CLIMATE-RELATED STRESSORS AND THEIR EFFECTS ON SABLEFISH (*Anoplopoma fimbria*) CARDIORESPIRATORY PHYSIOLOGY AND IMMUNOLOGY

by

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This thesis is dedicated to my family and friends
and especially Amir.

Abstract

Fish in aquatic environments worldwide are increasingly experiencing extreme abiotic conditions, including high temperatures and hypoxia, due to global warming and eutrophication related to anthropogenic activities. However, our understanding of how these climate-related stressors impact fish physiology is often limited, and many critical questions in this area of ecophysiology research remain unanswered. In this dissertation, I have addressed several knowledge gaps related to fish (teleost) cardiorespiratory physiology and immunology, using the sablefish (*Anoplopoma fimbria*) as a model species. The sablefish is an economically and ecologically important species in the North Pacific, and an emerging aquaculture species, and it has a unique life history as it encounters a wide range of temperatures and O_2 levels in the wild. My research shows that sablefish, as compared to Atlantic salmon (*Salmo salar*), are only slightly less tolerant of acute exposure to high temperatures (critical thermal maximum ~25°C), but much more tolerant of acute hypoxia (O_2 level at loss of equilibrium ~5% air sat.). However, I also discovered that acute hypoxia can substantially constrain the cardiorespiratory response and tolerance of sablefish when subsequently exposed to elevated temperatures. This finding has major ecological implications for fishes given that high temperatures and hypoxia often co-occur in the environment. Finally, I found that chronic hypoxia can impair the sablefish's adaptive immune response (as measured by total IgM antibody titers) to antigens of the pathogen *Aeromonas salmonicida*, a bacteria that frequently causes disease (furunculosis) in various aquaculture fish species. On the other hand, chronic hypoxia only had a limited effect on the innate immune response.

Thus, this research highlights that distinct branches of the fish immune system can respond differently to hypoxia. Overall, this thesis provides key ecophysiological data on the sablefish, and greatly improves our fundamental knowledge of the impact of environmental stressors on fishes. In addition, these results help us to understand, and predict, the consequences of climate change on fish species with importance to fisheries, aquaculture, and conservation efforts.

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Co-authorship Statement

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List of Abbreviations and Symbols

air sat.	Air saturation
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
A. sal.	*Aeromonas salmonicida*
AS	Aerobic scope
AS_T	Temperature-induced aerobic scope
bpm	Beats per minute
BSA	Bovine serum albumin
C_aO_2	O_2 content of arterial blood
CDS	Coding sequence
CFU	Colony forming unit
C_T value	Threshold cycle value
CT_{max}	Critical thermal maximum
C_vO_2	O_2 content of venous blood
DAPI	4′,6-diamidino-2-phenylindole
dELISA	Direct enzyme-linked immunosorbent assay
EC	Endogenous control
FACS	Fluorescence Assisted Cell Sorting
FBS	Fetal bovine serum
f_H	Heart rate
Fulton's K	Measure of condition factor
H chain	Heavy chain structure of immunoglobulin
Hb	Haemoglobin
Hct	Haematocrit
HRP	Horseradish peroxidase
Ig	Immunoglobulin
ip	Intraperitoneal
kPa	Kilopascals
L chain	Light chain structure of immunoglobulin
L-15	Leibovitz-15 media
LOE	Loss of equilibrium
M	Molar, mol L^{-1}
MCHC	Mean cell haemoglobin concentration
MIQE	Minimum Information for Publication of qPCR Experiments
MMR	Maximum metabolic rate
$\dot{M}O_2$	Mass-specific oxygen consumption
mRNA	Messenger RNA
MS-222	Tricaine methanesulphonate
MW	Molecular weight

n	Sample size
N_2	Nitrogen gas
O_2	Oxygen
OCLTT	Oxygen- and Capacity-Limited Thermal Tolerance
OD	Optical density
OMZ	Oxygen minimum zone
P	Probability
PaCA	Plasma-accessible carbonic anhydrase
PBS	Phosphate buffered saline
P_{crit}	Critical oxygen tension
pH	-log $[H^+]$
P_{O_2}	Partial pressure of oxygen
$\dot{Q}$	Cardiac output
QC	Quality control
qPCR	Real-time quantitative polymerase chain reaction
RBC	Red blood cell
RBM	Relative bulbar mass
RMR	Routine metabolic rate
ROS	Reactive oxygen species
RQ	Relative quantity
RVM	Relative ventricular mass
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
s.e.m.	Standard error of the mean
SMR	Standard metabolic rate
SNP	Single nucleotide polymorphism
$T_{arrhythmia}$	Onset temperature of cardiac arrhythmias
T_{crit}	Critical temperature
TOI	Transcript of interest
TX-R	Texas Red
U_{crit}	Critical swimming speed
V_S	Stroke volume of the heart
WBC	White blood cell
α	Probability of a Type I error
β-NHE	β-adrenergically stimulated sodium-proton exchanger

