (warmer months) recover from the ordeal of capture and to digest recently consumed bait. One week's recovery time was set aside for the fish caught in the colder months (March to June) as they were more docile in temperament.

2.4 Substrate

In the aquarium the smaller fish made strenuous but futile efforts to burrow into the beach-side sand provided. Pails of soft clay substrate were retrieved from the area in which the fish were captured and inserted into the tank. Some hagfish burrowed, assuming the horizontal, exposed-nostril position described by Strahan (1958) and illustrated in Figure 2.4. Small diameter plastic pipes, as recommended by Ota and Kuratani (2006), were also provided and the fish would often conceal themselves within them, usually in pairs.

2.5 Feeding regime

During their sojourn in the aquarium, the fish were offered a variety of food every 14 days followed by 10 days of fasting. The feeding regime was based on information provided by a study of trophic levels of feeding in various species of New Zealand Myxinidae (Zintzen et al., 2013). Inspection of the stomach contents and muscle tissue analysis using the stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) has shown that *N. biniplicata* are likely to feed regularly, consuming invertebrates and small fish, both alive and deceased (Zintzen et al, 2011; Zintzen et al, 2013).

Food offered included Mysis (*Mysida*), sardine (*Clupea sagax*), gurnard (*Chelidonichthys spinosus*), mussels (*Perna canaliculus*), prawn (*Penaeus esculentus*) and salmon (*Oncorhynchus tshawytscha*). Salmon was the preferred item of food.

2.6 Identification

As no more than 10 *N. biniplicata* were in the aquarium at any one time, the fish were not tagged and were instead identified by a variety of natural physical characteristics which included size and length; scars and pigmentation; missing slime pores and eggs which, when in an advanced stage of development – between stages 5 and 7 (Martini & Beulig, 2013) – were clearly visible through the flanks of the fish. Several fish caught in the summer months came into the aquarium with small segments bitten out of their tail fin but within 14 days the tails regenerated, and therefore, sections of missing tail cannot be reliably used as identifying marks.

2.7 Experimental set-up

Experiments were carried out in the wet labs at the University of Canterbury. Before every experiment the fish were caught and weighed (wet weight) and, depending upon the disposition of the animal, were rested for between 60 and 90 minutes. Initially respirometry experiments began with a 20-hour resting period, which was revised to 90 minutes for reasons explained in Chapter 4. The experimental set-up and equipment were modified and refined as research progressed. All experiments were carried out in the seawater to which the animals were acclimated.

2.8 Retrieval of specimens from the aquarium

The method of capture that involved the least amount of stress to the animals involved first gently nudging the selected fish with a thin metal rod. The rod was then carefully inserted beneath the body of the fish and the fish lifted a few millimetres off the floor of the tank. This had the effect of rousing but not alarming the fish which would usually then swim up to the surface. There the fish could be easily caught by placing both hands under the body, supporting the tail, and placing

the animal into a waiting bucket of seawater. If the fish were caught in this way, they did not become unduly alarmed and slime ejection was usually minimal or non-existent. Inverted fish are often sleeping fish and unless absolutely required for an experiment are best left for another time. Burrowed fish were never disturbed.

The best and most efficient method of settling a hagfish into the respirometer required a small piece of cling film that had been submerged, arranged over the respirometer, and weighed down by a small volume of water. Immediately upon inserting the fish into the respirometer the film was drawn over to form a seal. The hagfish responded to the soft, weighted film and settled quickly. No better method was found and neither fine netting, nor the acrylic lid of the respirometer induce the same rapid settling of the fish. In fact, if placed under netting, the hagfish will persistently probe and explore the mesh trying to escape. It may be that the soft, wet, and weighted cling film in some way replicated the feeling of contact with a fellow hagfish.

Despite their small mass an abruptly woken slender hagfish would eject a surprisingly prodigious quantity of slime and, if their last food was salmon, an oily, orange-coloured fluid.

2.9 Lighting

If the fish had recently eaten, they would remain unresponsive to light and lie blimp-like on the substrate. Otherwise, at any time of the day, if the hagfish tank was uncovered, the fish would respond, initially by slowly waving their heads, and then swimming, often at the surface. This being so, all experiments were undertaken in a dimly lit laboratory.

2.10 Circadian rhythm

Specimens of *N. biniplicata* are active nocturnaly. The LabChart trace recorded patterns of oxygen consumption between 9 am and 4 am. Experience proved that the

best time to catch and settle a specimen into a respirometer was between 5 am and 6 am when the fish were relaxed and quiescent.

2.11 Data collection and calibration of Firesting oxygen meter

Dissolved oxygen within the respirometer was measured with the Firesting oxygen meter (www.pyroscience.com). Before experiments began, the equipment was calibrated in freshwater and seawater at temperatures between 0 °C and 12 °C, in compressed air at 0 °C and with nitrogen gas and, in all conditions and at both high and low levels of dissolved oxygen, the measurement was accurate.

Data were recorded by AD instruments Powerlab 4/25T using LabChart software and sampled at 40/s with a range of 2 V and units of pressure being mmHg. An example of the reduction of dissolved oxygen by a specimen of *N. biniplicata* within a sealed respirometer is illustrated in Figure 2.3.

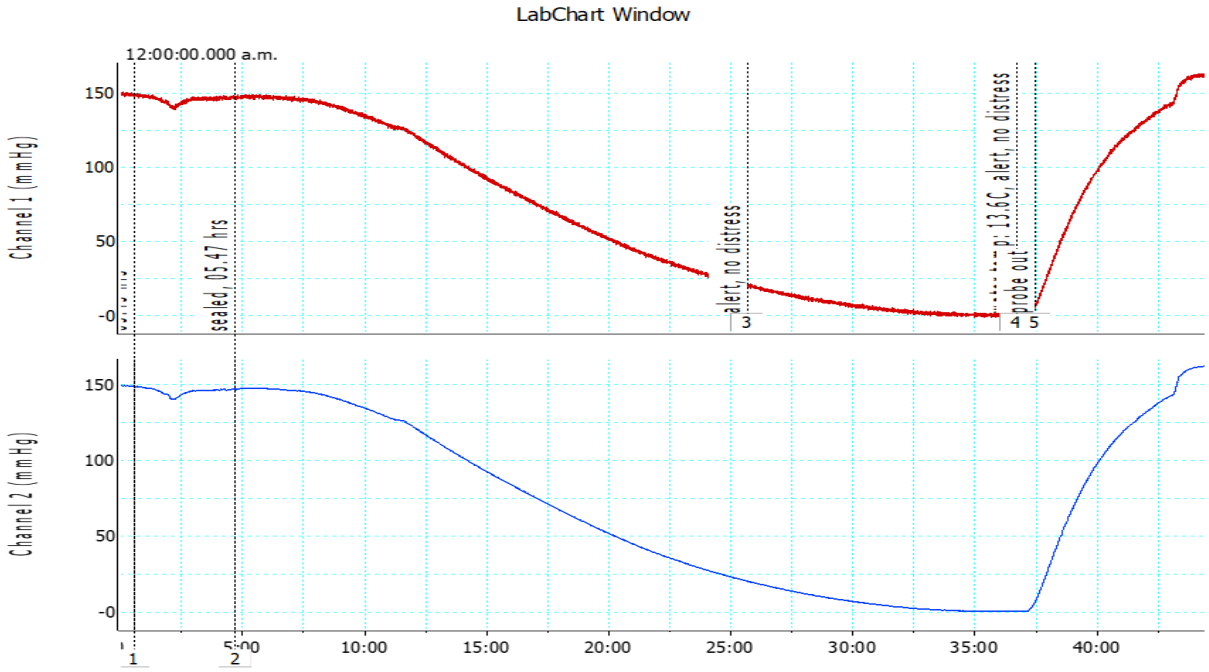

Fig. 2.3. Reduction of dissolved oxygen within a sealed respirometer. The y-axis shows the level of dissolved oxygen (mmHg), time is recorded along the x-axis. The red trace charts the reduction in oxygen tension. The blue trace is a filter. Data captured by Firesting oxygen probe and recorded by AD Instruments Power Lab and LabChart software.

2.12 Statistical Analysis

Data were analysed by Sigmaplot ver. 14. All data are presented ± as mean standard error (SE) unless otherwise stated. Statistical significance was assessed at an alpha level of $p < 0.05$ and 95% confidence intervals. Before statistical analysis, normality of data was examined by a Kolmogorov–Smirnov test and equal variance analysed by the Brown–Forsythe test. One sample t-test and non-linear regression to examine relationships between metabolic rate and hypoxia. One-way ANOVA was used to compare data within populations with a post hoc Holm–Sidak or Dunn's test recommended for non-normally distributed data.

Fig. 2.4. *N. biniplicata* in captivity. The fish in the centre has burrowed into soft clay substrate with nostril extruded.

Chapter Three

Ventilatory response to acute changes of temperature

3.1 Introduction

In aquatic ectotherms the rate of metabolism is inextricably linked to ambient water temperature, which, in turn, influences the concentration of dissolved oxygen. The solubility of oxygen is inversely correlated with water temperature and will diminish as water temperature rises (Fry, 1947; Fry, 1971; Jobling, 2008). In aerobes, oxygen powers the metabolism and is obtained from the surrounding medium (Nelson, 2016). In this way the environment exerts control over an organism's metabolism and can either enhance or limit metabolic rate (Fry, 1947; Fry, 1971). The temperature of an aquatic organism equilibrates with that of its environment at the confluence of gills and water, and fluctuations in water temperature will translate into an altered metabolic rate. Usually an increased external temperature will escalate the metabolic rate, whilst a lowered environmental temperature will have the opposite effect (Jobling, 1993; Jobling, 1994; Barrioneuvo & Fernandes, 1998; Holt & Jørgensen, 2018).

3.2. Metabolism and temperature

Energy metabolism is the sum-total of all biochemical and physiological processes required by a life form to maintain homeostasis (Schmidt-Neilsen, 1975; Jobling, 1993). In rising water temperature, aerobic organisms respond mechanically to the metabolic demand for additional oxygen by increasing ventilation and cardiac rate, and stroke volume. At the cellular level, the delivery of additional oxygen results in the accelerated synthesis of adenosine triphosphate (ATP) required to fuel increased chemical and physical metabolic output. Elevated environmental temperature causes

changes in the metabolic rate because the biochemical reactions that drive catabolism and anabolism (the metabolism of energy – diffusion, enzymatic rates, and thermal energy) are temperature reliant (Schmidt-Neilsen, 1975; Hochachka and Somero 2002). At the top end of the thermal range, haemoglobin has less affinity to bind oxygen to haemoglobin whilst mitochondrial synthesis of ATP is reduced at the lower limit of temperature that a species is acclimatised to (Somero & Hofman, 2008; Pörtner, 2010).

3.3 Thermal acclimation

Every species has a range of thermal tolerance, and beyond this the cardiovascular system cannot supply the oxygen demands of the tissues, and metabolic rate becomes impaired. Once an organism moves beyond its critical range of temperature, anaerobic metabolism and heat shock proteins can sustain life for a limited period but without acclimation the life-span of the organism is limited (Schmidt-Nielson, 1975; Pörtner, 2010).

The absolute aerobic scope (AAS) hypothesis explains the relationship between aerobic performance and temperature (Farrell, 2016). The parabolic curve in Figure 3.1.A illustrates optimal aerobic performance at the preferred temperature, while Figure 3.1.B shows how aerobic performance is deleteriously affected in relation to water temperature outside normal range and aerobic scope is narrowed to a range close to the thermal limits of the organism (Pörtner & Farrell, 2008; Clark, Jefferies, Hinch, Farrell, 2011; Clark, Sandblom, & Jutfelt, 2013). This hypothesis explains the link between the altered capacity of the respiratory organs and the cardiovascular system to deliver oxygen to the tissues when outside the normal thermal range of the organism of aquatic water breathers (Pörtner 2010). The oxygen capacity-limiting thermal tolerance (OCLTT) hypothesis suggests that an organism's capacity to supply oxygen to the body becomes limited when the physiological temperature reaches extremes and the scope for the species to grow and replicate is reduced

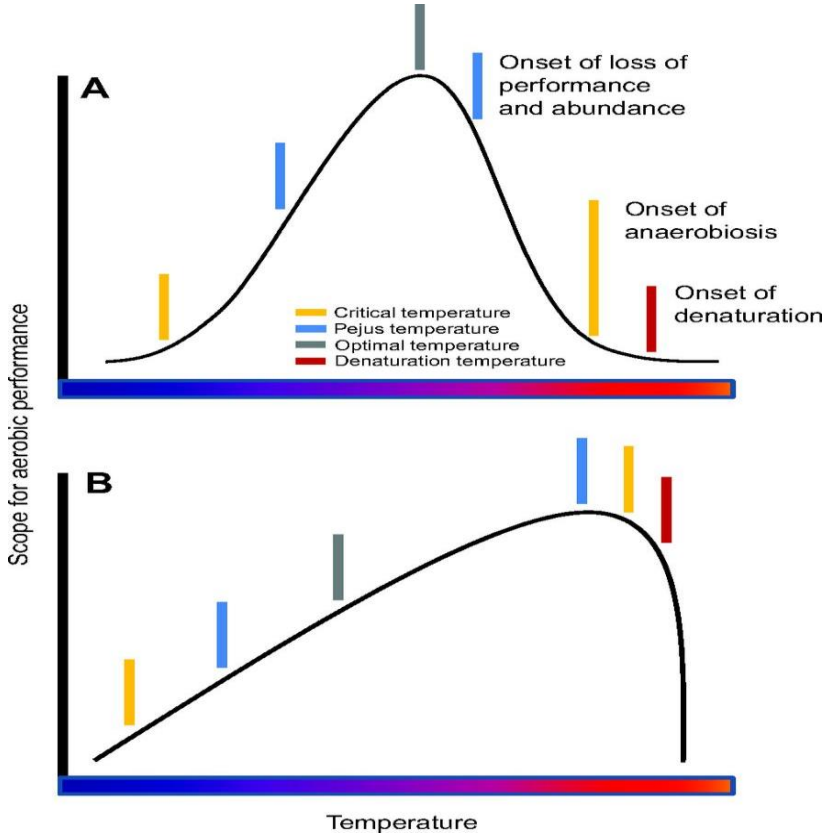

Fig. 3.1. Hypothetical curves illustrating aerobic scope of fishes with temperature. A) redrawn from Pörtner & Farrell, 2008 by Clark et al., 2013. B) aerobic performance responding to temperature from Clark et al., 2011; Clark et al., 2013.

within this environment (Pörtner & Knust, 2007; Pörtner 2010). The OCLTT hypothesis is useful in measuring performance although it may not identify the preferred temperature of a species but merely the aerobic scope at which lethal temperatures ensue (Fry, 1947; Clark et al., 2013).

The temperature co-efficient, Q_{10}, can be calculated to show if the physiological process being examined is dependent upon temperature. A higher metabolic rate will require increased oxygen which is supplied by anaccelerated ventilatory rate. A Q_{10} of 1 indicates the process is not dependent upon temperature, the higher the Q_{10} result is, the more closely dependent upon temperature the process (Fry, 1947;

Schmidt-Nielsen, 1975). A Q_{10} of between 2-3 indicates a two-to-three-fold increase in the process being studied and is not unusual in metabolic studies (Rao & Bullock, 1954). The van't-Hoff formula is used to calculate the Q_{10} effect (Fry, 1957).

Nonetheless, ectotherms that inhabit temperate waters often exhibit the ability to thermally acclimate and physiologically adjust and compensate for acute and prolonged variation in temperature (Cossins & Bowler, 1987). Compensatory metabolic adjustments may occur within minutes to weeks of exposure, depending upon the span of temperature change (Alderdice, 1976; Jobling, 1994). Thermal acclimation by individuals may eventually translate into thermal adaptation and changes in fitness within the population (Barrioneuvo & Fernandes, 1998; Sinclair & Roberts, 2005; Pörtner, 2010).

3.4 Temperature range of Myxinidae held in captivity

Myxinidae are aquatic, demersal ectotherms that inhabit the seabeds of temperate oceans where water temperature is cool and constant, and it is in this environment that they are optimally fit, and energy expenditure or the metabolic rate is most economical. (Adam & Strahan, 1963; Jobling, 1993; Martini, 1998b).

Myxinids are reported to tolerate a wider range of temperatures than eptatretids (Ota & Kuratani, 2006). In several instances *Myxine glutinosa* have been held in aquaria at 10 °C – 15 °C but for a long excursion into captive life, temperatures between 0 °C and 4 °C in this species are best (Fernholm, 1998). Gustafsen (1935) reported that specimens of *M. glutinosa* can bear a 5 °C alteration in temperature, from 10 °C to 15 °C, with no ill effects but the upper extremity of temperature range in this species is close to 20 °C. Sudden and significant changes in temperature are fatal to hagfish (Bloom, Östlund, & Fänge, 1963; Martini, 1998b; Ota & Kuratani, 2006). Whilst researching the effect of temperature on heart rate in *M. glutinosa,* Jensen (1961) recorded the heart rate of 3–4 bpm at 1 °C rising to 16 bpm at 13 °C and 30 bpm at 26 °C, with heart rate becoming erratic at 30 °C.

Species of eptatretids have been kept in aquaria at temperatures ranging between 14 °C and 22 °C, with *E. stoutii* being briefly exposed to 30 °C and surviving (Fernholm, 1998). Kench (1989) kept specimens of *E. hexatrema* in captivity for two years at temperatures between 14 °C and 17 °C. The migratory hagfish, *E. burgeri,* moves away from Koajiro Bay in Japan when water warms to above 20 °C, and water temperatures between 24 °C and 28 °C are probably terminal for this species (Fernholm, 1974). Heart rate and temperature are significantly correlated in the native hagfish, *E. cirrhatus*; heart rate of 16 bpm was recorded at 12 °C, rising exponentially to 48 bpm at 19 °C, at which point the hagfish exhibited extreme agitation (Coxon & Davison, 2011).

3.5 The effects of specific dynamic action

As well as thermal stress, ingestion, digestion and absorption of food also raise the metabolic demand for oxygen and this is described as specific dynamic action (SDA), (Jobling, 1993; Alsop & Wood, 1997; Chabot, Koenker, & Farrell, 2016). To avoid inaccurate results when measuring physiological functions, including ventilatory rate, time must be set aside to allow metabolic oxygen demand to return to pre-digestion levels (Fry, 1947). The effect of SDA on the ventilatory rate in *N. biniplicata* is illustrated in Figure 3.10. The basal metabolic rate (BMR) is the amount of energy expended within a given unit of time for the animal to function at rest. The ventilatory rate in specimens of *N. biniplicata* in the summer population was measured at 3, 5 and 7 days after feeding and found to be elevated. Myxinidae do not osmoregulate and this contributes to hagfish exhibiting the lowest routine metabolic rate in fishes; for these reasons specimens of *N. biniplicata* were rested for between 10 and 14 days after feeding to allow the system to return to BMR (Munz & Morris, 1965; Forster, 1989; Forster, 1990; Jobling, 1993).

3.6 Stroke volume

In cartilaginous fish, the increased demand for oxygen can be supplied by greater stroke volume which delivers a greater volume of water across the gills. In hagfish, both velar movement and muscular contraction of the velar chamber propel water to the gills (Hol & Johansen, 1960). Stroke volume, however, cannot be expanded and cellular requirement for additional oxygen can only be supplied by increasing the ventilatory rate (Malte & Lomhalt, 1998; Perry, Vulesevic, Braun, & Gilmour, 2009). This being so, the effect of acute changes in water temperature on ventilatory function in Myxinidae can be easily tested.

3.7 Respiratory anatomy of hagfish

Over millennia, Myxinidae have evolved a respiratory and ventilatory system that is distinct from that of all other vertebrates including bony and cartilaginous fish (Johansen & Strahan, 1963; Malte & Lomhalt, 1998; Janvier, 1999). In hagfish, gas exchange occurs within internal gills, and ventilation is powered by the movement of the velum. Water is inhaled through the median nostril and delivered to the velar chamber, itself a widening of the pharynx, via the nasopharyngeal duct (Strahan, 1958). The velum is formed of cartilage and is fixed midline to the roof of the velar chamber by the velar frenulum membrane and supported by the visceral skeleton. Inserted upon the skeleton are the posterior cranio-velar muscles that contract antagonistically against the spino-velar and the dorsal and ventral cranio-velar muscles. Bilateral and co-ordinated movement of this group of muscles control movement of the velum (Cole, 1907; Strahan, 1958). The velum takes the form of a two-sided scroll that is closed when the velum is in the ventral position and opens and extends laterally as the velum moves dorsally. The upstroke of the velum produces a change of pressure within the velar chamber, which draws water in through the animal's nostril, whilst propelling water within the chamber beyond the posterior pharynx (Strahan, 1958; Johansen & Strahan, 1963). The velar cycle has four stages, and velar rate is measured in beats per minute (Strahan, 1958). The external

respiratory anatomy is illustrated in Figure 3.2, the velar cycle in Figure 3.3 and a schematic of the anatomical structure of the velum in Figure 3.4.

Expelled from the pharynx by the velum, water enters the afferent gill ducts and then onto and over the gills, which in *Neomyxine biniplicata* are arranged bilaterally on either side of the pharynx in a parallel straight-seven formation (Bartels, 1998). The number of gills varies between species as does the method of evacuation of de-oxygenated water from the body. The gills are deep red in colour, the sagittal surface is convex and inclined towards the midline, overlapping the neighbouring forward gill. Each gill is encased within a pleural sac and gas exchange occurs within the enclosed gill lamellae (Johansen & Strahan, 1963). The gills contract during respiration, (Hol & Johansen, 1960; Coolidge, Hedrick, & Milsom, 2007), and this has been seen in specimens of *Neomyxine biniplicata.*

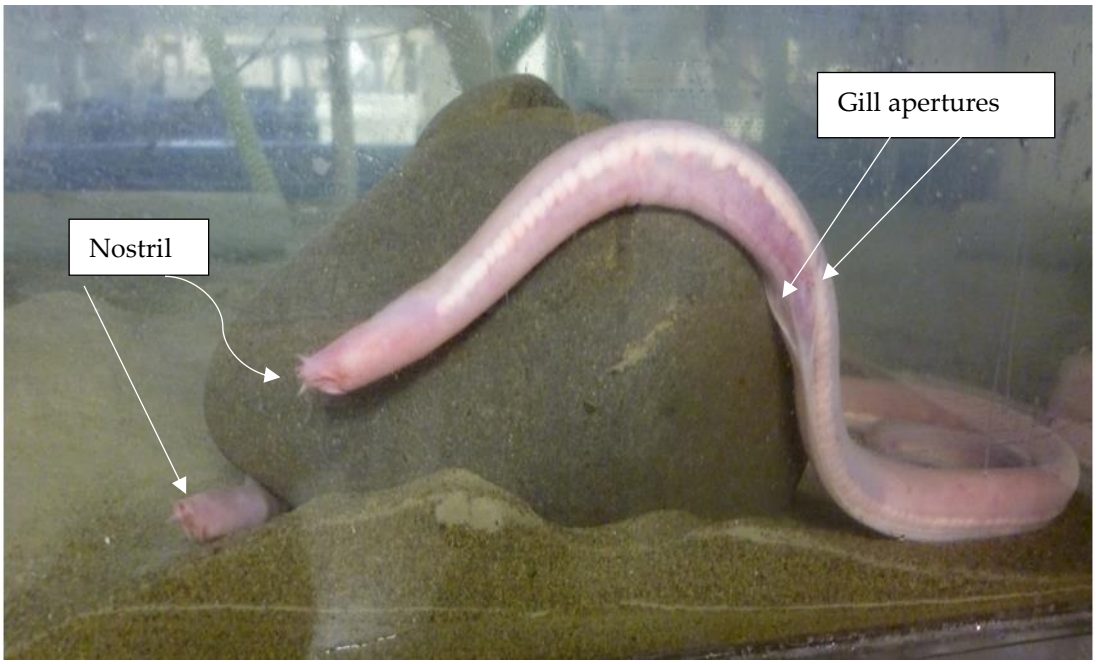

Figure 3.2. Nostril and bilateral gill apertures in *Neomyxine biniplicata*.

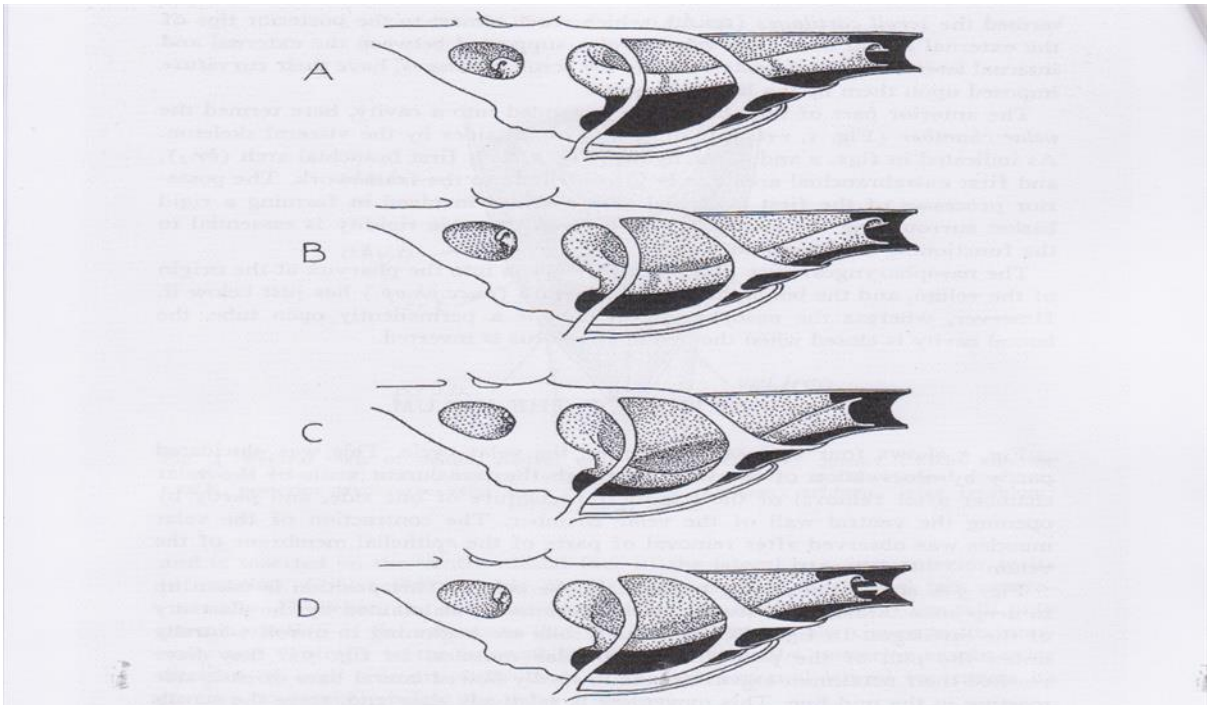

Fig. 5. Four successive stages in the velar cycle. The velar chamber is shown from the left side with the left pharyngeal wall removed. A, resting; B, velar scroll beginning to unroll; C, velar scroll unrolled to full extent; D, velar scroll beginning to move dorsally, arrow showing direction of water flow.

Figure 3.3. The velar cycle in *Neomyxine biniplicata*. From Strahan, 1958.

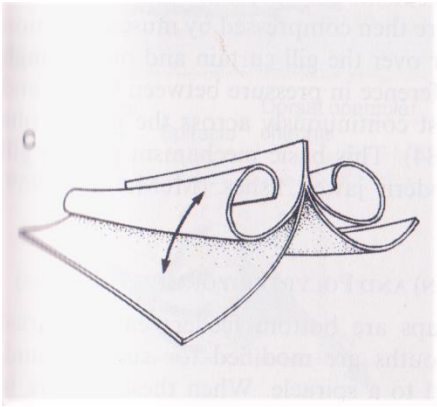

Figure 3.4. Schematic of a lateral view of the velum scrolling and unscrolling to move water through the pharynx. From Kardong, 2002.

3.8 Experimental series

The effect of acute alterations in water temperature on the ventilatory rate in specimens of *N. biniplicata* extracted from Pegasus Bay was examined. The fish were divided into two seasonal populations and were tested in a range of water temperatures for the effect of temperature and for any seasonal acclimatisation. The ventilatory rates of *N. biniplicata* and *E. cirrhatus* were compared. Ventilatory rates were compared between a wild fish, an acclimated fish, and a fish with SDA.

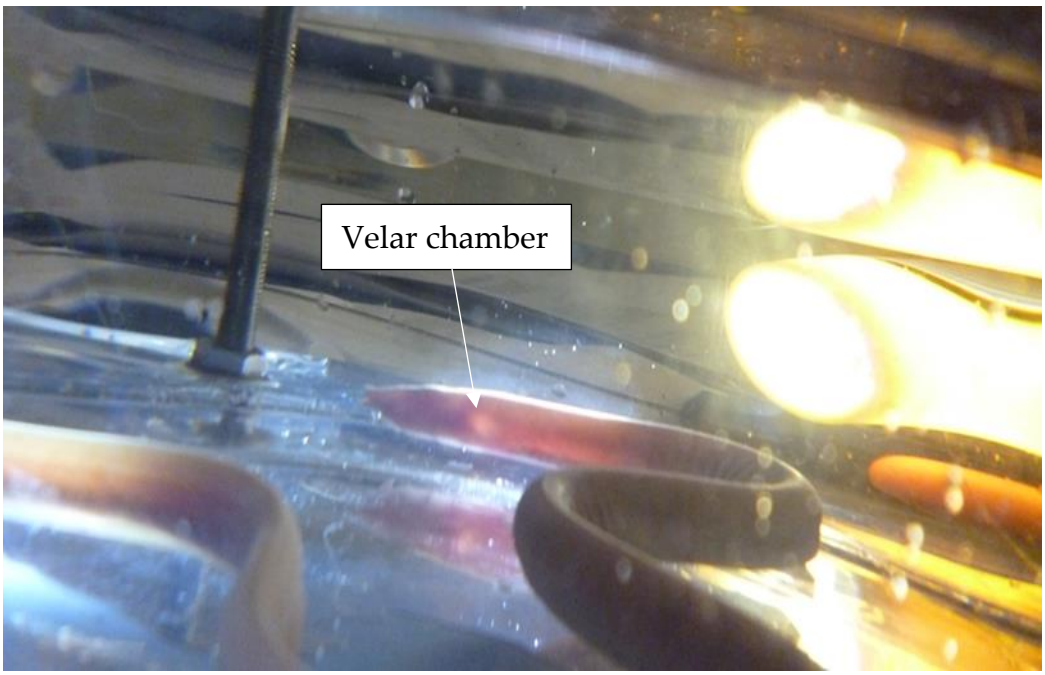

Figure 3.5. Illumination of the velar chamber in an unrestrained, resting specimen of *N. biniplicata*.

3.9 Material and Methods

Two seasonal populations of *N. biniplicata* were captured and held in the aquarium at the University of Canterbury, as described in section *2.3* in Chapter 2. The fish were weighed (wet weight) before every experiment. In resting and unrestrained *Neomyxine biniplicata*, movement of the velum is easily visible when illuminated, the velar chamber being translucent (Figure 3.5). Provided that the beam of light is not

directed on to the nostril, tentacles or vestigial eye tissue, the fish were unperturbed by, or unaware of, the light. The most convenient form of illumination was a torch as it allowed the researcher to move around the respirometer should the fish happen to move, rather than having to realign the respirometer within a fixed beam of light.

3.9.1 Effect of temperature on ventilatory rate

Each population of fish was exposed to acute changes in water temperature. The lower and upper limits were set at 5 °C and 20 °C and water temperature was altered in 1 °C increments every 40 minutes. Ultimately each animal determined the range of temperature it could tolerate. Ventilatory rate in response to acute alteration in water temperature was measured for 30 seconds with several measurements taken over the course of 30 minutes. The experimental methods were modified as inaccuracies with timing and water temperature became obvious.

3.9.2 Small experiments of interest but no statistical value

The effect of SDA on ventilatory rate was measured. The results were compared with the ventilatory rate of one acclimated specimen and one recently caught specimen of *N. biniplicata,* both from the summer population. The results of the ventilatory rates in the summer population was compared with that of *E. cirrhatus* from the Coxon and Davison study (2011).

3.9.3 Method I

Initial experiments with the summer population involved inserting a fish into a clear acrylic 1.2 L respirometer set on a bench. Each experiment started in the water temperature to which the fish were acclimated (12.5 °C), with a resting period of 90 minutes. In the first set of experiments the water temperature was increased by 1 °C every 40 minutes until the water had reached a temperature to which the animal registered distress by swimming and trying to escape. The water temperature was then immediately reduced and then gradually brought to acclimation temperature before returning the animal to the aquarium. Throughout the second set, the same protocol was followed but this time exposing the same fish to gradually colder water

decreasing the temperature every 40 minutes by 1 °C. All fish were rested for two weeks between the warm- and cold-water experiments.

Accuracy was hindered by the small volume contained within the respirometer and the ambient temperature within the laboratory. In the early experiments, water temperature was altered by the almost perpetual syringing of cold or warm seawater into the respirometer. Ice packs or an aquarium heater placed into a bucket of seawater were used to reach the desired water temperature. This method was somewhat random and mixing of the water within the respirometer was slow. It was difficult to maintain a stable water temperature and the constant movement of the water being added or removed by the syringe irritated the animals. The results obtained from this series of experiments have not been used in this report as they may not represent a resting velar rate at a precise temperature.

3.9.4 Method II

The second series of experiments used a water bath containing 30 L of seawater, the temperature of which was manipulated by a Hilea refrigeration unit with a range between 4 °C and 30 °C with an accuracy of $\pm$ 0.5 °C. Each fish was introduced into the respirometer, which was then submerged into the water bath. Once the resting period was complete the respirometer was lifted and placed onto the side of the water bath for velar monitoring. Careful placement of the respirometer did not disturb the fish and was certainly less irritating than the constant syringing of water. This method was an improvement on the bench-top respirometer and good results were obtained. The fish were generally passive and undisturbed. A piece of cling film floated above the respirometer and if the fish began to swim, the respirometer could be lifted, with the effect of the cling film sealing the vessel and so preventing the fish from escaping. Once the fish had settled, the respirometer could be re-submersed, and the cling film floated to the surface. This allowed for thorough mixing of the water whereas a fixed respirometer lid would have impeded rapid alteration of temperature.

The limitation of this method was the time required to achieve the desired water temperature in the upper and lower extremes. The experiments would run into several hours, keeping the fish in what may not have been ideal water temperatures. The aquarium heater and ice packs were still used to assist in bringing the temperature to the higher and lower extremes required for the experiment.

3.9.5 Method III

The third experimental set-up used two 30 L seawater baths (Figure 3.6). The temperature of the first seawater bath was maintained by two heat-exchangers, which were attached to pumps and lines set within a freshwater bath, the temperature of which was controlled by a Grant GD100 unit. The temperature of this bath was maintained at either 5 °C or 20 °C, depending upon whether a warm- or cold-water trial was being run. The temperature of the second seawater bath was maintained by the Hilea refrigeration unit at 12.5 °C. The fish were rested within the respirometer set inside the 12.5 °C bath for up to 90 minutes, depending upon the disposition of the fish. Once the experiment began, the respirometer was transferred to the second bath and held, immersed, for a matter of minutes until the water had mixed and the desired temperature was attained. This allowed for both thorough and acute temperature alterations. The respirometer was then lifted onto the side of the bath for velar monitoring where it remained for 40 minutes using cling-film to seal the respirometer, and so preventing any attempt at liberation by the animal.

The advantages of the final experimental arrangement were the rapid and thorough mixing of the water: less agitation of the water and so less disturbance to the fish. The ventilatory rate was counted for a period of 30 seconds and the result then multiplied to give the beats per minute (bpm). Measurements were taken every 5 minutes over a period of 30 minutes before the water temperature was altered although this could be extended if the fish had begun to swim. As the water temperature decreased below 10 °C, condensation obscured the fish and was

constantly wiped away with a soft cloth. At very low temperatures, velar movement could be counted for only 15 seconds.

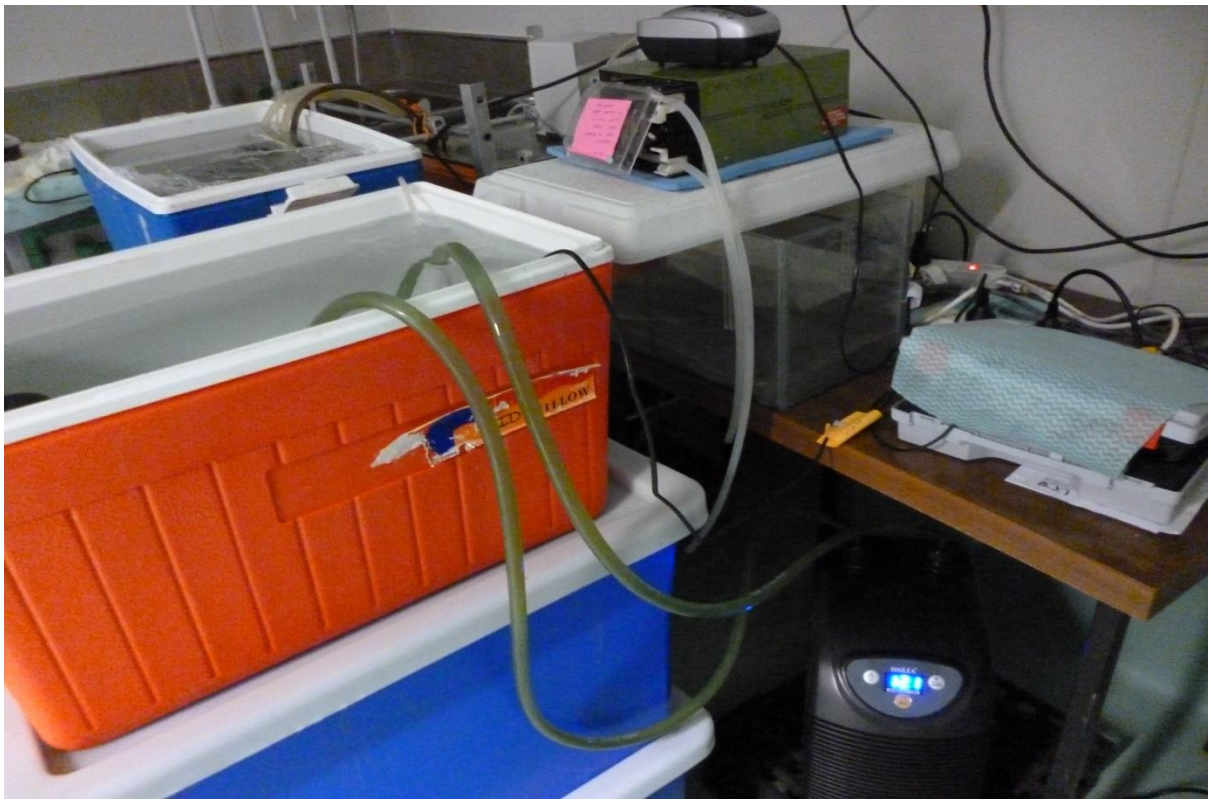

Fig. 3.6. Method III: monitoring the ventilatory rate, with respirometer submerged in the blue water bath.

3.10 Summer and Winter Populations

The summer population comprised six *Neomyxine biniplicata* (weight: mean ± SE 45.07 g ± 4.85 g; length: mean ± SE 448.33 mm ± 13.33 mm) which were caught in two expeditions in November and January. The winter population of six fish were caught in March and June (weight: mean ± SE: 47.70 g ± 4.56 g; length: mean ± SE 448.50mm ± 15.63mm).

3.11 Calculations and statistical analysis

Statistical analysis of data recorded of the ventilatory response to acute water temperatures changes were analysed by Sigmaplot ver. 14. Data are reported as mean ± SE unless otherwise stated. Normality of data was examined by Kolmogorov–Smirnov test and equal variance analysed by the Brown–Forsythe test. One-way ANOVA was used to compare data within populations. The relationship between temperature and ventilatory rate was analysed using one sample *t* test (Shapiro–Wilk) for normality, and non-linear regression and $\log_{10}$ transformation. Data were considered significant at alpha level of $p \leq 0.05$ and confidence levels of 95% to assess statistical significance.

Q_{10} values

Q_{10} values were calculated using the van't Hoff equation:

$R_2 > R_1$: reaction rate.

$T_2 > T_1$: temperature at which reaction rates are measured.

Q_{10}: reaction rate increases when temperature is raised by 10 °C.

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)}$$

3.12 Results

3.12.1 Water temperature

The range of temperature that each population of fish could endure being submersed in differed. The highest temperature tolerated by one fish in the summer population was 20 °C compared to 16 °C by a single winter fish. No fish from the winter population could bear being submersed into temperatures below 8 °C. Four fish in the summer population were able to tolerate water at 6 °C. The mean ± SE of

ventilatory rate against temperature of the seasonal populations is listed in Table 3.1 and graphed in Figure 3.7.

3.12.2 Ventilatory rate and temperature

Ventilatory rate increased in fish immediately after extraction from the aquarium but generally returned to a resting rate within 15 minutes, although some fish required closer to 30 minutes to regain their resting composure. Generally, upon introduction to the respirometer, the fish tended to settle quietly on the base within minutes. In specimens that had been retrieved from the aquarium, the immediate ventilatory rate could increase to above 100 bpm. Sporadic and erratic velar movement was seen in two fish that died within a few days of the trial and possibly this was indicative of the health of the fish. Resting velar rates were recorded between 6 °C and 20 °C in the summer population compared to 8 °C–16 °C in the winter population.

3.12.3 Ventilatory rates in the summer population

The combined velar rate of the summer population ranged between 7 bpm and 94 bpm compared to 10.5–67.5 bpm in the winter population. The highest velar rate recorded was 146 bpm, which was seen in a specimen that had recently ingested salmon. The mean ventilatory rates concealed the actual velar rates, especially in some of the summer fish at the lower temperatures. A complete cessation of ventilation, which could last for up to 20 minutes, was seen on a few occasions and this was in fish that were perfectly healthy. There were differences between the fish in both populations but mostly ventilatory rate increased in response to rising water temperature and slowed in water below 11 °C.

3.12.4 Ventilatory rates in the winter population.

In comparison, data from the winter population showed a steady rise in ventilatory rate between 8 °C and 10 °C. The ventilatory rate was steady between 10 °C and 12 °C, at which point velar rates increase exponentially until at 14 °C ventilation reached a plateau, and at this point all the fish vigorously tried to escape.