Chapter 1: **Introduction**

1.1. Climate-Related Stressors in the Aquatic Environment

In certain aquatic ecosystems, extreme environmental conditions such as high temperatures and low oxygen (O_2) levels, can occur naturally (Rabalais *et al.*, 2010, Oliver *et al.*, 2021). However, freshwater and marine habitats (especially coastal areas) around the world are increasingly experiencing elevated temperatures (Masson *et al.*, 2007, Wernberg *et al.*, 2011, Doney *et al.*, 2012, IPCC, 2021) and hypoxia (Gilbert *et al.*, 2005, Kemp *et al.*, 2005, Diaz *et al.*, 2008, Diaz *et al.*, 2009, Rabalais *et al.*, 2010, Doney *et al.*, 2012, Breitburg *et al.*, 2018, Claret *et al.*, 2018) due to global warming and eutrophication related to anthropogenic activities. Rapid annual warming throughout the water column has been recorded at various coastal sites [e.g., British Columbia, Canada (Masson *et al.*, 2007), Southern Australia (Wernberg *et al.*, 2011)], and acute warming events (heat waves) in lakes and oceans are becoming more frequent, severe, and longer-lasting (IPCC, 2014, Frölicher *et al.*, 2018, Oliver *et al.*, 2018, Oliver *et al.*, 2021, Woolway *et al.*, 2021). The warming of marine and freshwater systems also contributes to the development of hypoxic conditions, by leading to physical and biological changes that decrease O_2 solubility, and increase organismal respiration rates and stratification (Gilbert *et al.*, 2005, Diaz *et al.*, 2008, Diaz *et al.*, 2009, Stramma *et al.*, 2010, Doney *et al.*, 2012, Breitburg *et al.*, 2018). Eutrophication, the excess runoff of nutrients (e.g., nitrogen, phosphorous), typically from agriculturally-based fertilizers, further exacerbates the global spread of hypoxia (Kemp *et al.*, 2005, Diaz *et al.*, 2008, Diaz *et al.*, 2009, Stramma *et al.*, 2010, Doney *et al.*, 2012, Breitburg *et al.*, 2018). In some marine environments, naturally occurring deep-water oxygen minimum zones (OMZs; areas with O_2 levels <2 mg L^{-1}) have already expanded into shallower coastal waters (Stramma *et al.*, 2010).

Coping with high temperatures and hypoxia in the aquatic environment can be challenging for ectothermic animals such as fish, because of increased metabolic O_2 demands and a difficulty to meet metabolic O_2 requirements (Diaz *et al.*, 2009, Doney *et al.*, 2012, Currie *et al.*, 2014, Sampaio *et al.*, 2021). This can have subtle or sublethal effects on fish, but drastic consequences for fish populations are also possible, including mass mortalities. These have been documented for wild coastal fishes and fish held at aquaculture cage-sites (Diaz *et al.*, 1995, Diaz *et al.*, 2009, Doney *et al.*, 2012, Breitburg *et al.*, 2018, Burke *et al.*, 2020, Gamperl *et al.*, 2021). In the following sections (1.2.1-1.2.2), what is known about the effects of high temperatures and hypoxia on fish cardiorespiratory physiology and immunology, specifically, is briefly discussed. Open questions with respect to fish ecophysiology are also described, some of which are being addressed in this thesis (see Section 1.4).