3.12.5 Comparison of ventilatory rates between seasonal populations

A different pattern of ventilatory rates was exhibited by the seasonal populations. In the summer population, ventilatory rates increased very slowly between 6 °C and 10 °C at which point rates increased exponentially, flattening off at 12 °C–14 °C before showing a steady rise until rates reached a plateau at 16 °C and began to slow. In comparison, data from the winter population showed a steady rise in ventilatory rate between 8 °C and 10 °C. At 12 °C ventilatory rates increased exponentially until 14 °C where ventilation reached a plateau at which point all the fish vigorously tried to escape. A $\log_{10}$ transformation of the data (Figure 3.9) clearly shows a different ventilatory pattern between the two seasonal populations and a linear dependence upon temperature to 16 °C in the summer population compared with 14 °C in the winter fish.

3.12.6 Q_{10} values

Using the mean velar rates (Table 3.1), the Q_{10} values were calculated for each population. Using the entire range of temperatures that each fish was exposed to: 6 ^{o}C – 20 ^{o}C in the summer fish and 8 ^{o}C – 16 ^{o}C in the winter fish, Q_{10} values of between 2 -3 were calculated.

3.12.7 SDA

Ventilatory rates in the summer caught acclimated fish and the wild fish were similar until the water reached just above 12 °C. At this point the wild fish had a slightly higher ventilatory rate and tried to escape whereas ventilation could be measured at 14 °C in the acclimated fish. The fish with SDA exhibited higher ventilatory rates at all temperatures, doubling in rate at 10 °C with rates soaring to 120 bpm at 12 °C (Figure 3.11).

3.12.8 Comparison of ventilatory rates between N. biniplicata and E. cirrhatus

The comparison of ventilatory rates between *E. cirrhatus* (Coxon and Davison, (2011) and the two seasonal populations of *N. biniplicata* show that *E. cirrhatus* has a higher ventilatory rate across the range of temperatures (Figure 3.10).

3.13 Statistical analysis

Results show a positive relationship between temperature and ventilatory rate with the winter population exhibiting higher rates across the temperature range except at 12 °C where values are similar. The mean ± SE of ventilation rates are shown in Table 3.1 and Fig. 3.7. The relationship between temperature and ventilatory rate was analysed using one sample *t* test and non-linear regression shows a significant relationship between resting ventilation and temperature, $r^2 = 0.9827$, $P < 0.001$ (summer population), $r^2 = 0.8836$, $P < 0.001$ (winter population), (Fig. 3.8). Log_{10} transformation of the data shows a significant relationship between temperature and ventilatory rate: summer population, $r^2 = 0.09718$, $P = < 0.001$; winter population, $r^2 = 0.8133$, $P = <0.001$, (Fig. 3.9).

Table 3.1. Velar rates at each temperature, mean ± SE

Temp.	Summer population velar rate		Winter population velar rate	
(°C)	Mean	± SE	Mean	± SE
20	36.00			
18	45.33	8.37		
16	49.10	9.26	57.00	
14	37.75	8.35	56.67	13.38
12	34.00	3.00	37.75	11.87
10	22.50	7.92	37.59	7.20
8	17.64	6.06	31.10	5.74
6	15.00	5.12		

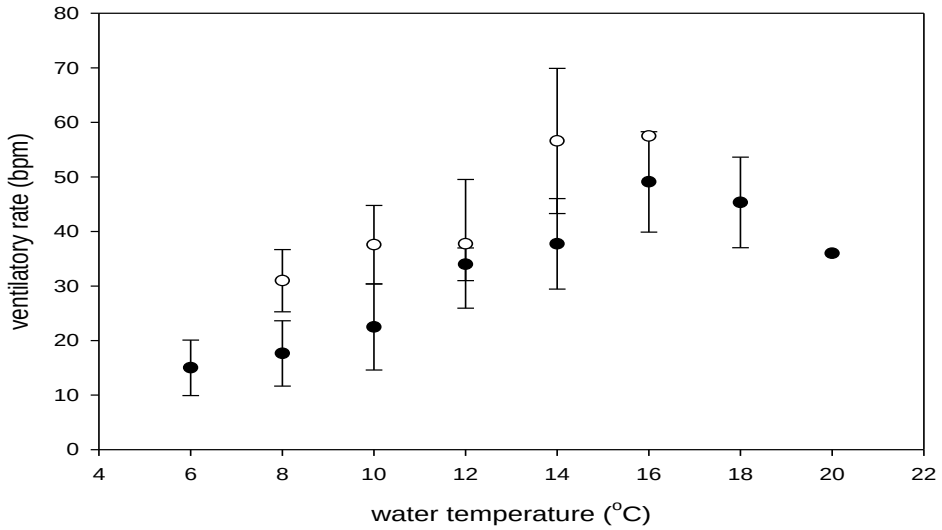

Fig. 3.7. Resting velar rates, mean ± SE, comparison between summer (black dot) and winter (open dot) populations of *Neomyxine biniplicata*, n = 12. Summer population: range 7–94 bpm; winter population: range 10.5–78 bpm.

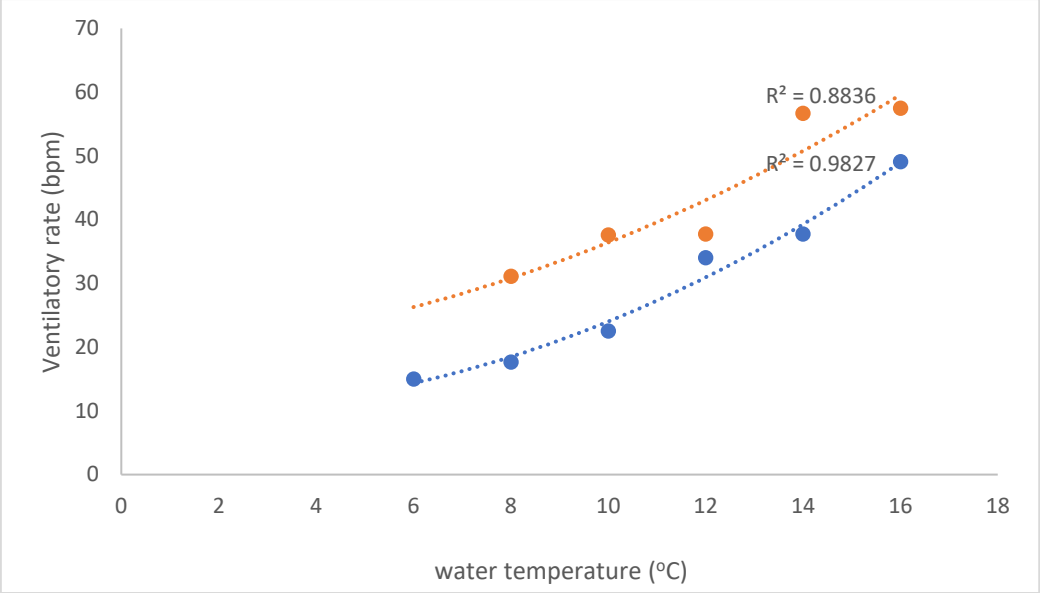

Fig. 3.8. Non-linear regression of ventilation rates in summer population (blue dot), n = 6, winter population (orange dot), n = 6.

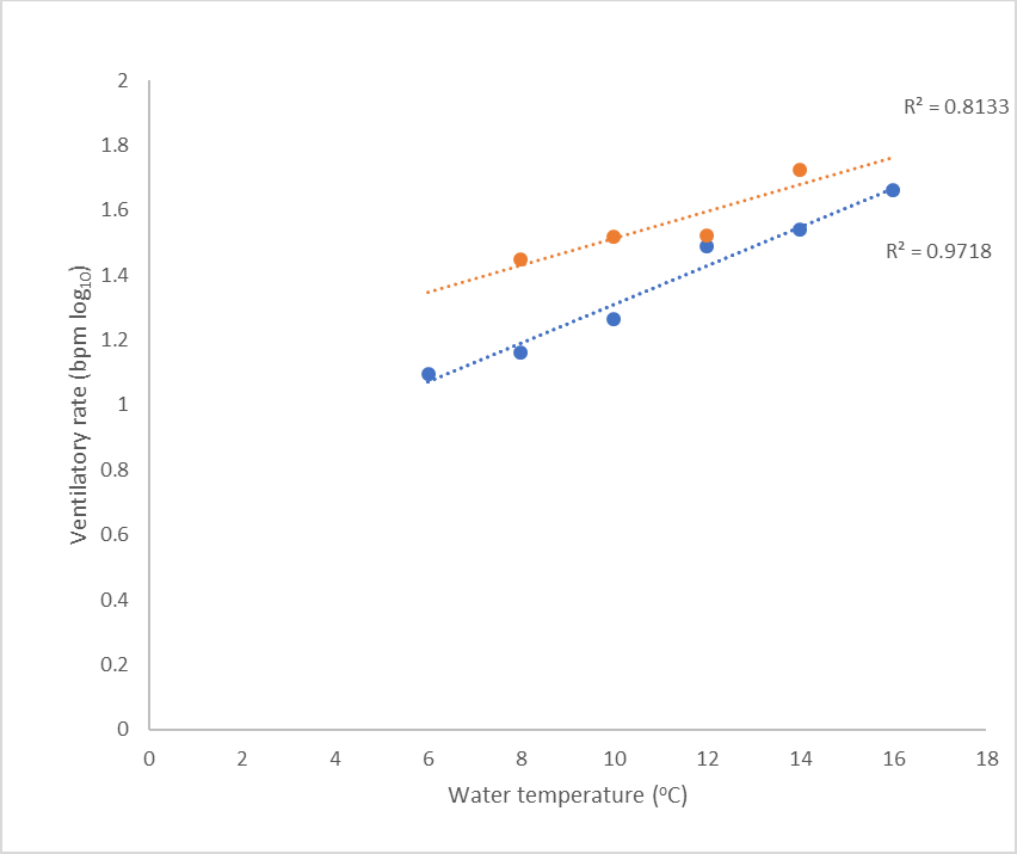

Figure 3.9. Log_{10} transformation of ventilatory rates response to acute temperature changes: summer population (blue dot) n = 6, winter population (orange dot) n =6.

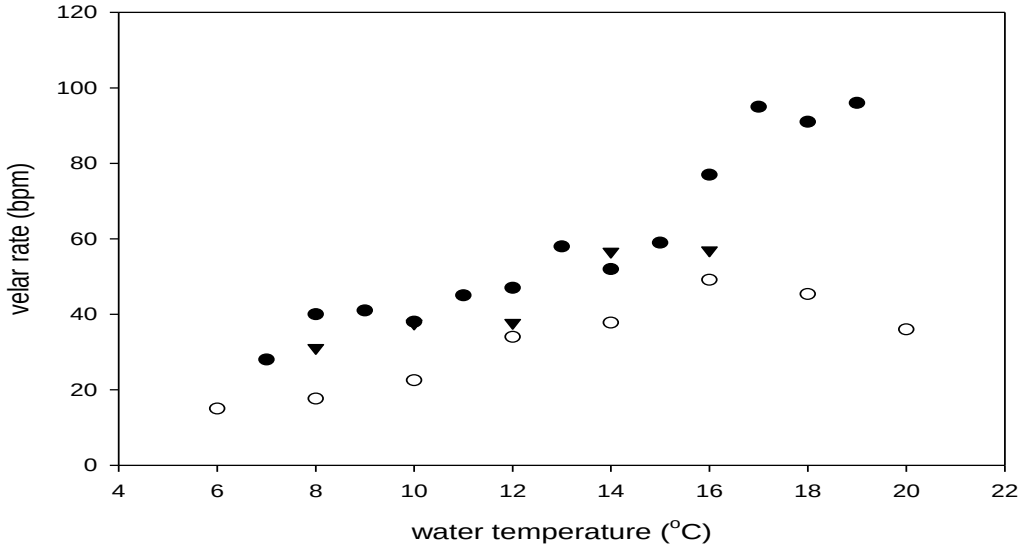

Fig. 3.10. Water temperature and velar rate (mean bpm): comparison of two New Zealand hagfish: *N. biniplicata*: Summer population, open dot; Winter population, black triangle; *E. cirrhatus*: closed dot, data modified from Coxon & Davison (2011).

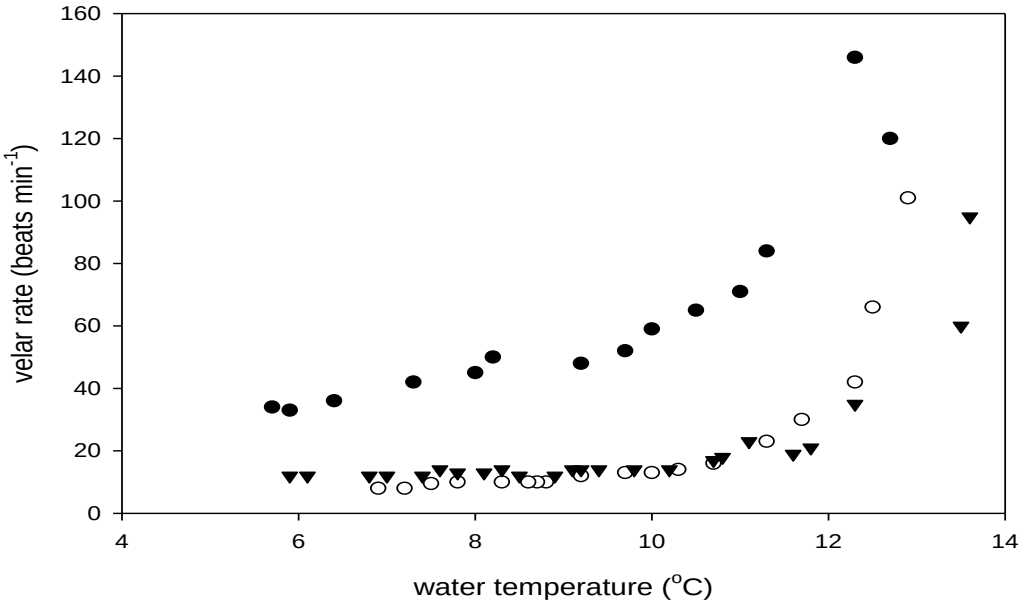

Fig. 3.11. Velar rate (mean bpm) in resting *N. biniplicata*, open dot: new and unacclimated fish; black triangle: acclimated fish; black dot: acclimated fish with SDA, n = 3.

3.14 Discussion

This is the first study of ventilatory rate in *Neomyxine biniplicata*. By utilising the relatively placid nature and translucent velar anatomy of this species of Myxininae, this experiment has been accomplished very simply without restraining or implanting electrical monitoring devices. Only a small piece of cling film was used to both pacify the fish and prevent them from moving out of the respirometer and into the water bath.

3.14.1 Response to acute temperature changes and differences between seasonal populations

Ventilation in response to acute alteration in water temperature differed between the summer and winter populations of *N. biniplicata*, as did the range of water temperatures tolerated.

The summer population was able to bear immersion into wider range of temperatures, between 6 °C – 20 °C, although only one fish, the smallest fish in the study, 14.63 g in mass, could be submersed into water at 20 °C and this was only for a few minutes. In water below 10 °C, ventilation was generally under 15 bpm and in some fish the musculature around the velar chamber contracted and became dark red in colour making velar movement very difficult to observe. Some fish, in temperatures below 8 °C, produced a very fine veil of slime about their body and some became quite shiny and stiff to the touch. Mostly, fish did not attempt to escape from colder temperatures, which was different to their response to warmer water where they would explore the top of the respirometer with their tentacles before the imperative to escape became more urgent and they began to swim.

In contrast, the winter population could only tolerate water temperatures between 8 °C and 14 °C, with only one specimen able to withstand immersion into water at 16 °C and that was only for a matter of a few minutes. Only four of the winter fish were able endure water at 14 °C and again that was only briefly. Despite the distress caused by immersion in warm water, very few fish released slime.

In both seasonal populations there is a positive relationship between temperature and ventilatory rate (Figure 3.6). The ventilatory rate in the winter fish was higher across the range of temperatures measured. In the summer population ventilatory rate reached a plateau at 16 °C before declining. Ventilatory rate rose exponentially at 14 °C and measurements at temperatures warmer than this could not be obtained in the winter fish. The plateau indicates that the cardiorespiratory system has reached capacity and can no longer supply oxygen to the tissues at the extreme physiological limits and metabolic performance has become impaired as illustrated in Figure 3.1 B (Pörtner & Farrell, 2008, Pörtner 2010; Clark et al., 2013). A Q_{10} of 2 - 3 was measured in both populations of fish illustrating that ventilatory rate is tied to water temperature and metabolic rate through oxygen requirement and in hagfish increased oxygen is delivered to the tissues via an increased ventilatory rate (Rao & Bullock, 1954; Fry, 1947; Schmidt-Neilson, 1975; Perry, Jonz, & Gilmour, 2009).

3.14.2 Acclimatisation to seasonal temperatures

The seasonal populations of *N. biniplicata* are showing acclimatisation to seasonal temperature changes, with the summer fish being able to navigate a wider range of temperature. This may be related to temperature of the sea floor in the area in which the populations of *N. biniplicata* were caught. It also brings up the question of why the winter fish were not better acclimatised to cooler water temperatures or why are they acclimatised to such a narrow temperature range?

Three possibilities emerge: (1) The physical condition of the fish has affected results. (2) The lowest temperature encountered by these fish, in this region, is close to 10 °C in the cooler months, elevating slightly during spring and summer time. (3) Another possibility, given the rarity of the animals during the winter months, is that they migrate to deeper waters that are more stable in temperature during winter (Fernholm, 1974).

Two studies of the seasonal effect on hagfish condition are available. Specimens of *M. glutinosa* caught in the Bay of Fundy, Canada, showed natural periods of fasting

during winter (Foster & Moon, 1986), whilst specimens of *E. cirrhatus* show the greatest increase in weight occurs during summer and autumn (Martini & Beulig, 2013). In both instances it was thought that food may not be readily available in winter, which may be illustrated by lower weights in fish caught in the spring. *E. burgeri* is the only species of Myxininae known to migrate to deeper waters between July and October in the northern hemisphere summer. Whether this is to escape warmer waters or for breeding is unknown (Fernholm, 1974).

Behaviour towards food differed between the summer and winter populations of *N. biniplicata.* Fish caught in the warmer months always responded to food being dropped into the tank even if they were not hungry. The fish caught in the cooler months often did not appear to notice the arrival of food and in one instance removed themselves to the other side of the tank when a slice of sardine (*Clupea sagax*) was dropped into the tank close to where they were resting. Also, dissection of deceased specimens of *N. biniplicata* from the winter population revealed an entirely empty gut. This behaviour may be a seasonal response, or the winter fish are fasting, as seen in other species, and are not in such good physical condition. Certainly, the summer fish were more vigorous, ate enthusiastically and, on occasion, slimed with wild abandon.

Neomyxine biniplicata are a demersal fish that inhabit the sea floor at depths between 35 m and 396 m within waters to the east coast of New Zealand (Roberts et al., 2015). The specimens that were conscripted for this research were drawn up from depths between 20 m and 22 m. The summer population seem to be acclimatised to water of 12 °C–14 °C with a mean velar rate of around 30 bpm but lower in individual fish. Although the velar rate fell below 20 bpm at 10 °C, behaviour of the fish and the contraction of the velar anatomy may indicate that this species of Myxininae, caught in this location, does not normally encounter water temperature much below 12 °C. By comparison, the winter population of fish, which may have been fasting, seem to be acclimatised to water temperature that does not move far from 12 °C.

The smallest fish in the study, 14.63 g, had a ventilatory rate comparable to that of the larger fish at between 6 °C and 16 °C and a slower ventilatory rate at 18 °C to 20 °C and was the only fish in the study to cope with immersion at 20 °C. In their study of *E. stoutii*, Munz and Morris (1965) found no significant relationship between size and metabolic rate and concluded this may indicate that species of Myxinidae are unable to acclimate to altered water temperature and there is no selective advantage conferred by doing so, considering the stable water temperatures inhabited. Their specimens of *E. stoutii* were pulled up from depths of 70 m compared to 20 m inhabited by the specimens of *N. biniplicata* in this study. The ventilatory rate is higher in the winter population than in the summer population at all temperatures that could be tested. Therefore, it may be that the seasonal populations of *N. biniplicata* are exhibiting seasonal acclimation within a range of 1 °C–2 °C. This may be because of the relatively shallow depth they naturally inhabit, or it could be that the winter fish, which seemed to be fasting and were less robust in condition, found the acute temperature alterations a greater challenge.

The behaviour of these fish in water temperatures below 10 °C may indicate that they do not encounter water of this temperature in the ocean at the depths inhabited. It is possible that *N. biniplicata* occupying deeper regions of the ocean floor may exhibit a different range of temperature to which they are acclimatised. Considering the difficulties in procuring specimens of *N. biniplicata* for this research, it may be that this species, in the cooler months, migrates away from the shore to deeper water that is more stable, or slightly warmer. The Japanese hagfish, *E. burgeri*, removes from seasonally warm waters, and this may indicate that *N. biniplicata,* as with *E. stoutii (*Munz & Morris, 1965), have a limited potential to thermally acclimate.