1.2. Effects of High Temperatures and Hypoxia on Fish Physiology

1.2.1. Cardiorespiratory Physiology

The metabolic response of fishes to high temperatures, and how it relates to their thermal tolerance limits, is the focus of a major scientific effort and is currently being actively debated (e.g., see Clark *et al.*, 2013, McKenzie *et al.*, 2016, Jutfelt *et al.*, 2018, Pörtner *et al.*, 2018, Lefevre *et al.*, 2021). One of the prominent concepts in this discussion is the Oxygen- and Capacity-Limited Thermal Tolerance (OCLTT) (for reviews, see Pörtner, 2010, Pörtner *et al.*, 2017). It postulates that the O_2 consumption ($\dot{M}O_2$) of a resting, post-prandial, fish [i.e., its standard metabolic rate (SMR)] increases exponentially with temperature, while the fish's maximum metabolic rate (MMR) is

highest at a species-specific optimum temperature (Figure 1.1A). Because of these different temperature-dependent relationships, SMR and MMR become equivalent at a certain high temperature, and aerobic scope (AS; MMR–SMR) is essentially zero. Survival beyond this temperature is limited (e.g., by the capacity for anaerobiosis), and eventually the fish's thermal tolerance limit is reached, which is often measured as the critical thermal maximum (CT_{max}); the temperature at loss of equilibrium. Thus, AS has been proposed as the primary factor that determines the thermal tolerance of fishes. However, a scientific consensus about the OCLTT concept is lacking, given that there is both evidence that supports (e.g., Eliason *et al.*, 2011, Anttila *et al.*, 2013, Kelly *et al.*, 2014) and contradicts (e.g., Ekström *et al.*, 2016, Ern *et al.*, 2016, Lefevre, 2016) this hypothesis.

There are other theories about the thermal tolerance and metabolic physiology of fishes that are being discussed in the scientific community. An example of an alternative view to the OCLTT concept is the idea of Multiple Performances – Multiple Optima (MPMO) (Clark *et al.*, 2013), which argues that different physiological functions can have different optimal temperatures. This idea acknowledges that AS is not necessarily the fundamental physiological process driving thermal tolerance in fishes, and that other physiological processes can also be the limiting factor in a thermal challenge. The 'plastic floors and concrete ceilings' hypothesis (Sandblom *et al.*, 2016) is related to the OCLTT concept, but its main premise is that resting cardiorespiratory functions (e.g., SMR) ('floors') are predicted to be phenotypically flexible while maximum cardiorespiratory capacities (e.g., MMR) and CT_{max} ('ceilings') show limited thermal plasticity. Finally, the Gill Oxygen Limitation (GOL) paradigm (Pauly, 1981) is a relatively long-standing

theory that proposes that body size increases proportionally more than gill respiratory surface area, and that therefore, constraints in gill O_2 supply and AS limit the body size of fishes. This mechanism has been used to as a potential explanation for declines in body size of fishes in warming environments (e.g., see Audzijonyte *et al.*, 2019, 2020) and the negative relationship between CT_{max} and body size that appears to exist across and within fish species (McKenzie *et al.*, 2021).

The fish's metabolic response to hypoxia, and its link with hypoxia tolerance, has been less explored than the response/tolerance to high temperatures in fishes. Although, recently the validity of the use of the metric P_{crit} (critical O_2 tension) has also become a topic of discussion (Wood, 2018, Reemeyer *et al.*, 2019, Regan *et al.*, 2019, Ultsch *et al.*, 2019, Seibel *et al.*, 2021). The P_{crit} is the O_2 level at which SMR or routine metabolic rate (RMR; which takes into account low levels of random activity) starts to decline due to a switch from O_2 regulation to conformity (i.e., when $\dot{M}O_2$ becomes directly dependent on the O_2 level; see Figure 1.1B for details). It has been proposed that a low P_{crit} and SMR/RMR are important predictors of high hypoxia tolerance in fishes (e.g., see Farrell *et al.*, 2009b, Mandic *et al.*, 2009, Claireaux *et al.*, 2016, Killen *et al.*, 2016).

With regard to upper thermal tolerance, cardiac function appears to be amongst the key physiological factors/systems that determine AS and the temperature limits of fishes (Wang *et al.*, 2007, Farrell *et al.*, 2009a, Pörtner, 2010, Eliason *et al.*, 2011, Eliason *et al.*, 2017, Farrell *et al.*, 2017). The fish's response to acute high temperatures typically includes increases in heart rate (f_H) and cardiac output ($\dot{Q}$; the amount of blood pumped/min) in order to deliver more O_2 to the tissues, and thus, meet the fish's elevated metabolic demands (e.g., see Gollock *et al.*, 2006, Eliason *et al.*, 2017, Farrell *et al.*,