3.14.3 Ventilation in other species of Myxinidae

Few studies of ventilation in Myxinidae have been attempted. Slime production seems to be an obstacle. The dark pigmentation of certain species of hagfish

including *E. cirrhatus*, the common hagfish, would likely prevent clear observation of the velum although not all specimens are solid in colour (Martini & Beulig, 2013).

Two studies of the effect of temperature on ventilatory frequency in Myxinidae exist: the study of *Eptatretus cirrhatus* (Coxon & Davison, 2011) and the work on *Myxine glutinosa* (Steffensen et al., 1984) Two studies of ventilatory frequency of *M. glutinosa* in captivity have been published (Gustafsen, 1934; Strahan, 1958), and there is one study of the effect of hypoxia on ventilatory frequency in *E. stoutii* (Perry, Vulesevic et al., 2009), which is discussed in Chapter 4.

3.14.4 Ventilatory rate in the native E. cirrhatus

In *E. cirrhatus*, a species that inhabits water at depths ranging between 1 m and 922 m (Zintzen et al., 2015), Coxon and Davison (2011) used electromyography to record ventilation in response to acute alterations in temperature. The fish were an order of magnitude larger in mass, and ventilatory rate was recorded approximately as graphed in Figure 3.9. Specimens of *E. cirrhatus* demonstrated an exponential rise in ventilatory rate, which stabilised at 17 °C, approximately 2 °C above the point at which the ventilatory rate in *N. biniplicata* reached a plateau. At 17 °C the ventilatory rate in *E. cirrhatus* was 90 bpm compared to 45 bpm in *N. biniplicata.* The data suggests that the natural thermal range of these specimens of *E. cirrhatus* is likely to be between 8 °C and 16 °C with *N. biniplicata* inhabiting water temperatures between 10 °C and 15 °C.

3.14.5 Ventilation in M. glutinosa

By observing velar pulsations visible beneath the skin close to the snout, Gustafsen (1935) recorded a ventilatory rate of 25 bpm–30 bpm in captive *M. glutinosa* held at 6 °C to 8 °C. Also, in *M. glutinosa* held at 8 °C, Strahan (1958) recorded 11 bpm–15 bpm by counting the respiratory current in animals as they inhaled and exhaled methylene blue. The conditions under which Gustafsen (1935) obtained ventilatory data may not have been conducive to providing a close proximation of a resting rate; however, the Strahan (1958) data is similar to that of specimens of *N. biniplicata*.

Ventilatory frequency in response to altered temperature was studied in *M. glutinosa* using flow transducers (Steffensen et al., 1984). Results from these experiments show that ventilation increased with rising water temperature (18 bpm at 7 °C, 70 bpm at 15 °C and 76 bpm at 20 °C, mean ± SE) and this was the outcome of thermal stress. The fish in the Steffensen study were close in mass to *N. biniplicata* and the ventilatory frequency at 7 °C is similar but higher at 15 °C and 20 °C, although not dissimilar to the rate measured in some individual fish. Specimens of *M. glutinosa* were caught at 20 °C and held for a period of three weeks prior to experimentation at 7 °C and 15 °C.

3.14.6 Effect of SDA on ventilatory rate in N. biniplicata

In Figure 3.10. the effect of SDA on ventilatory rate is clearly illustrated. The ventilatory rate more than doubled after feeding. In the acclimated fish and the newly caught fish, the velar rate was very similar below 10 °C and this may be because this species of fish does not usually encounter water of this temperature in the ocean. Above 11 °C, the resting velar rate in the acclimated fish increased at a slower rate and is lower than that of the recently caught fish, which exhibit both a higher resting velar rate and an exponential rise in velar rate at and above 13 °C. The fish that had been in captivity for several weeks may be illustrating the effects of long-term acclimation or were less disturbed by activities within the aquarium.

3.15 Summary

Specimens of *Neomyxine biniplicata,* in both seasonal populations, alter ventilatory rate in response to acute temperature changes; hyperventilating above 10 °C-14 °C whilst below 10 °C, the ventilatory rate falls under 15 bpm. The ventilatory range of all fish was 0 bpm to 146 bpm. The water temperature of 12 °C, the temperature at which the fish were held, may replicate that of the natural environment. The mean resting ventilatory rate is close to 20 bpm. A water temperature of 16 °C in the summer population (14 °C in the winter population) is the top of the thermal range in this species of hagfish where environmental temperature has reduced the capacity

of the respiratory and cardiovascular systems to deliver oxygen to the tissues and this has deleteriously affected the metabolic rate (Schmidt-Neilson, 1975; Pörtner, 2010).

The fish caught within the warmer months were in good physical condition and were able to be immersed into a wider range of water temperatures compared to the winter population. The reason for the disparity in ventilation rates and range of temperature between the populations may be that the winter fish were in fasting condition and could not physically navigate a wider range of temperatures because of reduced physical condition and exhibited a higher ventilatory rate as the metabolic strain imposed by acute temperature changes was greater.

The Q_{10} values of between 2 – 3 were measured in both populations and these values show that increased environmental temperature accelerates the metabolic rate and the demand for more oxygen is met through an increased ventilatory rate (Rao & Bullock, 1954; Fry, 1947; Schmidt-Neilson, 1975; Perry, Jonz et al., 2009). The effect of SDA was measured in one fish and was found to increase ventilatory rate substantially with at least a doubling of velar rate (bpm) across all temperatures.

The difference in ventilatory rates measured in the seasonal populations of *N. biniplicata* shows that seasonal acclimatisation occurs within a very narrow range of temperatures - 1 °C to 2 °C – which probably reflects small seasonal fluctuations in the water temperature of the bay. Bearing in mind that *E. burgeri* migrate into deeper water during the summer months it may be that *N. biniplicata* do not possess the ability to acclimate to water temperatures below 10 ^{o}C and prefer to relocate to an area within Pegasus Bay that is more stable in temperature.
Monitoring velar movement is a useful indicator of the water temperature to which the fish is acclimated, and possibly, the health of the fish. It also gives a clear indication of the tolerable range of temperature before subjecting fish to experiments that may require an overnight acclimation and prevent the submersion of fish into water temperatures, that may have deleterious effects, for many hours.

Cling film maybe an environmentally obnoxious product but it is indispensable when attempting to pacify and settle indignant hagfish.

Chapter Four

Routine metabolic rate and critical oxygen tension in *Neomyxine biniplicata:* two experimental methods

4.1 Introduction – Metabolic rate

The metabolism of an organism encompasses all the energy required to support homeostatic balance and maintenance of the systems (Jobling, 1993). Chemical energy is supplied to the cells by the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) (Schmidt-Nielsen, 1975, Dzal et al., 2015). In aerobic life-forms, oxygen is used as the terminal electron acceptor in the electron transport chain to synthesise ATP (Mitchell, 1961; Mitchell, 2011). The synthesis of ATP is regulated by its cellular concentration, increasing or decreasing in response to metabolic demand (Clarke & Fraser, 2004). All stages of the life history, the surrounding environment, temperature and diet influence energy metabolism and the metabolic rate (Schmidt-Nielsen, 1975; Clarke & Johnston, 1999). The physiology and behaviour of aquatic ectotherms are controlled primarily by temperature and restricted by the level of dissolved oxygen in the environment (Fry, 1947; Fry, 1971). The aerobic metabolic scope concept was designed by Fry to describe and analyse how the environment regulates aquatic ectotherms through metabolism (Claireaux & Chabot, 2016).

4.2 Oxygen consumption as a proxy for metabolic rate

In organisms reliant upon oxygen, the metabolic rate is the total oxygen consumption (MO_2) per unit of body mass per unit of time (Schmidt-Nielson, 1975). This being so, oxygen uptake can be measured and used as a substitute to understand the metabolic rate and how it may be affected by environmental variations (Fry, 1971; Steffensen, 1989; Nelson, 2016).

Metabolic rate can be calculated using a variety of units – here we use mg O_2 kg^{-1} hr^{-1} – and the rate measured may be routine, standard or active (Fry, 1971; Fry & Hart, 1984; Steffensen, 1989). The routine rate of metabolism (RMR) is the mean rate of metabolism in a resting fish sheltered from external perturbations, and the actual rate obtained will depend upon which part of the circadian and diurnal cycle the measurements are taken in. The standard metabolic (SMR) is an approximation of the absolute minimum metabolic rate the subjects being post absorptive and inert. An active (Fry, 1971), or maximal (Claireaux & Chabot, 2016), metabolic rate (MMR) is measured in swimming fishes. At SMR, ATP is supplied for maintenance of the systems, whilst MMR is an estimation of the maximal rate at which ATP can be supplied to the systems throughout activities that extend beyond homeostasis (Claireaux & Chabot, 2016). Although, and particularly in the daytime, hagfish may tend towards comatose, spontaneous movement may occur and here we measure the routine metabolic rate. Amongst vertebrates, hagfish have an extremely low metabolic rate, which, in part, extends from environmental osmoconformation (Munz & Morris, 1965; Robertson, 1963; Steffensen, 1984; Forster, 1990).

One difficulty of using metabolic oxygen consumption as a proxy for metabolic rate when exposing fish to hypoxic and anoxic conditions is that the metabolic pathways used during anaerobic respiration will be concealed and not included in the calculated rate (Nelson et al., 2016).

4.3 Specific dynamic action

Metabolic rate is influenced by the ingestion, digestion and absorption of food. The metabolism converts ingested food or stored reserves into energy and oxygen is required for the process (Nelson, 2016). To meet the physiological requirements of metabolising and assimilating nutrients, oxygen consumption increases, and the process is described as specific dynamic action (SDA). The extent and period of SDA is dependent upon the amount of food consumed, the surrounding temperature and the size and species of animal (Jobling, 1993; Boyce & Clarke, 1997). Prior to the

ventilatory series detailed in Chapter 3, the effect of SDA on ventilation rates was monitored. Ventilatory rates were found to be elevated 7 days post feeding and 10 to 14 days were set aside to allow the metabolic rate to settle to a post-absorptive state.

4.4 Environment, temperature and metabolic rate

As it is with all sentient beings, the metabolic state of the myxinid is both influenced by, and reliant upon, the surrounding environment (Fry, 1971). Whilst homeostasis is the perpetual flux between systems to maintain a steady-state within an organism, that organism is subjected to constant physiological change from the natural cycles of life: movement, the ingestion and absorption of food; growth and reproduction, daily and seasonal temperature changes (Jobling, 1993). The OCLTT hypothesis suggests that the aquatic ectotherm's capacity to supply oxygen to the tissues is compromised at the extremes of the temperature range of the species (Pörtner, 2010).

As ectotherms, the metabolic rate of myxinids is manipulated by the temperature of water in which they are immersed. Alterations in temperature will bring about changes in metabolic rate that quickens and slows in response to heating or cooling of the surrounding environment, and this will either increase or slow biochemical and enzymatic reactions within the body (Jobling, 1993; Clarke & Johnston, 1999). A raised metabolic rate will require increased oxygen that is supplied by accelerated ventilation, and the effect can be calculated using the van't Hoff Q_{10} equation (Fry, 1947; Schmidt-Nielsen, 1975). In response to an increase in temperature of 10 °C a rise of two- to threefold in metabolic rate is not unusual (Schmidt-Neilsen, 1975), as demonstrated by the ventilatory experiments in Chapter 3.

The solubility of oxygen in water is inversely related to temperature (Henry's Law) and as water temperature increases so oxygen solubility is reduced (vanLoon & Duffy, 2007). In most aquatic ectotherms the increased biological demand for oxygen is driven by elevated water temperature and is responded to by hyperventilation, hagfish being unable to increase stroke volume through constraints of the ventilatory

anatomy (Lomhalt & Johansen, 1979; Malte & Lomhalt, 1998; Perry, Vulesevic et al., 2009); this was demonstrated by specimens of *N. biniplicata* and described in Chapter 3.

4.5 The state and effect of hypoxia

Hypoxia is the state of a medium characterised by low oxygen saturation whilst anoxia is the complete absence of oxygen. There is no precise point at which water becomes hypoxic (Seibel et al, 2016). Depending upon barometric pressure, fully oxygenated seawater has a dissolved oxygen tension (PO_2) of ~150 mmHg (Claireaux & Chabot, 2016). For aquatic ectotherms, severely hypoxic conditions are considered to lie in the range 30–10 mmHg (Malte & Lomhalt, 1998).

The metabolic rate alters in response to the oxygen tension of the surrounding environment. If oxygen is freely available, aquatic ectotherms regulate intake and are independent of environmental oxygen (Mandic, Todgham, & Richards, 2009). When the level of oxygen saturation declines below a level that is particular to a species, the fish are forced to conform and use oxygen at the minimum sustainable level of metabolic oxygen consumption (Steffensen, 1989; Nelson, 2016).

4.6 Critical oxygen tension (P_{crit})

The transition between regulating oxygen use and conforming is termed the critical partial pressure of oxygen or critical oxygen tension (P_{crit}). The critical oxygen level is the point at which the availability of oxygen is insufficient to complete normal metabolism and oxygen consumption is not independent of oxygen tension (Pörtner, Heisler, & Grieshaber, 1985; Seibel et al., 2016). At oxygen saturation below the critical tension, the animal begins to conform, finally switching to anaerobic metabolism which, before becoming unsustainable, alters the acid–base balance and homeostatic equilibrium (Perry & Tzaneva, 2016). When under acute hypoxic constraint, fish can increase gill surface area and ventilatory rate and improve their oxygen extraction from the blood but eventually this becomes unsustainable (Perry,

Vulesevic et al., 2009). The tolerance of aquatic ectotherms to hypoxic conditions has been shown to decrease as environmental temperature increases. In a study of Crucian carp (*Carassius caraassius*), Yang, Cao, & Fu, (2015) found that higher temperatures were positively correlated with higher P_{crit} values. A lower P_{crit} has been usually associated with a greater tolerance of hypoxia. Although this does not stand across all aquatic ectotherms, it is a useful comparison of the response of the metabolism to hypoxic conditions (Chapman, Chapman, Nordlie, & Rosenberger, 2002; Mandic et al., 2009; Speers-Roesch et al., 2013).

4.7 Myxinidae sub-familial differences to hypoxia

Myxinids and eptatretids occupy different habitats and although both experience hypoxia and anoxia when enclosed within a decaying carcass, myxinids are considered to be better adapted to conditions of low oxygen and this is indicated by a lower P_{crit} (Davie, Forster, Davison, & Satchell; Forster, 1989; Forster et al., 1997; Forster, 1998; Malte & Lomhalt, 1998; Drazen, Yeh, Friedman, & Condon, 2011). In contrast, hyperoxia may be harmful (Mamum, Focken, & Becker, 2013), and instances of *N. biniplicata* becoming trapped in a column of rapidly flowing oxygen bubbles have been observed and described in Chapter 5.

4.8 Respirometry

Respirometry is an established method of measuring and quantifying the metabolic rate in aquatic organisms (Clarke et al., 2013; Svendsen et al., 2016). The experimental animal is contained within a closed chamber and oxygen consumption is measured and quantified. The reduction of the partial pressure of oxygen and the capacitance of oxygen in the water allows for the calculation of the metabolic rate which must be reliable and repeatable (Steffensen, 1989; Clarke et al., 2013). The resting period must be sufficient to allow the fish to recover from the stress of transfer and correspond to a time of naturally low activity (Claireaux & Chabot, 2016).

4.8.1 Respirometry – conditions, considerations and conventions

During this research, a substantial amount of time was devoted to designing and building an experimental set-up that would allow the hagfish to produce consistent data whilst in a sedentary and resting state. The results obtained from the initial conventional 24-hour acclimation period were inconsistent and subverted by the habit of the animals and the transfer of heat through friction by the peristaltic pump (Parker, Boggs & Blick, 1969).

4.8.2 Open vs closed system

The open system allows for the removal of waste products and re-oxygenation of the chamber. This method measures the difference between the rate of flow and the gas content of the water (Ege & Krogh, 1914; Steffensen, 1989). Within the closed system the experimental subject controls the rate at which hypoxia is induced and waste products accumulate, and the effects of these cannot be separated from those of hypoxia (Steffensen, 1984; Steffensen et al., 1989; Rosewarne, Wilson, & Svendsen, 2016). Trials within a closed system are of shorter duration and result in a greater decrease in oxygen saturation which may affect measurements (Clark et al. 2013). However, only in anaerobic conditions does CO_2 become a problem, and reduced oxygen is a limiting factor, not increased CO_2 (Fry, 1971). In the case of *N. biniplicata*, and other hypoxia-tolerant organisms, these conditions may replicate benthic conditions and the combination of reduced oxygen and increased CO_2 may be ecologically relevant to myxinids (Mandic et al., 2009; Gillis et al., 2015; Snyder et al., 2016).

4.8.3 Background respiration and leakage

Microbial colonisation of surfaces and lines can significantly interfere with results. Water temperature, the experimental set-up and cleaning practice have a bearing on background respiration (Fry, 1947; Clark et al., 2013; Rodgers, Tenzing, & Clark, 2016; Svendsen, Bushnell, & Steffensen, 2016). Depending upon the partial pressure of the gas and water inside a sealed respirometer, air bubbles will either take up or

leak oxygen into the water and must be removed before the vessel is sealed (Fick, 1855; Rodgers et al., 2016). The seal of the respirometer was tested with blue food colouring and found to be secure. Tubing that connected the respirometer to the peristaltic pump may have absorbed oxygen to some extent. However, in Method II all tubing was eliminated and this, combined with accurate temperature control and rested hagfish, make the second experimental method in this chapter the preferred set-up when examining *N. biniplicata* under confined conditions.

4.8.4 Cleaning and maintenance of respirometry equipment

After each experiment a quantity of the seawater (30 L) was renewed, unless the fish had released slime or obvious waste products, in which case the entire system was drained, dismantled, scrubbed, and dried. Every 10 days the entire system was cleaned with Chlor-o-gene non-surfactant bleach, rinsed multiple times, and dried. Biofilm that formed within the lines was disturbed by the insertion of tubing of a narrower diameter and then flushed out. To detect background respiration the equipment was run empty after cleaning and no evidence of microbial respiration was detected.

4.8.5 Respirometer – shape and volume

To capture data that is physiologically relevant and representative of the species under investigation, the experimental conditions must be conducive to complete relaxation, and the morphology of the fish must be considered (Svendsen et al, 2016). Because of the technical difficulties associated with hagfish – slime and the extreme anatomical flexibility – there is very little respiratory data available (Perry, Vulesevic et al., 2009). The shape and size of the respirometer must be such that it comfortably accommodates the fish. To avoid an extended period of confinement the volume of water should be minimal. Unless water is mixed within the respirometer, stratification of oxygen and carbon dioxide will occur and introduce error into the calculation of metabolic rate (Fry, 1971). It is also important that the method of mixing the water within the respirometer does not interfere with, or irritate, the

animal (Clark et al., 2013). It is equally important that the method of circulating the water does not introduce heat into the system (Parker, et al., 1969).

Trials with a tubular respirometer proved to be unsuccessful. Initially it seemed a good idea to replicate the plastic pipes in which the fish rest inside the aquarium. However, the fish did not settle for long and were continuously turning around and moving along the tube, and although the volume of water was considerably reduced, which in turn reduced the time the fish were confined to the respirometer, the magnetic stirrer did not function properly inside the tube and water was not properly circulated.

Hagfish settle quickly and comfortably within a circular respirometer and assume a coiled position around the perimeter of the vessel. The magnetic propeller whirls about in the centre without bothering the animal. Whether they were attracted to it, or it was just chance, the fish often positioned themselves directly beneath the oxygen probe. The respirometer used in all hypoxia experiments was 0.540 L in volume which allowed the fish to comfortably rest and move about whilst not being so large that experiments would not extend beyond a few hours. Trials with an internal pump were unsuccessful, the fish being very sensitive to noise and vibration, and attempts to install a baffle failed dismally.

4.8.6 Acclimation to the respirometer

Before the respirometer is sealed the fish must rest inside the vessel and this will allow the animal to return to a usual pattern of respiration (Claireaux & Chabot, 2016). The resting period should correspond to a time where the fish are normally inactive and be of long enough duration to enable the fish to recover from the anxiety associated with capture; a period of 24 hours recovery is often observed (Fry, 1971; Clark et al., 2012).

Circadian and diurnal rhythm alter metabolic oxygen consumption, and lighting within the laboratory can also have an effect (Clark et al., 2013; Svendsen et al., 2016).

There are several reports of nocturnal activity by hagfish held in aquaria (Gustafsen, 1935; Kabasawa et al., 1993; Fernholm, 1974). Although *N. biniplicata* were not observed at night in the aquarium, the LabChart trace (Figure 4.1) shows long periods of rhythmical and elevated oxygen consumption. *Neomyxine biniplicata* are sensitive to light, and all experiments were run in a dimly lit laboratory. Specific dynamic action (SDA) will interfere with oxygen consumption and therefore fish must be in a post-absorptive state and the length of time for this to occur will vary between species (Jobling, 1981; Claireaux & Chabot, 2016). In *N. biniplicata* 10–14 days post feeding was adequate.