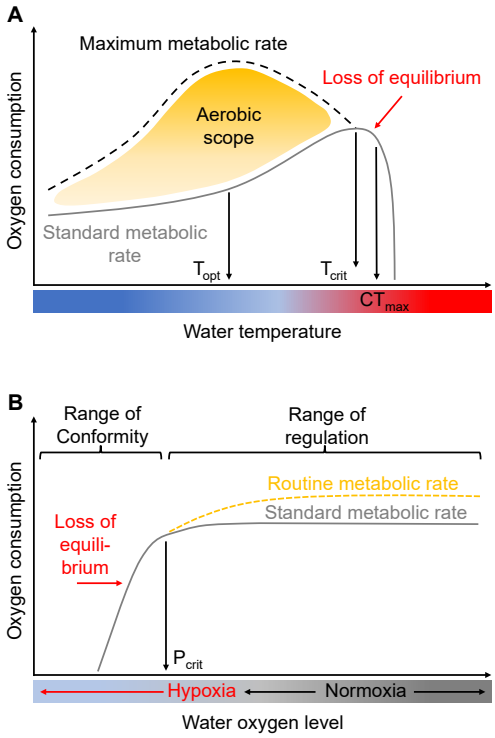

Figure 1.1. Conceptual overview of fish oxygen consumption ($\dot{M}O_2$) as influenced by acute changes in (A) water temperature or (B) oxygen (O_2) level. See Pörtner (2010) for information on the Oxygen- and Capacity-Limited Thermal Tolerance (OCLTT) hypothesis, and Farrell *et al.* (2009) for examples of descriptions of the concepts in panels A and B, respectively. (**A**) The $\dot{M}O_2$ of a resting, post-prandial, fish (i.e., its standard metabolic rate, SMR) increases exponentially with temperature, until reaching the critical temperature (T_{crit}). Whereas the fish's maximum metabolic rate (MMR) is at its highest at the fish's optimal temperature (T_{opt}), and decreases as temperature is increased or decreased. The result of these two different temperature-dependent relationships is that MMR and SMR are equivalent at the fish's T_{crit}, and thus, aerobic scope (AS; MMR–SMR) is zero at this temperature. Beyond this point, survival is time-limited and defined by the ability to utilize anaerobic metabolism and metabolic depression, and eventually the fish loses equilibrium (LOE). The temperature at this point is defined as the fish's critical thermal maximum (CT_{max}). (**B**) The SMR and routine metabolic rate (RMR; which takes into account low levels of random activity) are relatively stable during normoxia and moderate hypoxic conditions. Under severe hypoxia, however, $\dot{M}O_2$ decreases in a linear fashion with P_{O_2}. The ranges of regulation and conformity are when a fish's MO_2 is independent of, and dependent on, the water O_2 level, respectively. The breakpoint between regulation and conformity is defined as the critical oxygen tension (P_{crit}). Below the P_{crit}, water O_2 levels can no longer support tissue O_2 demand, and survival time is determined by the fish's capacity for anaerobic metabolism and metabolic suppression. Eventually, the fish loses equilibrium, which is followed shortly thereafter by death. The O_2 level at LOE is normally recorded as the lowest oxygen level that can be tolerated by the fish.

2017). This is in contrast to the cardiac response to acute hypoxia, as low O_2 levels often result in a regulated decrease in f_H, a response known as bradycardia (e.g., see Farrell, 2007, Petersen *et al.*, 2010, Gamperl *et al.*, 2014). Prior to this thesis research, it was not known how hypoxia/hypoxic bradycardia would impact the fish's capacity to enhance cardiac function when exposed to high temperatures, and the consequences of acute hypoxia on thermal tolerance limits (CT_{max}) had only been examined in a single study (Ern *et al.*, 2016). Considering that these stressors frequently co-occur in coastal marine environments (Kemp *et al.*, 2005, Breitburg *et al.*, 2018) and aquaculture cage-sites (Burt *et al.*, 2012, Stehfest *et al.*, 2017, Burke *et al.*, 2020), and in freshwater systems (Del Rio *et al.*, 2019, McDonnell *et al.*, 2021), it is critical that we understand their combined effects on fish physiology and their tolerance limits.