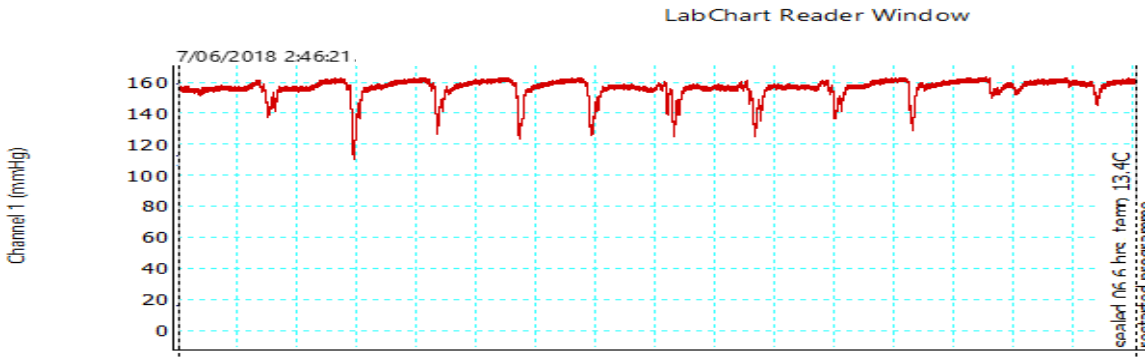

Figure 4.1. Example of activity and oxygen consumption by specimen of *N. biniplicata* during overnight acclimation in an unsealed respirometer. Oxygen pressure within the respirometer is measured in mmHg (y-axis), the red trace indicates oxygen consumption throughout the night during acclimation time.

4.9 Experiments Objectives

The inter-familial differences in hypoxia tolerance between the Eptatretids and Myxinids have been established (Strahan, 1958; Strahan, 1963b; Fernholm, 1998). No data exists of the metabolic rate and P_{crit} in *N. biniplicata*. In these experiments we sought to elucidate the routine metabolic rate and critical oxygen tension at three temperatures in specimens of *N. biniplicata* while testing and comparing two different experimental arrangements.

4.10 Materials and Method

Dissolved oxygen within the respirometer was measured by Firesting fibre-optic oxygen meter (Pyro-science; www.pyro-science.com), and rate of oxygen consumption was captured by AD Instruments Power Lab 4/25T with LabChart software.

4.10.1 Metabolic rate: Method 1: 20-hour overnight rest, single water-bath, peristaltic pump at 12 °C, n = 7

A single water bath was used in this series of experiments and the temperature was controlled by a Hilea refrigeration unit. Aerated water was circulated within the system by a peristaltic pump. The subject was weighed (wet weight) and inserted into the respirometer of 0.54 L volume, less the mass of the animal, 20 hours before each hypoxia experiment began. During the overnight resting period, oxygenated water was circulated through the unsealed respirometer by the peristaltic pump. The respirometer was sealed between 5.30 and 6.30 am when the fish were usually inactive. As the respirometer was entirely submerged, any accumulated air bubbles were easily removed (Figure 4.2).

4.10.2 Metabolic rate: Method II: 90 minute early-morning resting period, magnetic stirrer. at three temperatures: 11 °C (n = 7); 12 °C (n = 10); 13 °C (n = 7).

The experimental water bath set-up as described in section *3.9.5*, Chapter 3 was retained but modified to suit the requirements of respirometry (Figure 4.3). Depending upon the ambient temperature of the laboratory, the freshwater bath was held at either 1 °C or 2 °C below that of the temperature at which the experiment was being run. This was to compensate for heat taken up by the lines attached to the heat exchangers. The temperature of the blue water bath was maintained by the heat exchangers attached to a Grant GD100 unit set in the freshwater bath and preset to the temperature at which the hypoxia experiment was to be run. The orange water bath was held at 12 °C and into this bath the respirometer was placed. The blue water bath was set 22 cm below the orange water bath and the two were connected

by a gravity-fed siphon and the pump that drove the magnetic stirrer. Offsetting the levels of the baths allowed water to be circulated by the siphon and pump between the water baths in perfect equilibrium.

Ninety minutes before the respirometer was to be sealed, the fish was weighed (wet weight) and introduced into the respirometer. Generally, the fish settled within minutes and no slime was released. If a fish was inclined to release slime it was usually when first collected from the aquarium. A small piece of cling-film was used to prevent the animal from escaping into the water-bath. Once the fish had settled, the respirometer was submerged into the 12 °C water bath to complete the resting period. Ten minutes before the end of the resting period the refrigeration unit was reset to the experimental temperature that corresponded to the temperature of the blue water bath. The temperature of both baths quickly equilibrated when the gravity-fed siphon and the pump driving the magnetic stirrer were operating. The main reason for this more complicated arrangement of water baths was to reduce noise by placing the pump to the propeller into the blue water bath. An extra benefit was the stability of the water temperature.

Once the resting period was complete, the respirometer lid was screwed down and the system sealed. The fish reduced oxygen within the respirometer at will, although to prevent the fish going into anaerobic respiration, experiments were terminated when the oxygen tension fell below 19 mmHg, and the fish slowly returned to acclimatised temperature in fully oxygenated water. The results obtained by experiments in Method I and Method II were used to assess the P_{crit} where osmoregulation gives way to osmoconformity (Coolidge et al., 2007). An example of data captured by the Firesting oxygen probe and LabChart software is shown in Figure 4.4.

4.10.3 Time course of oxygen depletion at 12 °C: comparison of Method I and Method II, and at three temperatures (Method II).

The time to consume oxygen within the respirometer was compared between Method I and Method II. The experimental set-up of Method II was used to compare the effect of temperature on oxygen consumption at three temperatures: 11 °C, 12 °C, 13 °C and in four fish with probable SDA at 11 °C.

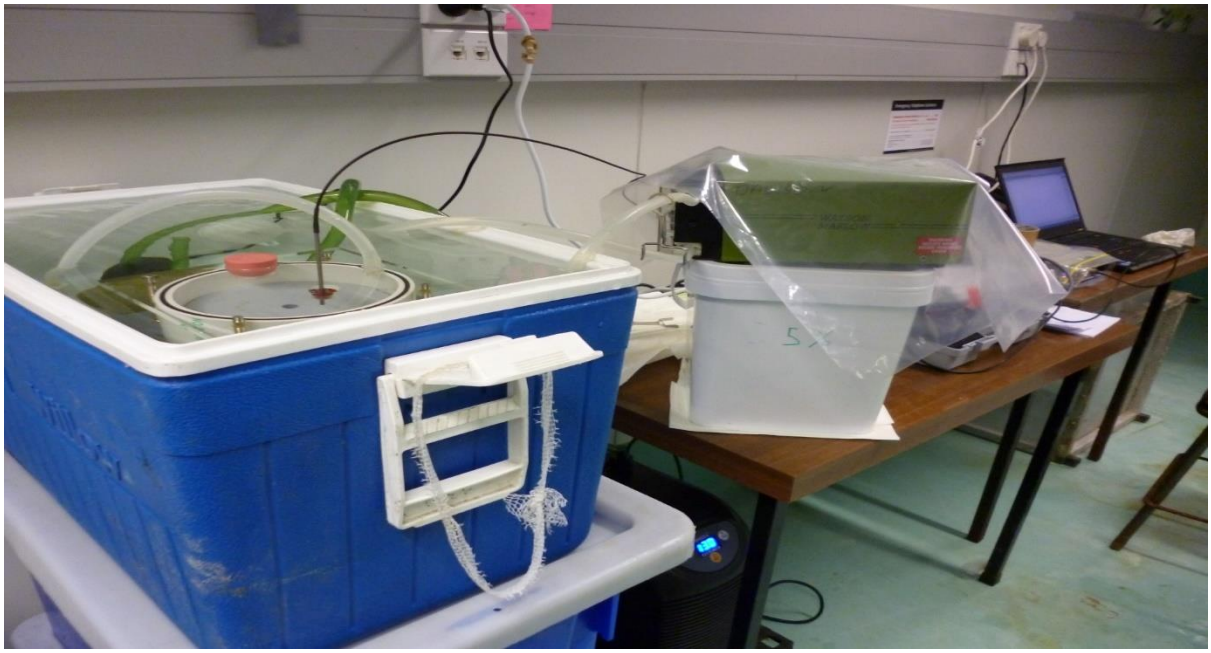

Fig. 4.2. Method 1 experimental set-up of one water bath, temperature maintained by Hilea chilling unit, oxygenated water circulated by peristaltic pump; Firesting oxygen probe and LabChart capturing data through AD Power Lab.

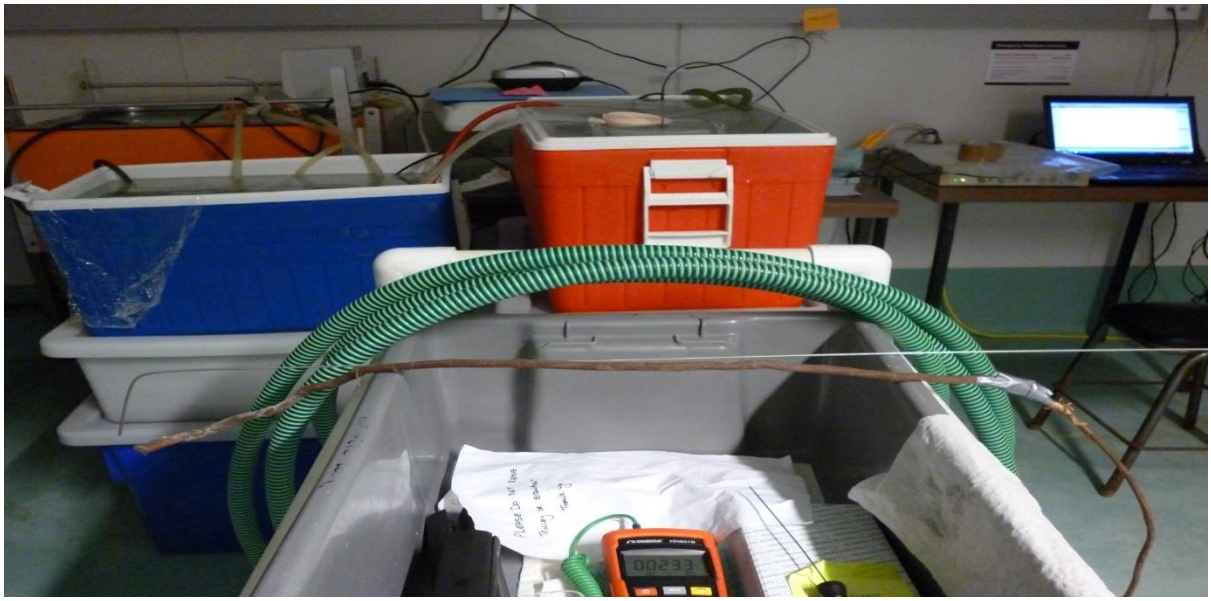

Fig. 4.3. Method II experimental set-up of two seawater baths connected by gravity-fed syphon; one freshwater bath maintaining temperature of the blue bath by two heat exchangers. The temperature of the orange bath is maintained by the Hilea chilling unit. The magnetic propeller is set on top of the submerged respirometer with the pump driving the propeller set in the blue bath. Firesting oxygen probe and AD Instruments Power Lab and LabChart software capture data.

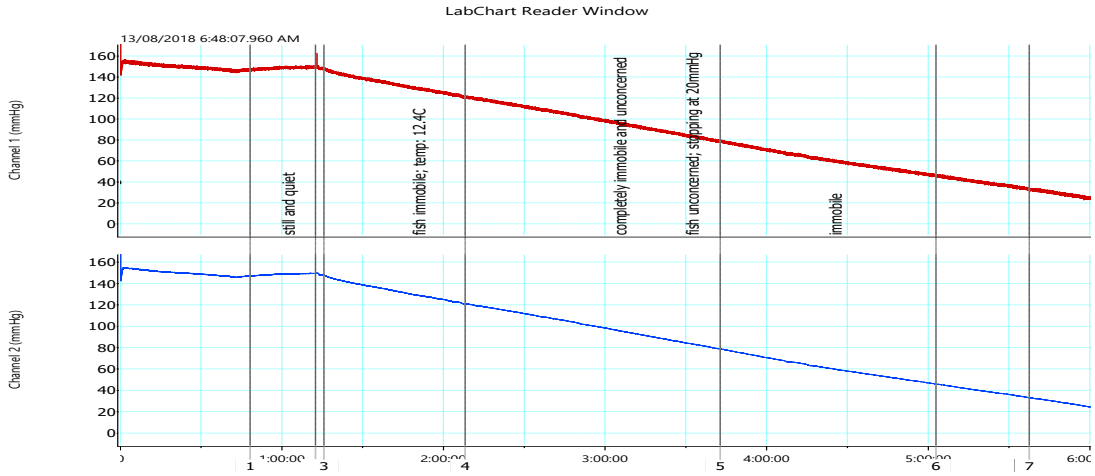

Figure 4.4. Example of LabChart oxygen consumption after 90 minutes of acclimation. Oxygen tension (mmHg) is shown on the y-axis and time (hours) on the x-axis. The red line (top) charts consumption of oxygen over time. The blue line (bottom), is a filter.

4.11 Fish Statistics

4.11.1 Method I with overnight acclimation and peristaltic pump

Ten specimens of *N. biniplicata* were used in this series of experiments. The mean weight: 55.75 g ± SE 3.84 g; range 35.58 g: maximum: 70.21 g, minimum: 34.83 g.

4.11.2 Method II, 90-minute rest with magnetic stirrer

Three populations of *N. biniplicata* were exposed to three different water temperatures:

13 °C: n = 7, mean weight: 46.71 g + SE 5.26 g; range 42.47 g, maximum: 67.02 g, minimum: 24.55 g.

12 °C: n = 10, mean weight: 48.62 g + SE 2.14; range 20.85 g, maximum: 60.60 g, minimum: 39.75 g.

11 °C: n = 7, mean weight: 50.353 g + SE 1.582 g; range: 11.620 g, maximum: 45.35 g, minimum: 48.03 g.

4.12 Statistical analysis and calculations

4.12.1 Statistical analysis

Data recorded by Lab chart of oxygen consumption were analysed by Sigmaplot ver. 14. Data are reported as mean ± S.E unless otherwise stated. Normality of data was examined by Shapiro–Wilks test and equal variance analysed by the Brown–Forsythe test. To test for statistically significant differences, one-way repeated measures ANOVA with all pair-wise Bonferroni post-hoc multiple comparison of critical oxygen consumption was applied. The relationship between temperature and metabolic rate was analysed using one sample *t* test and non-linear regression. Data were considered significant at alpha level of $p \leq 0.05$ and confidence levels of 95% to assess statistical significance (Behrens & Steffensen, 2007; Svendsen et al., 2016).

4.12.1 Calculation of oxygen consumption

The metabolic consumption of oxygen (MO_2) was calculated using the following equation

$$\Delta PO_2 = [(V_r - V_t) \times \Delta C\ wO_2] / \Delta_t \times M_f)$$

The ΔPO_2 = the change in oxygen partial pressure (mmHg) across the period of measurement.

ΔC= oxygen capacitance of seawater at the given temperature ($\mu mol\ L^{-1}\ mmHg^{-1}$).

$V_r - V_t$ = volume of water in the respirometer less the volume of the animal (L).

wO_2 (31.999) = the molecular mass of oxygen

Δ_t = the duration of the measurement (h).

M_f = the mass of the animal (g).

4.12.3 Determination of critical oxygen tension

To calculate P_{crit}, the basal oxygen requirement at routine metabolic rate or the mean rate of oxygen consumption above 120mmHg and in normoxic conditions was determined. MO_2 was plotted as a function of dissolved oxygen (Svendsen et al., 2016). (The same thing can be achieved by a segmented regression in Sigma Plot). To find the increments of PO_2 where MO_2 is significantly different to the routine MO_2, repeated measures ANOVA with Bonferroni post-hoc comparison was used to decipher the points at which metabolic oxygen was different to oxygen consumption at normoxic levels. A linear regression was fitted through these data and forced through the origin. Routine oxygen consumption was extrapolated across the PO_2 range and the P_{crit} was defined by the intercept of routine oxygen consumption and the linear regression (Behrens & Steffensen, 2007; Svendsen et al., 2016). Data were considered significant at alpha level of $p \leq 0.05$ and confidence levels of 95% to assess statistical significance.

4.13 Results

Comparison of experimental set-ups in Method I and Method II at 12 °C

4.13.1. Method I: overnight acclimation, water movement by peristaltic pump

Oxygen consumption by the fish was erratic with little correlation between temperature and resting metabolic rate: non-linear regression, $R^2 = 0.2384$ compared to $R^2 = 0.8439$ in the 90-minute acclimation group (Figure 4.7). At the time of sealing the respirometer, oxygen consumption was around 25 mg kg^{-1} hr^{-1} and a decline in oxygen tension occurred very slowly for the first hour then increased markedly without the fish swimming (Figure 4.5). There is no data point for 70mm Hg because the fish were consuming oxygen very rapidly at that point. The mean MO_2 varied between 15 and 68 mg O_2 kg^{-1} h^{-1}. From the data collected, the P_{crit} cannot be determined because the RMR could not be calculated. The metabolic oxygen consumption was established to be 25 mg kg^{-1} h^{-1} although this is unlikely to be a routine or standard metabolic rate. Data is shown in Table 4.1.

4.13.2 Method II: 90 min. early morning acclimation, water movement by magnetic propeller at three temperatures: 11 °C, 12 °C, 13 °C.

In comparison, oxygen consumption in the fish that had been rested for a period of 90 minutes at 12 °C before sealing the respirometer reduced dissolved oxygen at a steady rate: non-linear regression, $R^2 = 0.8439$, $P < 0.001$. (Figures 4.7, 4.10), and metabolic oxygen consumption was calculated to be 17 mg kg^{-1} h^{-1}.and the P_{crit} was 27.83 mmHg (Figures 4.6).

At 13 °C mean MO_2 was 23 mg kg^{-1} h^{-1} ($r^2 = 0.6868$); P_{crit}: 38 mmHg, compared to MO_2 at 11 °C of 15 mg kg^{-1} h^{-1} ($r^2 = 0.7807$) with P_{crit} at 48mmHg (Figure 4.10). The data collected in the hypoxia trials at 11°C (Figure 4.9) and 13 °C (Figure 4.8) suffer from a lack of points at the lower end and is very likely showing a type 2 error. The data has passed Shapiro–Wilks normality tests and Brown–Forsythe equal variance tests ($p < 0.001$) but repeated measures have not found a difference where a difference

exists because there are not enough data points at the lower end of oxygen tension. The means are statistically different at 20 mmHg, but error bars are tiny because of the low number of data points.

In the 11 °C, 13 °C and overnight acclimation hypoxia trials, the data points at 70 mmHg are unusual: higher at 13 °C, lower at 11°C whilst the fish acclimated overnight at 12 °C were consuming oxygen so rapidly that no data point could be measured. This may indicate stress, although *Neomyxine biniplicata* do not necessarily communicate stress by movement during hypoxia. The results of the hypoxia trial at 11 °C and 13 °C can be only explained in terms of speculation and more work needs to be done to verify data as data collection may not represent the situation. The RMR is likely inflated due to the low number of data points at the lower levels of oxygen tension and more work needs to be done to verify these results.

4.13.3 Time course of oxygen depletion at 12 °C: comparison of Method I and Method II and at 3 temperatures: - 11 °C, 12 °C, 13 °C and in four fish with probable SDA.

The time taken to deplete oxygen within the respirometer was compared across the two experimental methods and the three temperatures (Figures 4.10, 4.11, 4.12). Oxygen consumption is related to environmental temperature, increasing with higher temperatures and slowing as the water cools (Fry, 1947; Fry, 1971), and experimental methods have an effect (Clark et al., 2013). The time to reduce the oxygen tension from normoxia (152 mmHg) to 20 mmHg (severely hypoxic conditions) differed between experimental methods and across a very narrow temperature range. Temperature did not affect the ability of the fish to withstand severe hypoxia and most fish were resting and still at 20 mmHg or severely hypoxic conditions. At 12 °C the overnight acclimated fish reduced oxygen tension to 20 mmHg over the course of 400 minutes compared with 240 minutes in the fish acclimated for 90 minutes (Figure 4.11). At 11 °C the fish required 450 minutes to use available oxygen compared with 230 minutes at 13 °C (Figure 4.12). The fish with probable SDA, also acclimated to 11 °C, used available oxygen within 200 minutes

compared with 450 minutes in the unfed fish (Figure 4.13). The P_{crit} was not measured in the fish with possible SDA although tolerance of hypoxia was not affected, with the fish reducing oxygen tension to below 20 mmHg.

Table 4.1. Hypoxia: comparison of resting MO_2 in *N. biniplicata*. * indicates probable inexact measurement.