Acclimation to one environmental stressor may, in certain cases, improve the tolerance to another related stressor ('cross-tolerance'; e.g., see Sinclair *et al.*, 2013). Given that high temperature and hypoxia are both O_2-limiting conditions, it is conceivable that long-term exposure to hypoxia can improve thermal tolerance, and vice versa, because of shared cellular pathways/adaptive mechanisms (Ely *et al.*, 2014). However, research findings about this type of cross-tolerance in fishes have been equivocal to date (e.g., see Anttila *et al.*, 2015, McBryan *et al.*, 2016, Motyka *et al.*, 2017, Levesque *et al.*, 2019, McDonnell *et al.*, 2019, Collins *et al.*, 2021, Del Rio *et al.*, 2021, McDonnell *et al.*, 2021). For example, a study in steelhead trout (*Oncorhynchus mykiss*) showed that chronic hypoxia (acclimation to 40% air saturation for >3 months) modified the cardiorespiratory response to acute warming under normoxic conditions (Motyka *et al.*, 2017), by lowering the maximum $\dot{Q}$ and enhancing O_2 extraction, but did not change

CT_{max}. Exposure of Chinook salmon (*Oncorhynchus tshawytscha*) embryos to hypoxia (50% air sat.) during development also did not improve their normoxic CT_{max} as fry (Del Rio *et al.*, 2021), and conversely, rearing embryonic zebrafish (*Danio rerio*) at elevated temperatures (from 27 to 32°C) did not confer enhanced hypoxia tolerance during the larval stage (measured as P_{crit} and survival) (Levesque *et al.*, 2019). Whereas, hypoxia acclimation of pugnose shiner (*Notropis anogenus*) (2 weeks at 40-56% air. sat.) was recently found to increase their CT_{max} under hypoxic conditions (McDonnell *et al.*, 2021), and warm acclimation of Chinook salmon embryos (10 to 14°C) improved hypoxia tolerance (time to LOE) (Del Rio *et al.*, 2021). Overall, these acclimation studies indicate that chronic hypoxia can induce a significant level of phenotypic plasticity (i.e., the flexibility of animals with the same genetic background to develop different phenotypes depending on their environment; West-Eberhard, 1989) in fish, and this warrants further investigation.

1.2.2. Immunology

Similar to cardiorespiratory function, the fish's immune function can be affected by abiotic stressors including high temperatures and hypoxia (Yada *et al.*, 2002, Bowden, 2008, Abdel-Tawwab *et al.*, 2019). Immunity is a host's main mechanism of defence against infections by pathogenic bacteria, viruses, and eukaryotic parasites, and thus, it plays an important role in determining the health and disease status of fishes (Zaccone *et al.*, 2009). This applies to wild stocks, and in aquaculture where disease control is a major concern (Semple *et al.*, 2020, Soto-Dávila *et al.*, 2020). As in other vertebrates, fish immunity has both an innate (non-specific) and adaptive (specific/acquired) branch;

although fishes have a key evolutionary position given that they are the most ancient group with adaptive immunity akin to mammals (Nakanishi *et al.*, 2018). For instance, fishes posess immunoglobulins (Igs) as well as B and T lymphocytes, which form the basis for humoral and cell-mediated immunity, respectively, with the capacity for immunological memory (Kaattari *et al.*, 2009, Nakanishi *et al.*, 2018). However, adaptive immunity in fishes appears to have limitations compared to that in mammals, so the response to infectious agents is based for a large part on innate immune defences such as the complement cascase, antimicrobial peptides, and phagocyte respiratory burst activity (Tort *et al.*, 2003, Semple *et al.*, 2020).

The study of the interactions between the animal host, pathogen/parasite and environment (also known as ecoimmunology) is a relatively new, but rapidly developing, research field (e.g., see Brock *et al.*, 2014, Downs *et al.*, 2014, Schoenle *et al.*, 2018, Forbes, 2020, Scharsack *et al.*, 2021). It has long been known that environmental temperature can strongly modulate immunological processes in fishes because of their poikilothermy (Yada *et al.*, 2002, Bowden, 2008, Nakanishi *et al.*, 2018), however, the potential impact of hypoxia has received less attention.