Resting period	Temperature (°C)	MO_2 mg kg^{-1} h^{-1}	P_{crit}	r^2
Overnight	12	22.5	-	0.2384
90 min	12	17	27	0.8439
90 min	13	25*	38*	0.6868
90 min	11	15*	48*	0.7807

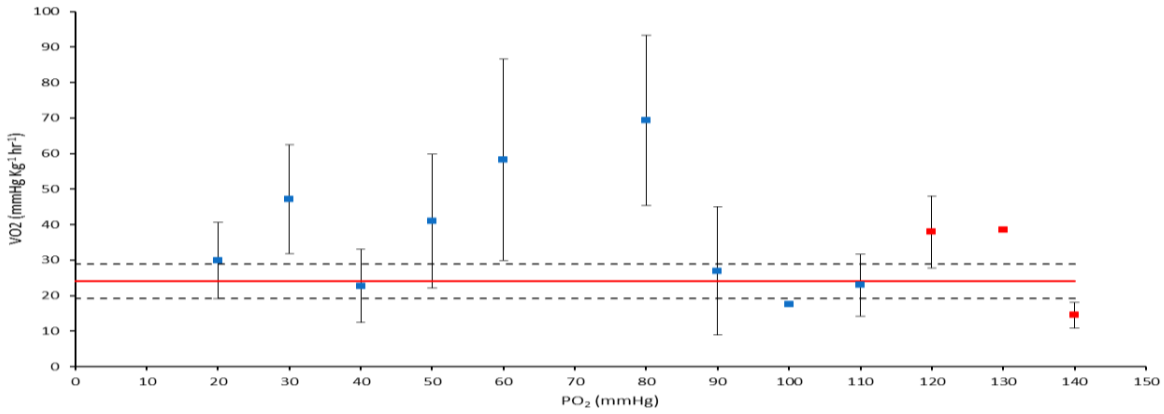

Figure. 4.5. Oxygen consumption, 12 °C, overnight acclimation with peristaltic pump, n = 7. The red points indicate normoxic conditions; blue: gradually diminishing levels of dissolved oxygen.

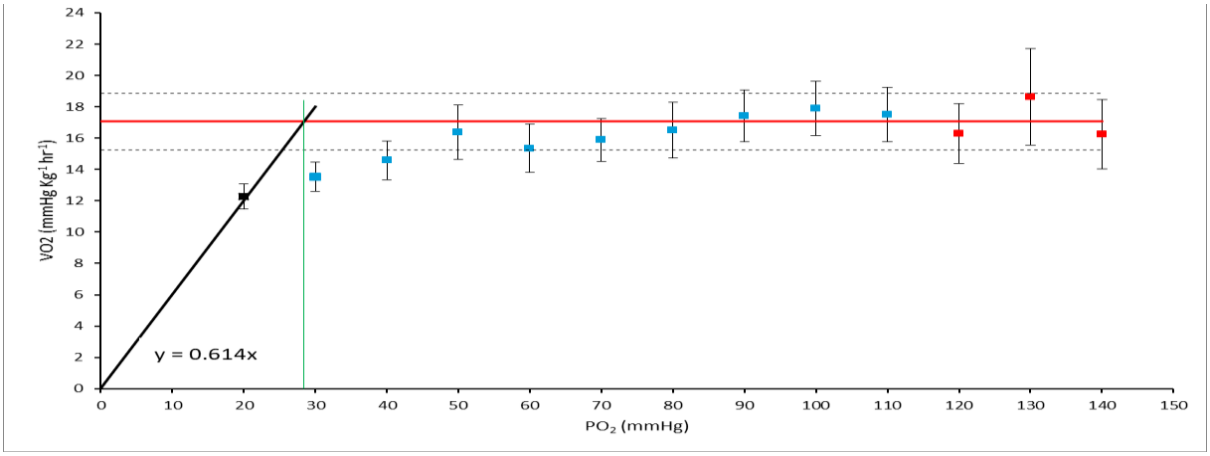

Figure 4.6. Oxygen consumption, 12 °C, 90-minute early morning acclimation, magnetic propeller. The green line indicates P_{crit}; n = 10. Red points = normoxia; blue: hypoxia.

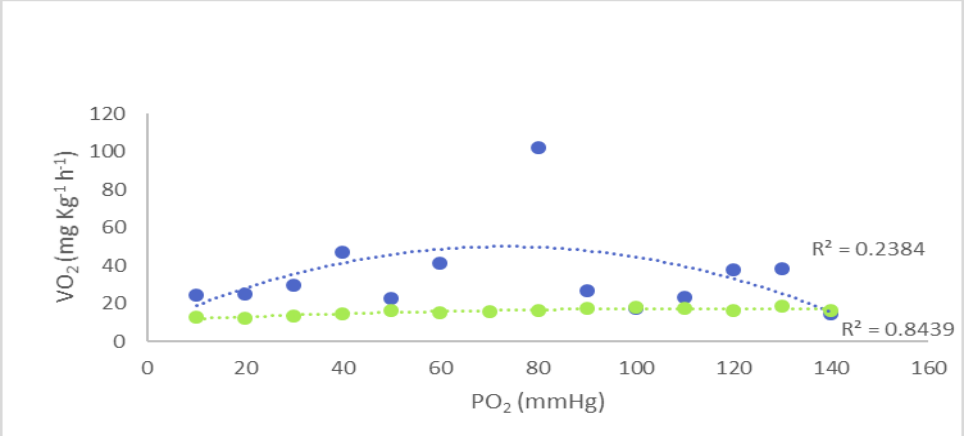

Fig 4.7. Oxygen consumption, 12 °C, comparison between overnight acclimation: R^2 = 0.2384 (dark blue dot), n = 7, 90-minute early morning acclimation: R^2 = 0.8439 (lime dot); n = 10.

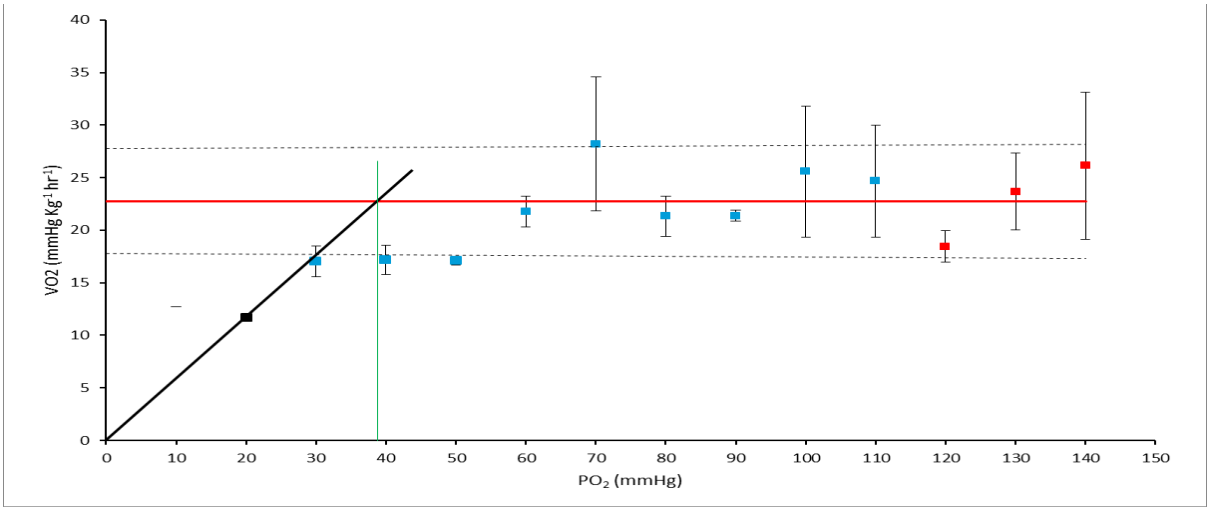

Figure 4.8. VO_2 vs PO_2, 13 °C, 90-minute early morning acclimation. The green line indicates P_{crit}; n = 10. Red points indicate normoxia; blue: hypoxia – also in Figure 4.9.

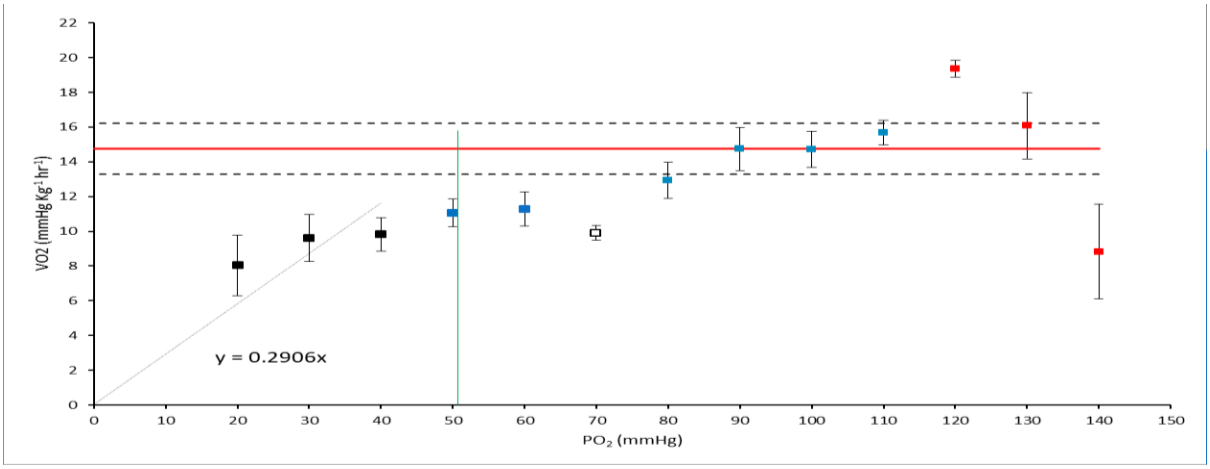

Figure 4.9. VO_2 vs PO_2, 11 °C, 90-minute early morning acclimation. The green line indicates P_{crit}; n = 7

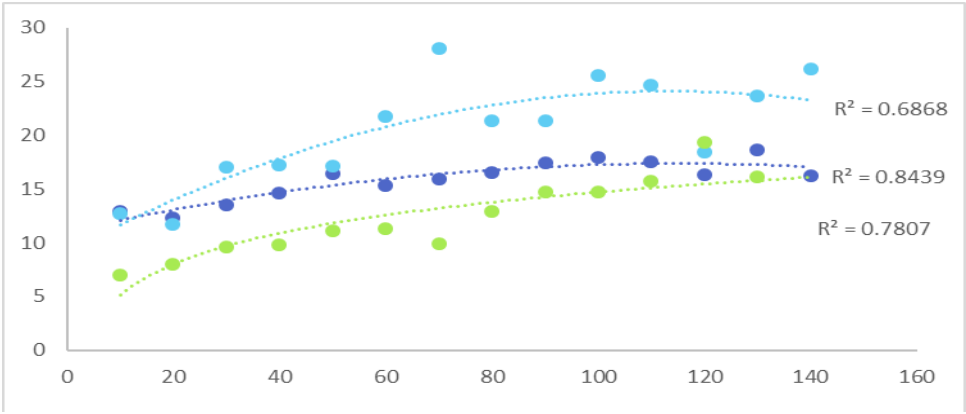

Figure 4.10. Method II, 90-minute acclimation, metabolic rate at 11 °C, (lime dot: r^2 = 0.7807); 12 °C, (mid-blue dot: r^2 = 0.8439); 13 °C (light-blue dot: r^2 = 0.6868); n = 7.

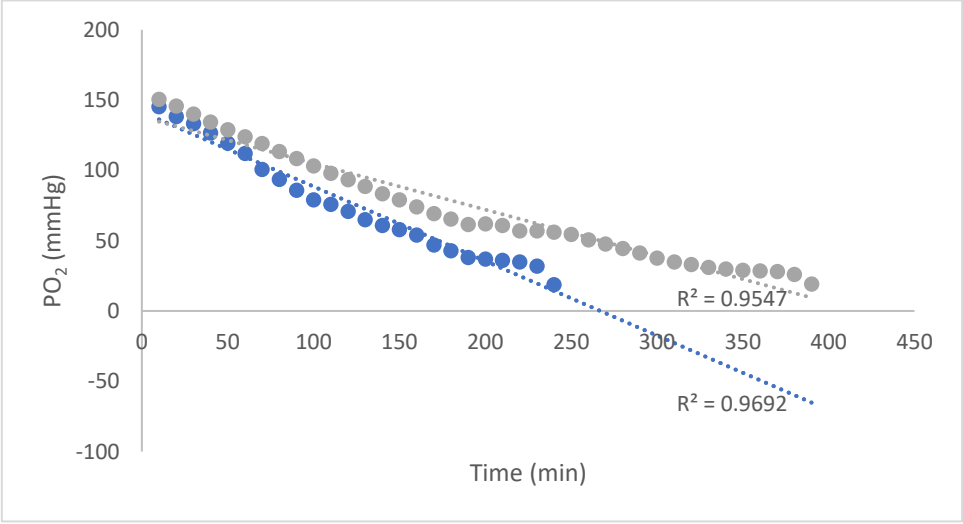

Fig. 4.11. Time course of reduction in ambient oxygen tension at 12 °C, comparison between overnight acclimation (blue dot), n = 7, and 90-minute acclimation (grey dot), n = 10.

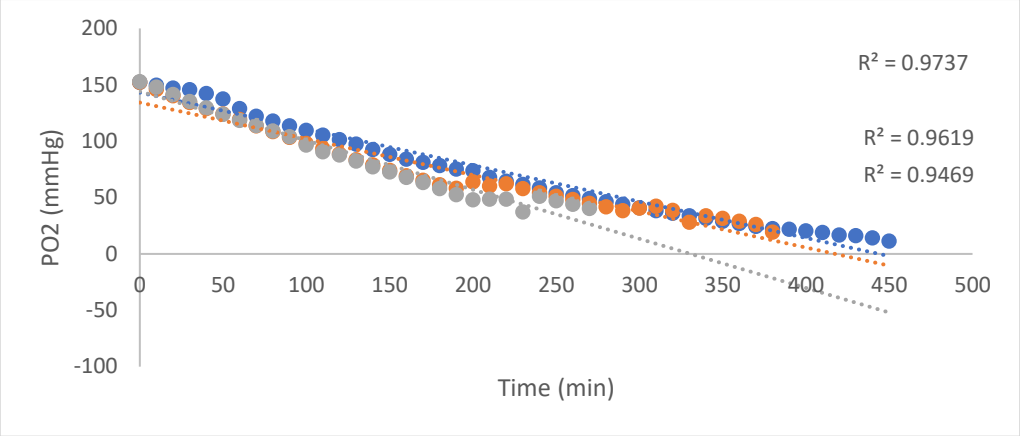

Figure 4.12. Mean PO_2 vs time at 3 temperatures: 11 °C (blue dot), n = 7; 12 °C (orange dot), n = 10, 13°C (grey dot), n = 7.

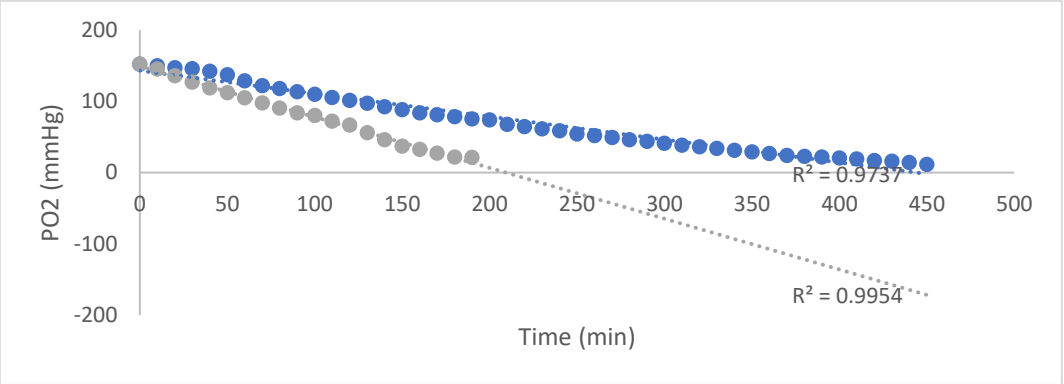

Figure 4.13. Mean PO_2 vs time at 11 °C. Comparison of fish with probable SDA (grey dot), n = 4 vs unfed fish (blue dot), n = 7.

4.14 Discussion

4.14.1 Oxygen consumption at start of all hypoxia experiments

During the 90-minute resting period (Method II), and before the respirometer was sealed, oxygen consumption was close to 8 mg O_2 kg^{-1} h^1 (not shown in graphs). As soon as the respirometer was sealed, and in fully oxygenated water, oxygen consumption increased, fluctuating between 18–39 mg O_2 kg^{-1} h^1 (Figures 4.5, 4.6, 4.8, 4.9). This increase in oxygen consumption was caused by the action of sealing the respirometer, as no matter how gently the respirometer lid was screwed down, the hagfish would increase oxygen consumption in response. As well as the vibration introduced into the system by the action of sealing the respirometer, the oxygen

pressure in the respirometer elevated slightly upon closure. This was exacerbated by the action of the peristaltic pump. Directly upon sealing the respirometer with the peristaltic pump operating (Method I), oxygen pressure within the respirometer was often recorded at 160 mmHg. The fish reacted to the elevated pressure by swimming and with escape behaviour and in several instances the experiment was terminated through the release of slime. It was clear that elevated oxygen tension, and probably that of other but unmeasured gases, disturb the fish and they are aware of the change in pressure. The results obtained by Method II show that the hagfish settle quickly after closure of the respirometer but the introduced vibration and slight elevation of pressure within the respirometer during closure remain an unresolvable flaw in this method.

4.14.2 Overnight acclimation vs 90-minute resting (Method I vs Method II)

Method I: Although the temperature was set at 12 °C, the action of the peristaltic pump introduced heat via friction into the system and the water temperature was increased by up to 2 °C inside the respirometer (Parker, et al., 1969). It was only quite late in the experimental series that this was realised, because the temperature of the water bath remained stable. The fish recovered from the prolonged exposure to the increased temperature, but the resulting data was not convincing and a gradual decrease in oxygen tension, over the course of the experiments, was not demonstrated. In the overnight acclimation group the mean oxygen consumption fluctuated markedly. This occurs even without the fish swimming. Even so, the fish reduced the oxygen tension to 20 mmHg and metabolic oxygen consumption was calculated to be 25 mg kg^{-1} h^{-1} although this figure is unlikely to be an accurate representation of metabolic rate.

This compares to a metabolic rate of 17 mg kg^{-1} h^{-1} measured in the 90-minute acclimation group (Method II). The fish acclimated for 90-minutes reduced the oxygen tension at a steady rate until a PO_2 of 20 mmHg was reached and the experiments were terminated. The time taken to deplete oxygen differed between

the overnight and 90-minute acclimation groups: 240 minutes compared to 400 minutes respectively. The demeanour of the fish during the 90-minute acclimation experiments, and the length of time taken to reduce oxygen tension, suggests the fish were rested and ventilating at a usual rate and the routine metabolic rate at 12 °C at one atmosphere in *N. biniplicata* is 17mg kg^{-1} h^{-1}. There are three possible reasons for this difference.

1. The first is the peristaltic pump pressure and heat introduced through friction (Parker, Boggs & Blick, 1969). The peristaltic pump was used in Method I to circulate oxygenated water through the system during the acclimation period and after the respirometer was sealed to prevent stratification within the respirometer. The peristaltic pump seemed to be an improvement on the small internal pump used in the initial experiments and not detailed in this report. However, after 20 hours acclimation with the peristaltic pump circulating oxygenated water, the temperature within the respirometer was measured at ~14 °C which, as the ventilatory experiments in Chapter 3 revealed, is at the high end of this species' thermal range. Installing insulation foam around the lines was not helpful. The oxygen trace showed the animals, although perturbed by the increase in pressure within the respirometer, after the initial 20 minutes, were ventilating at a very low rate in these conditions and during the first hour within the respirometer, oxygen tension declined only by a few mmHg.

2. The Lab Chart trace (Figure 4.1) shows rhythmical oxygen consumption and consistent movement of the fish during the night, and this was not an isolated case. That the respirometer was unsealed during the night, for the animals to reduce the oxygen to the extent shown, reveals no small exertions on the part of the hagfish. Hagfish are known to have a nocturnal habit (Gustafsen, 1935; Kabaswa et al., 1993; Fernholm, 1974) and the resting period should correspond to a period of naturally low activity (Claireaux & Chabot, 2016).

3. The series of experiments on the effect of temperature on ventilatory rate

(Chapter 3) showed that *N. biniplicata* are a social species and, with some fish, a resting ventilatory rate could only be obtained by introducing two fish into the respirometer. The ventilation series was carried out during the daytime and possibly the fish become very anxious during the night if alone. Exhausted hagfish are unlikely to return a routine metabolic rate.

An accumulation of waste products is an unlikely cause of the inconsistent data because the respirometer was immersed into 30 L of seawater and was unsealed during the night, so any waste would have been diluted. It is probable that the inconsistent data produced by this series of experiments is a combination of introduced heat and the communal habit of the fish. Acclimating the fish overnight has caused the fish to become perturbed and overheated – either through the chosen method of circulating the water, or the social habit of the fish, or a combination of both – and the routine metabolic rate was not achieved.

4.14.3 90-minute acclimation at 11 °C and 13 °C

The results acquired from the 90-minute acclimation of the hagfish show that a short early morning period of rest may be the sensible option with this species of hagfish. However, the data produced from the 11 °C and 13 °C series of experiments is not convincing, as it suffers from too few data points at the lower end of oxygen tension. The experimental series was compromised by the very small population number and the extended time required for the fish to rest and recover from any SDA. Also, the effects of continued exposure to severe hypoxia and the unknown recovery time to recover from such, and a desire not to over-exert the fish by continual exposure to severe hypoxia resulted in there not being enough time to repeat the experiments.

4.14.4 P_{crit}

The P_{crit} indicates the point at which aerobic metabolism is relinquished and anaerobic respiration ensues because the level of dissolved oxygen within the environment is too low and the gills cannot take up and transport enough oxygen through the blood to the tissues (Pörtner & Grieshaber, 1993; Pörtner, 2010). As

discussed in Chapter 1, a difference in the tolerance and behaviour to hypoxia and anoxia has been identified within the sub-family members of the Myxinidae. Species of eptatretids: *Eptatretus cirrhatus* (Forster,1989; Axelsson et al., 1990; Coxon & Davison, 2010) and E. *hexatrema (*Kench, 1989) were found to have a P_{crit} of 45 mmHg – 50 mmHg although specimens of *E. stoutii* have been reported to tolerate low levels of dissolved oxygen and did not attempt to escape severe hypoxia until oxygen tension reached 22 mmHg (Cox et al., 2010). The myxinid sub-family has been found to have a higher tolerance of hypoxia with specimens of *M. glutinosa* demonstrating a P_{crit} of 11–22 mmHg with no attempt to escape (Perry et al., 1993). A lower P_{crit} is usually associated with a greater tolerance of hypoxia and, although this does not stand across all aquatic ectotherms, it is a useful comparison of the response of the metabolism to hypoxic conditions (Chapman et al., 2002; Mandic et al., 2009; Speers-Roesch et al., 2013).

In this series of experiments specimens of *N. biniplicata* demonstrated a high tolerance of hypoxia, being completely unperturbed and lying quietly at 20 mmHg. There were instances of oxygen tension being reduced below 10 mmHg and in one case, 0 mmHg was recorded. No attempts to escape were made in these conditions of severe hypoxia, which is different to the response of *N. biniplicata* when confronted with water temperatures above 14 °C. It may be said that this species of myxinid is unconcerned by water of low oxygen tension but becomes perturbed by water temperature beyond the thermal range that it usually inhabits. The P_{crit} at 12°C in *N. biniplicata* is 27 mmHg. At this point the fish have abandoned regulation of oxygen and become oxyconformers. The P_{crit} of 27 mmHg, compared with P_{crit} of 50 mmHg in eptatretids, indicates this species is very well adapted to hypoxic conditions (Perry, Vulesevic et al., 2009).

More work needs to be done to confirm the metabolic rate and critical oxygen pressure at 13 °C. At this temperature the P_{crit} increased and this may be in line with the results of a study of Crucian carp where higher temperatures were shown to be

positively correlated with higher P_{crit} values (Yang et al., 2015). The P_{crit} at 11 °C was 48 mmHg and this elevated value may be an aberration, because metabolic rate slows in response to submersion in cooler temperatures (Jobling, 1993). However, these results may replicate those of the winter population in Chapter 3, Figure 3.6 and 3.7, where the fish display an elevated ventilatory rate at 11 °C and another series of experiments needs to be carried out to confirm these results.

4.14.5 Reduction of oxygen tension vs time

The length of time required to reduce oxygen tension within the respirometer differed markedly between experimental Methods I and II. The overnight acclimation group reduced oxygen tension from 152 mmHg to 20 mmHg within 240 minutes, compared with 400 minutes in the early morning acclimation group – an increase of 61%. This shows that overall the fish subjected to overnight acclimation were ventilating at a higher rate, and therefore it is more likely that an active metabolic rate rather than routine metabolic rate was recorded (Fry, 1971; Claireaux & Chabot, 2016). The RMR of the overnight acclimation group has been calculated at 25 mg kg^{-1} h^{-1} although oxygen consumption of 40–70 mg Kg^{-1} h^{-1} was recorded throughout the course of the experiment. This may indicate that, through a combination of factors, the hagfish were not rested, and overnight acclimation may not be in the best interests of the solitary hagfish (Figure 4.13).

4.14.6 The effect of temperature on metabolic rate

The rate of metabolic oxygen consumption was affected by the slight alteration in water temperature 1 °C either side of 12 °C. Metabolic rate increased to 38 mg O_2 kg^{-1} h^{-1} at 13 °C from 17 mg O_2 kg^{-1} h^{-1} at 12 °C reducing to around 15 mg O_2 kg^{-1} h^{-1} at 11 °C (Figures 4.5, 4.8, 4.9). Oxygen consumption was highest in the group (Method I, Figure 4.5) acclimated overnight and exposed for a longer period to elevated water temperatures. However, this small alteration in

Figure 4.14. Companionable *N. biniplicata*

temperature indicates that this species of Myxinidae is vulnerable to temperature alterations and this is in line with the effect of acute temperature alterations on ventilation explained in Chapter 3. Climate induced thermal stress is likely to increase but as *N. biniplicata* are not confined to lakes, the species may be able to migrate and take advantage of the natural topography of the sea floor, travelling to regions of stable temperature (Fernholm, 1974; Narum, Campbell, Meyer, Miller, & Hardy, 2013). Given the rarity of the *N. biniplicata* during the cooler months, this may be the case.

4.14.7 Comparison of metabolic rate with other species of Myxinidae

Oxygen consumption was measured at 17 mg O_2 kg^{-1} h^{-1} at 12 °C (Method II, Figure 4.6) in *N. biniplicata*. This compares to 18.24 mg kg^{-1} h^{-1} recorded in *Myxine glutinosa* at 7 °C and 27.2 mg kg^{-1} h^{-1} at 15 °C (Steffensen et al., 1984). The fish in the Steffensen study were 38.4g $\pm$ 3.8g in mass and very similar in mass to our specimens of *N. biniplicata*. Also, in *M. glutinosa*, Lesser et al. (1996), recorded oxygen consumption of 21.76 mg kg^{-1} h^{-1} at between 6 °C – 7 °C. The opportunity to test oxygen consumption below 11 °C in *N. biniplicata* did not arise although from the results of the ventilation

experiments in Chapter 3 it is not likely the fish would have been exposed to temperatures below 8 °C.

In *Eptatretus cirrhatus* oxygen consumption at 11 °C was 9.92 mg kg^{-1} h^{-1}, (Forster, 1990). *Eptatretus cirrhatus* is one magnitude in size greater than *N. biniplicata* (Coxon & Davison, 2011), and larger fish will tend to have a lower metabolic rate than fish of a smaller mass (Jobling, 1993). In *E. stoutii* oxygen consumption was measured 12.8 mg kg^{-1} h^{-1} at 5 °C with no significant difference in oxygen consumption seen in animals acclimated between 4 °C and 10 °C. However, oxygen consumption increased to 26 mg kg^{-1} h^{-1} at 15 °C (Munz & Morris, 1965).

M. glutinosa (Steffensen et al., 1984; Lesser et al., 1996) and *N. biniplicata* (this study) seem to be the only two species of myxinid in which oxygen consumption and metabolic rate have been measured. Myxinids are generally considered to consume oxygen at a higher rate than eptatretids and this is likely a product of evolutionary divergence and the different ecological niche occupied by the sub-family members (Forster, 1998; Forster et al., 1992; Forster, 1998; Malte & Lomhalt, 1998). It is interesting that oxygen consumption measured in one specimen of *E. deanii* in situ at 1230 m in depth (119.4 atmospheres) and at 3 °C was calculated to be 3mg kg^{-1} h^{-1} (Smith & Hessler, 1974). Palmgren (1927) was convinced that bringing hagfish to the surface under pressure would improve survival rate. He contrived a complicated hydraulic apparatus but later decided that water pressure had no bearing on survival of hagfish. As *N. biniplicata* react to a slight elevation in pressure upon closure of the respirometer it would be interesting to explore the effect of water pressure on metabolic rate.

4.14.8 Sensing changes in oxygen tension

It was often observed that when oxygen tension within the respirometer had declined to around 80 mmHg, specimens of *N. biniplicata* reacted by either inverting or gently oscillating as if mixing the water about them. In their study of *E. cirrhatus,* Coxon & Davison (2011) recorded that ventilatory rate increased as oxygen tension

within the respirometer declined to 50 mmHg. Forster et al. (1992) also reported specimens of *E. cirrhatus* to be very sensitive to oxygen tension of 50 mmHg, to which the fish reacted with great agitation. Many species of fish respond to hypoxic conditions by hyperventilation, which increases the mean water to blood oxygen diffusion gradient, culminating in a higher arterial oxygen tension (Munz & Morris, 1965; Forster, 1990). Increased ventilation during exposure to hypoxia indicates that the fish can both detect changes in oxygen tension and regulate ventilatory activity in response (Lomhalt & Johansen, 1979; Coolidge, Ciuhandu, & Milsom, 2008; Dzal et al, 2015). It is not unusual to see an increase in metabolic oxygen consumption at intermediate levels of dissolved oxygen in fish and this can produce an overestimation of the P_{crit} in fish (Svendsen, 2016; Claireaux & Chabot, 2016).

In fish, the hyperventilatory response to hypoxia is facilitated by oxygen sensing chemoreceptors that detect oxygen status of the internal and external environments (Milsom, Sundin, Reid, Kalinin, & Rantin, 1999; Perry, Vulesevic et al., 2009). In zebrafish (*Danio rerio*) the branchial oxygen chemoreceptors are thought to be neuroepithelial cells placed where the cells can sense changes in the internal and external environment (Coolidge et al., 2008). So far, no studies of branchial neuroepithelial cells in hagfish have been undertaken (Perry, Vulesevic et al., 2009).

4.14.9 Effect of SDA

At 11 °C the four fish suspected of having SDA used the available oxygen within 180 minutes compared with 450 minutes in fish that were post-absorptive; an increase of 40%. The metabolic rate was not calculated, but the decreased time to consume oxygen indicates a raised metabolic rate (as all other conditions were stable) most likely to supply oxygen at a higher rate to aid digestion (Jobling, 1993; Nelson, 2016).

4.14.10 Adaptation to hypoxic conditions

Physiological adaptation to life in hypoxic conditions includes a low metabolic rate and the mechanisms to efficiently extract oxygen from water enabling the animal to regulate consumption at a low level of oxygen tension (Childress & Seibel, 1998;

Brauner & Berenbrink, 2007). Anaerobic metabolism is an essential component of systems support in animals that routinely encounter environmental hypoxia, and in hagfish this is supported by extensive stores of glycogen (Sidell, Stowe, & Hansen, 1983; Hansen & Sidell, 1983; Davison et al., 1990, Forster, 1991).

Hypoxia is a natural state within the benthos and although oxygen minimum zones (OMZ) are increasing through human activity (Breitburg et al., 2009), OMZ are not a modern phenomenon. A study of geochemical Paleo-redox proxies and organic-walled fossils found evidence of early Cambrian OMZ, which may have been expanded by the activity and continuous oxygen consumption by metazoan animals (Bianchi, Galbraith, Carozza, Mislan, & Stock, 2013; Guilbaud et al., 2018).

4.14.11 Summary

This small and unfinished study of respiration in *N. biniplicata* finds the species well adapted to hypoxia with P_{crit} of 27 mmHg at 12 °C. At 12 °C the metabolic rate was measured at 17 mg O_2 kg^{-1} h^{-1} compared 18.24 mg kg^{-1} h^{-1} recorded in *Myxine glutinosa* at 7 °C and 27.2 mg kg^{-1} h^{-1} at 15 °C (Steffensen et al., 1984), and 21.76 mg kg^{-1} h^{-1} at 6 °C – 7 °C (Lesser et al.,1996). Metabolic rates are generally higher than those measured in eptatretids (Munz & Morris, 1965; Coxon & Davison, 2011) and may reflect the difference in mass of the animals and adaptation to different substrates.

Response to reduced oxygen tension of 70 mmHg was often met by inversion or gentle oscillation, and to oxygen tension of 20 mmHg, or severe hypoxia, by indifference. The same cannot be said of the reaction of *N. biniplicata* to acute alterations in temperature, where increased ventilatory and metabolic rate were exhibited when the fish were removed from a very narrow range of temperature that seems to be centred around 12 °C. However, as *N. biniplicata* are an ocean-dwelling species and not confined to a closed system of water, the option to migrate exists (Narum et al., 2013). Whether *N. biniplicata* possess the physiological ability to adjust to a wider range of environmental temperature in the longer term could be explored.

However, as a species of hagfish with a lineage that extends to Cambrian era (Pough et al., 2013) the ability of *N. biniplicata* to adapt and endure should not be underestimated.

Chapter Five

Neomyxine biniplicata in captivity

5.1 Introduction

For some obscure reason, hagfish seem to elicit feelings of revulsion in many humans. The given name is not helpful and when attached to a soft and squidgy, eyeless serpentine form that can, without hesitation, discharge a hydraulic blast of adhesive slime, the subtle charms of hagfish are often left unadmired.

Ross (1963) dismissed hagfish as "extremely regressed vertebrate worms with a life both empty and obscure". However, the work of various researchers has rehabilitated this rather disparaging line of thinking and studies of the complex chemosensory and olfactory systems of hagfish (Greene, 1925; Jensen, 1966; Braun & Northcutt, 1998; Jørgensen, 1998) and these observations of *N. biniplicata* in captivity have revealed the hagfish to be cognisant of their fellow hagfish, and sensitive to sound and vibrations within their immediate surroundings and beyond. The ecological importance of hagfish to benthic ecology has been recognised (Cole, 1913; Dayton & Hessler, 1976; Martini, 1998b; St Martin, 2001), whilst the astonishing longevity of the Myxinidae family, and the phylogenetic relationship of these basal vertebrates to modern vertebrates continues to fascinate and perplex researchers (Forey & Janvier, 1993; Forey & Janvier, 1994; Donoghue & Keating, 2014; Brazeau & Friedmann, 2015). Unfortunately, hagfish are also appreciated on an industrial scale such that various species are becoming imperilled by overfishing (Gorbmann et al., 1990; NEFSC, 2003; Ellis et al., 2015).

For decades various species of hagfish have been kept in aquaria for research purposes, mainly the common native hagfish, *Eptatretus cirrhatus* and the Atlantic hagfish, *Myxine glutinosa,* and there is an extensive accumulation of information

describing habit and behaviour in captivity (Gustafsen, 1935; Strahan, 1958; Strahan, 1963a). The research detailed in Chapters 3 and 4 required 12 months to complete, and specimens of *Neomyxine biniplicata* were held in the aquarium at the University of Canterbury for the duration. This may be the first instance of this species of hagfish being observed for some length of time and it may be of interest; it certainly was to us involved in the research.

5.2 Seasonal condition of fish

Although the mean weight of the seasonal populations of *N. biniplicata* was not dissimilar, there was a clear seasonal difference in the condition and behaviour of the fish (summer weight: mean $\pm$ SE 45.07 g $\pm$ 4.85 g; winter weight: mean $\pm$ SE: 47.70 g $\pm$ 4.56 g). The fish caught in the warmer months were healthy and robust and felt sturdy when handled. These fish were interested in food and always registered its arrival into the tank by head waving and launching towards it enthusiastically if hungry. In comparison, the fish caught during the cooler months appeared to be fasting and did not often react to food. The winter fish felt limp and were less inclined to release slime. The intestinal tract of deceased specimens of the winter population was empty.

5.3 Sex ratio, length and weight; eggs, and slime pores

The sex ratio of the specimens of *N. biniplicata* that came into the aquarium between November 2017 and June 2018 was: female 61%; male 16%; unknown 22% (Figure 5.1). The female fish were both longer and heavier than the fish identified to be male and sex unknown shown in Table 5.1.

Table 5.1 Length and mass of *N. biniplicata* caught between November 2017 and June 2018, n = 19.

	Length (mm)	+ SE	Mass (g)	+ SE
Male	439.667	2.728	43.067	5.068
Female	467.083	11.150	56.789	2.868
Unknown	421.0	20.421	44.843	15.155

The fish that were caught between November and March were gestating eggs in various stages of development. Hagfish arriving in late June did not visibly appear to be carrying eggs. Using the stages of sexual maturation in *E. cirrhatus* (Figures 1–2) from Martini and Beulig (2013) as a guide and approximation, egg development, in various fish, was between Stage 3 through to Stage 7. Females in Stage 3 contained numerous round white-coloured eggs but, as the stages of egg development progressed, egg numbers became fewer in number, oval shaped and yellow in colour as described and illustrated in *E. cirrhatus* by Martini and Beulig (2013). Well-developed eggs were arranged in-line and horizontally within the abdominal cavity (Figures 5.8, 5.11). Stage 7 eggs can be clearly seen through the lateral wall of the flanks. In one female in an advanced stage of gestation, the eggs appeared to rearrange within the abdominal region adjacent to the cloaca and assume a vertical rather than horizontal position. It was hoped that the eggs were to be laid but nothing came of it. Stages of male sexual maturation were less easily discerned and some fish that may have been male have been listed as sex unknown.

Not all specimens were examined for eggs or slime pores but of the fish examined all conformed to morphometrics described in Zintzen et al. (2015). Slime pores had the appearance of opaque white beads and were not easily counted along the tail

section. Small sections of slime pores could be absent, and the number of pores were often not laterally symmetrical in an individual fish. The length of specimens of *N. biniplicata* vs number of slime pores is illustrated in Figure 5.2. The slime pore numbers vary individually and do not correlate with the length of the fish. Neither is the length of a hagfish indicative of weight or *vice versa*. None of the fish in captivity were below 400 mm in length, even the 14.63 g fish (Figure 5.3).

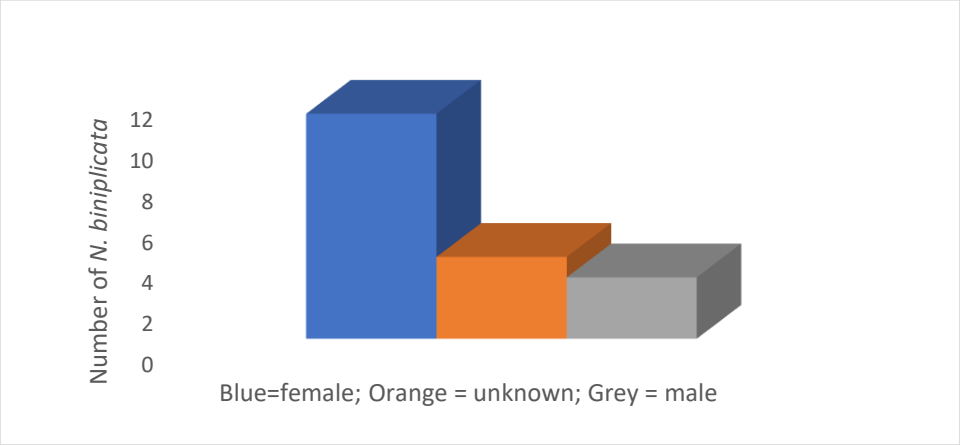

Fig. 5.1 Sex ratio of *N. biniplicata*, n = 19

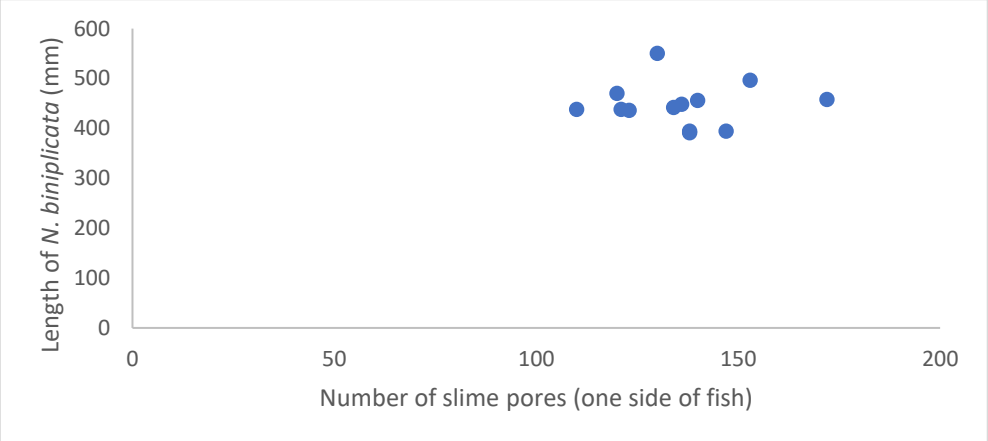

Fig. 5.2. Length of *N. biniplicata* vs number of slime pores, n = 12.

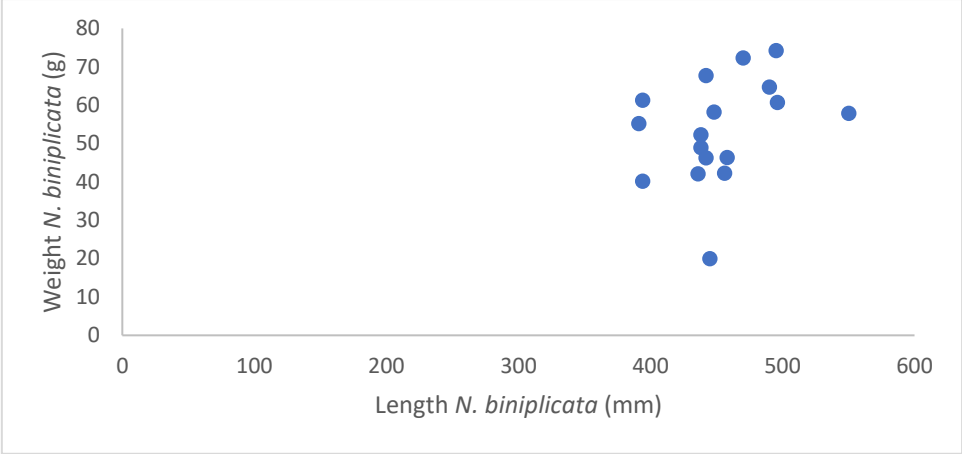

Fig. 5.3. *N. biniplicata:* length vs weight, n = 17

5.4 Settling into captivity, community, and communication

On introduction to the aquarium the hagfish swam about in the tank, often colliding with the acrylic walls which they always approached head up and tried to slither upwards. After a while they sank to the bottom and lay draped across whatever happened to be below them. Often, after the first night in the aquarium, recently ingested bait was egested. After several weeks in the aquarium, three fish caught in the warmer months, egested peritrophic membranes, as described by Hardisty (1979), containing what looked like freeze-dried salmon remains.

N. biniplicata are a sociable species. (Figures 5.4, 5.5). As described in Chapter 3 the very small fish would not settle alone within the respirometer. After a few days in captivity the fish seemed to form a community and usually slept squashed together behind rocks or squeezed into pipes. Despite having no external eyes, the hagfish regularly found their way into the pipes. When a hagfish moved across or over another hagfish they seldom reacted. However, when other life forms – worms or crabs – drifted on to them, the hagfish reacted with some small irritation, undulating their body but not often moving away.

The plastic pipes were usually occupied by two fish at a time, which entered a pipe and, despite the narrow diameter, were able to turn over within a pipe and emerge

together at the other end (Figure 5.4). The very small fish (14 g) was the only fish to persistently burrow. Often the hagfish undulated the body without swimming, in the same manner as a leech does. Whether this was to move water around the body or to remove parasites, or some form of communication is unknown. As discussed in Chapters 3 and 4, *N. biniplicata* have a keen sense of hearing and are sensitive to light.

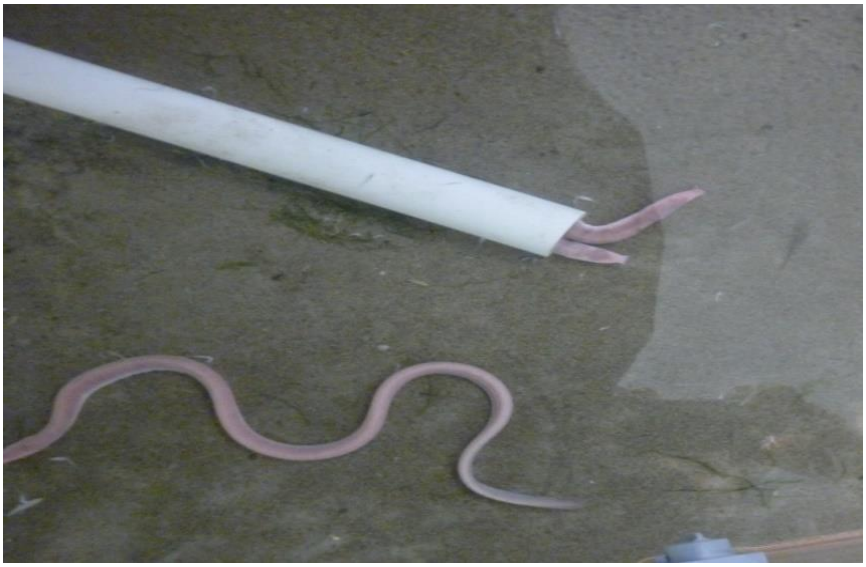

Figure 5.4. *N. biniplicata* in captivity.

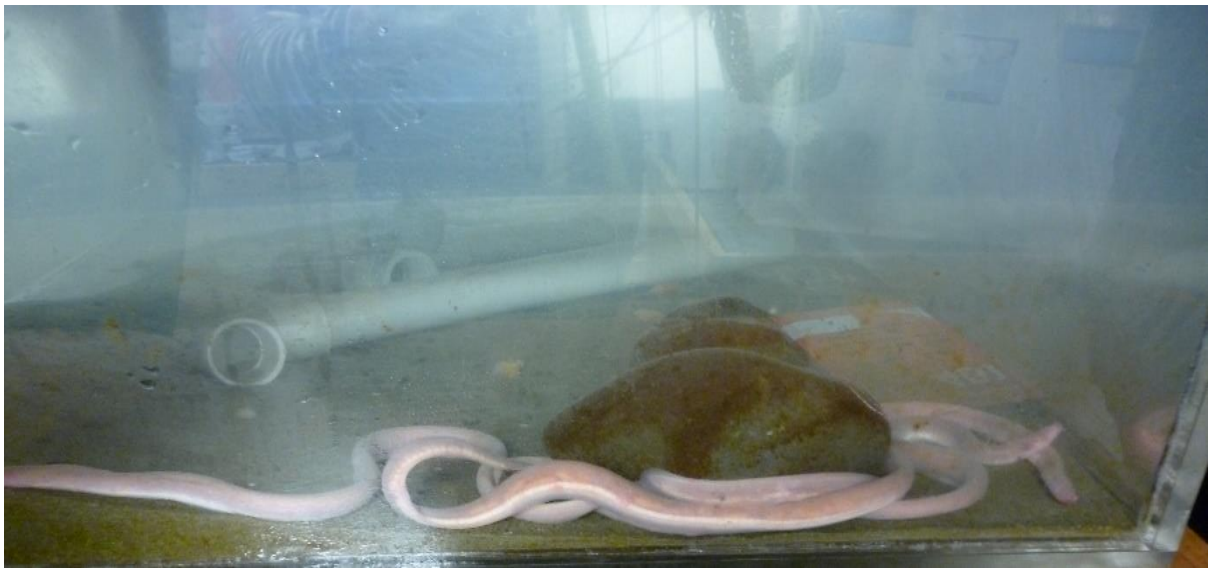

Figure 5.5. *N. biniplicata*: co-habitation

5.5 Food

Both seasonal populations were offered a variety of fish and shell fish. Salmon interested the hagfish but only if they were hungry, which happened every two weeks or so (Figure 5.6). If the fish had recently eaten, then food would remain untouched on the sand. On one occasion live mysids were introduced and although were never actually observed to have been eaten, over a few weeks the mysids gradually disappeared. On several occasions, the hagfish were observed tracking over the sand moving their heads side to side with tentacles touching the sand. It may have been they were hunting for worms (Martini, 1998b). A small vagrant common bully (*Gobiomorphus cotidianus)* that may have come into the aquarium with the hagfish disappeared from the tank and shortly after, four unfed hagfish returned elevated levels of oxygen consumption which were interpreted as SDA. It may be that the hagfish attacked and ate the fish, as described by Zintzen et al., (2011).

The smallest specimen of *N. biniplicata* (14.63 g) was observed unsuccessfully attempting to bite into a large piece of salmon. Possibly communal living is beneficial to juvenile hagfish which may exist on scraps until large and strong enough to bite into larger prey or carrion. This small fish was fed weekly and was observed to be regularly tracking over the sand apparently in search of food. After several weeks in the aquarium, the small hagfish knotted up and succeeded in tearing a slice of salmon away from a large piece (Figure 5.7). No specimen of *N. biniplicata* was observed to eat putrefying fish, and uneaten food was promptly removed from the tank. The fish caught during the warmer months gained some weight during captivity.

Figure 5.6. A specimen of *N. biniplicata* attacking a slice of fresh salmon.

Fig 5.7. Action photo of specimen of *N. biniplicata* knotting up to bite a piece of salmon

5.6 The fatal attraction of rapidly flowing oxygen bubbles

There were three instances of the hagfish becoming trapped in the rapidly flowing stream of oxygen bubbles. It was observed that the fish were attracted to the oxygen stone and even if the bubbles were flowing slowly the fish would often arrange themselves within the stream (Figures 5.8, 5.9). However, if the bubbles were

flowing rapidly the fish would become trapped, rise vertically, and seem to be unable to swim out of the stream. Hyperoxia can be disastrous to fish, as it effectively lowers ventilation, resulting in an elevation of carbon dioxide in the blood which results in a respiratory acidosis, although hagfish can buffer carbon dioxide to an extent (Olsvik et al., 2006; Clifford et al, 2018).

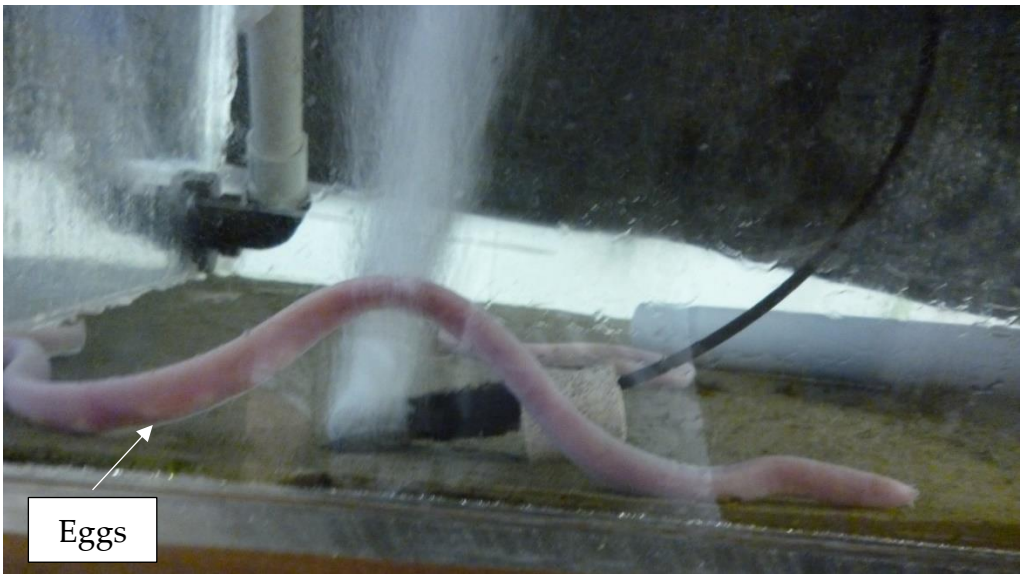

Fig. 5.8. *N. biniplicata*: gravid female arranged in the bubble column.

Figure 5.9. Swimming in the bubble column

5.7 Anatomy, heart, and gill contraction

Deceased specimens were subjected to a basic dissection where data on sex, length, number of slime pores and weight was collected. Even 12 hours after death and the

process of decay had begun, the branchial heart, and sometimes the gills, remained contracting. In three specimens the gall bladders were blue in colour (Figure 5.10).

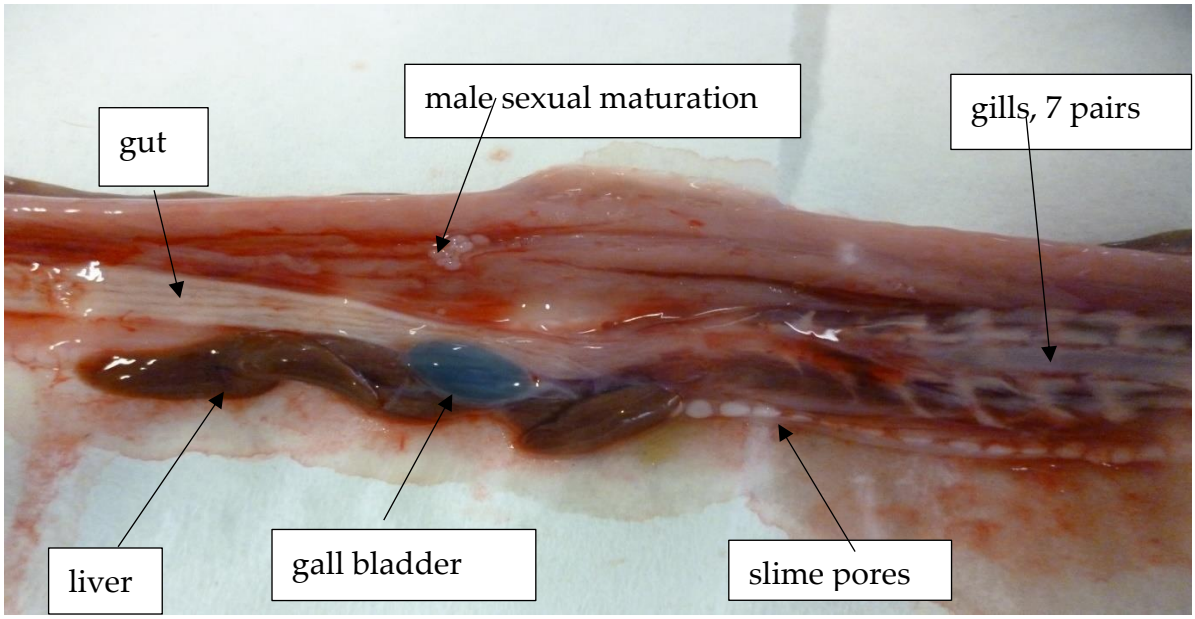

Fig 5.10. Dissected specimen of *N. biniplicata.*

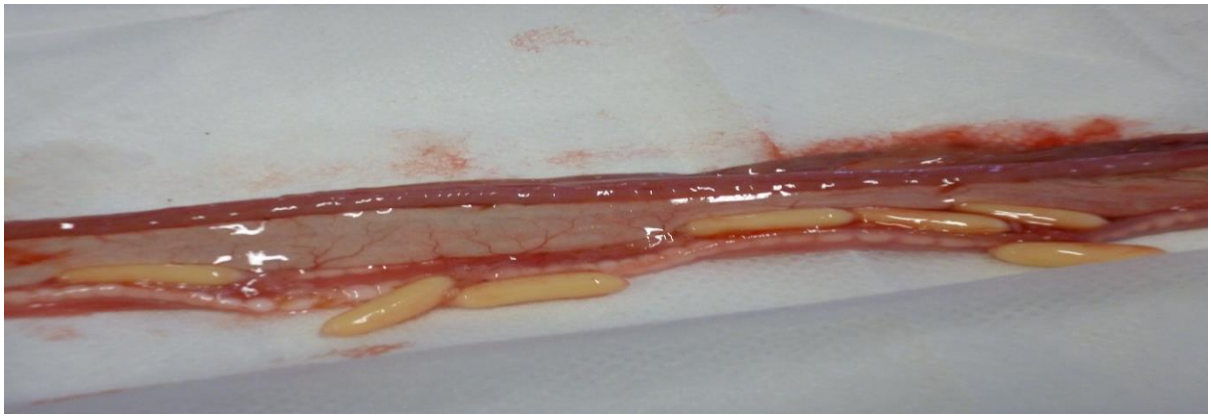

Figure 5. 11. Specimen of *N. biniplicata* containing Stage 7 eggs (approximately).

5.8 Summary

Neomyxine biniplicata are social creatures, cognisant of their fellow hagfish and able to discern them from other marine species such as crabs. The species is acutely

sensitive to sound and light. Juveniles may benefit from cohabitation within a group, especially until mature enough to tackle larger items of food. It seems likely that this species actively hunts for prey, given the mysterious disappearance of the small common bully (*Gobiomorphus cotidianus)* and mysids from the tank (and the equally mysterious but coincidental rise in metabolic rate in four fish). Of the fish in this study, female *N. biniplicata* were longer and heavier than the males, which were longer and heavier again than the animals of unknown sex.

Chapter Six

Conclusions, limitations of research and future enquires

6.1 Conclusions

This study set out to test and begin to understand the respiratory physiology of *Neomyxine biniplicata*, the slender hagfish which is endemic to the waters around the east coast of New Zealand. Aside from the discovery and description of the fish (Richardson, 1953), and morphometric data (Struthers et al., 2015), this is the first study of ventilation and routine metabolic rate (RMR) in this species of hagfish. Observations of the habit of the fish were also made and some basic anatomical data were collected.

6.2 Ventilation – Chapter 3

Neomyxine biniplicata respond to acute changes in environmental temperature by altering the ventilatory rate. The ventilatory range, measured in all fish, was between 0 bpm and 146 bpm and the fish responded to water temperatures above 12 °C by increasing ventilatory rate, whilst below 10 °C the ventilatory rate falls under 15bpm. The hagfish were studied in two seasonal populations and the thermal range differed with the winter fish occupying a narrow range of temperatures, between 11 °C–14 °C, compared to the summer population where the thermal range was extended to between 10 °C and 16 °C. Across the temperature range, ventilatory rate was higher in the winter fish. Studying the ventilatory rate in fish exposed to acute changes of environmental temperature revealed the fact that this species of hagfish, although occupying a very narrow range of temperatures, does acclimatise seasonally. *N. biniplicata* were difficult to find in the cooler months and it may be that the fish make a seasonal migration away from water below 10 °C. The fish caught during the warmer months were in good physical condition whilst the fish caught

during winter-time seemed to be fasting and refused food. Monitoring the ventilation rate allowed us to understand the thermal range of this hagfish, caught in Pegasus Bay at a depth of 20m, and this gave us a clear indication of the range of tolerable temperature before embarking upon metabolic rate experiments where overnight acclimation is conventional.

6.3 Routine metabolic rate, P_{crit} and experimental methods – Chapter 4

When quantifying oxygen consumption (metabolic rate) in fish, it is conventional to confine the fish for 24 hours before measurements begin and this allows the fish to recover from the stress of capture. The fish did not respond well to solitary confinement and difficulties with the method of water circulation produced data where the routine metabolic rate could not be established. However, monitoring oxygen consumption in fish confined overnight allowed us to understand the nocturnal habit of this species. The fish settled quickly during an early morning acclimation period of 90 minutes and the routine metabolic rate was established to be 17mg Kg^{-1} h^{-1}. More work needs to be done at 11 °C and 13 °C to increase data collection at lower levels of oxygen tension. The fish exhibited sensitivity to water temperature altered by only 1 °C, increasing oxygen consumption at 13 °C and decreasing at 11 °C. This is in line with the ventilatory series described in Chapter 3 where the rate of ventilation increased or decreased as the environmental temperature rose and fell respectively.

The period taken to consume available oxygen slowed in response to cooler temperature and increased in water that was warmer. In fish with probable SDA oxygen consumption was 40% more rapid compared to that measured in post-digestive fish. Experimental method had a bearing on oxygen consumption with the overnight acclimated fish consuming oxygen 61% more rapidly than 90-minute acclimated fish at the same temperature. The results obtained by Method II and the extended period required to reduce oxygen tension suggests the fish were resting. However, the experimental method is flawed in that the fish experience and react to

a slight increase in pressure when the respirometer is sealed. Regardless of the method, the fish were unperturbed by severe hypoxia which is in line with findings for *M. glutinosa*, the only other member of the myxine sub-family to be examined for adaptation to hypoxic conditions (Steffensen et al., 1984; Lesser et al., 1996). The critical oxygen tension where regulation is abandoned for a conforming pattern of respiration was determined to be 27 mg kg^{-1} h^{-1} at 12 °C (90-minute rest). Because of the lack of data at the lower end of oxygen tension, the P_{crit} at 11 °C and 13 °C needs to be re-examined. However, the P_{crit} was higher at 11 °C which may be in line with the results of the ventilatory series where the ventilatory rate, in the winter population, increased at 11 °C.

6.4 Observational and anatomical studies – Chapter 5

Neomyxine biniplicata were found to be a social species that could discern their fellows from crabs or other fish. These fish are sensitive to sound, vibration and light and exhibited circadian rhythm. This species is discriminating and displayed a seasonal difference in response to food. In the warmer months the *N. biniplicata* ate every two weeks and preferred salmon above other fish offered. The juvenile fish seemed unable to bite into large pieces of salmon and may benefit from communal living. Of the 19 fish examined, the females were longer (mm) and heavier (g) than the males which were again longer and but of similar weight to the sex-unknown fish. The sex ratio was female 61%; male 16%; unknown 22%. Although provided with clay substrate retrieved from the capture site, only the very small fish sought to burrow. Frankenstein-like, the branchial heart, and occasionally the gills, remain contracting several hours after a fish has expired.

6.5 Limitations of research

This research was limited by the small population number and the extended time required for the fish to recover from SDA. Further research would benefit from keeping two populations of *N. biniplicata* side by side which would allow for a more

productive use of time. For metabolic studies, it should be remembered that fish caught during the warmer months are stronger and healthier and further metabolic experiments may want to concentrate on these fish. The experimental set up needs to be further refined particularly with respect to the increase in pressure seen immediately upon sealing the respirometer. Although Clark et al. (2013) suggest that new species should be confined to the respirometer for up to 48 hours before metabolic rates are measured, it is difficult to see how that could be achieved in a social species that seeks physical contact with fellow hagfish and detects companions by touch rather than sight.

6.6 Future enquiries

Although *N. biniplicata* is very well adapted to withstand hypoxic conditions, further research is required to confirm the P_{crit} at a wider range of temperatures in this species. Regardless of temperature, behaviour and the rate of oxygen consumption alter at an oxygen tension of 70mmHg and examination of the gills for chemosensing neuroepithelial cells would be interesting. As the thermal range of this species seems to be very narrow, especially in the fish caught in winter, it maybe these fish move away from cooler water temperatures. Research into the possible migratory habit of this species could be investigated and this could be carried out in the laboratory by exposing the fish to areas of different temperature along a confined tubular set-up where the fish could actively move away from temperatures found to be unpleasant. Given the very low metabolic rate measured in *E. deanii* at 1230 metres depth (Smith & Hessler, 1974), and the increase in metabolic rate recorded upon closure of the respirometer, experiments with a hyperbaric chamber seem attractive but logistically bothersome.

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