Some studies have shown that maintaining defences against pathogens/parasites (immunocompetence) imposes a significant (and detectable) energetic cost in animals (Schoenle *et al.*, 2018). Thus, it is possible that hypoxic conditions (which can limit energy availability and AS) restrict the fish's capacity to mount a robust/effective immune response against infectious agents. Studies that have assessed the effect of acute hypoxia on immune function in a variety of teleost species, broadly support this idea (Yada *et al.*, 2002, Bowden, 2008, Abdel-Tawwab *et al.*, 2019). Acute hypoxia appears to suppress

both innate and adaptive components of immune function, such as serum complement and bactericidal activity, immune-relevant gene expression in the head kidney, and serum Ig titres, and it has been shown to increase disease susceptibility (Walters *et al.*, 1980, Boleza *et al.*, 2001, Evans *et al.*, 2003, Welker *et al.*, 2007, Rodríguez *et al.*, 2016). On the other hand, findings about the effect of chronic hypoxia (i.e., hypoxia for weeks/months) on fish innate and adaptive immunity are far from conclusive (e.g., see Kvamme *et al.*, 2013, Gallage *et al.*, 2016, Magnoni *et al.*, 2019, Martínez *et al.*, 2020, Zanuzzo *et al.*, 2020, Krasnov *et al.*, 2021, Schäfer *et al.*, 2021). Chronic hypoxic conditions may be an important stressor affecting the immune function of fishes because of the difficulty or inability of fish to avoid/escape them (as compared to acute hypoxia). Therefore, further research is required to clarify how chronic hypoxia may act as an immunomodulator in fishes, and it is thus, a major focus of this thesis.

1.3. Sablefish

1.3.1. As a Model in Fish Ecophysiology

In the fields of cardiorespiratory physiology and immunology, salmonid species (e.g., trout, char, salmon) are often used as a model for the teleost fish's response to high temperatures or hypoxia; which is likely related to their major economical importance to fisheries and aquaculture worldwide (McKenzie *et al.*, 2016). Further, despite the fact that there are >30,000 fish species globally (FishBase, 2021), ecophysiological research in this area has been restricted to only tens of marine fish species (Seebacher *et al.*, 2015, Lefevre *et al.*, 2016, McKenzie *et al.*, 2016). Therefore, to better understand how the cardiac, metabolic and immune function of fishes are affected by climate-related

stressors, it is essential to investigate a larger number/diversity of species (McKenzie *et al.*, 2016). In this thesis, the sablefish (*Anoplopoma fimbria*; superorder Acanthopterygii, order Scorpaeniformes, family Anoplopomatidae) is used as a model/study species. This species has several unique characteristics, and potential benefits, which make it worth studying: (i) it is not closely related to salmonids (i.e., they are members of the more ancient superorder Protacanthopterygii) and is a member of the largest group of teleosts (spiny-rayed fishes; class Actinopterygii); (ii) it has a unique life history and encounters a wide range of temperatures, O_2 levels and depths in the wild; and (iii) it supports significant capture fisheries and is an emergent aquaculture species. In the following sections (1.3.2-1.3.4), more information is provided about the life history, and the fishery for and aquaculture of sablefish.

1.3.2. Life History

The sablefish (Pallas, 1814), also known as black cod (FAO, 2021), is a long-lived deep-water species with a broad bathymetric and geographic range in the North Pacific ocean. The oldest individual recovered from British Columbian waters was aged at 92 years (DFO, 2021b). Sablefish can be found in relatively warm surface waters and in cool waters at depths over 1,500 m, and it is widely distributed along the continental shelf of the Eastern and Western North Pacific (from Japan, through the Bering Sea, to Southern California) (Moser *et al.*, 1994, FAO, 2021). On the west coast of North America, sablefish are pelagic or live in-shore as juveniles, and become bathydemersal as adults (Eschmeyer *et al.*, 1983, Mason *et al.*,1983, Rutecki *et al.*, 1997). It is also a highly migratory species (DFO, 2013, 2021b, Hanselman *et al.*, 2015, Jasonowicz *et al.*, 2017),

and may undergo daily vertical migrations, possibly in response to the movements or location of their prey (which mainly consists of fish, cephalopods, and krill) (Doya *et al.*, 2014, Goetz *et al.*, 2017, Sigler *et al.*, 2019). Another unique characteristic of sablefish is its ability to inhabit OMZs off the coast of California (Moser *et al.*, 1994) and British Columbia, where it is among the most abundant macrofauna observed (Doya *et al.*, 2017). Many pelagic, high-oxygen-demand fishes are unlikely to survive long-term in this extremely hypoxic environment (Stramma *et al.*, 2012).

1.3.3. Fisheries

The sablefish is an economically important species that supports significant fisheries in several countries, but in particular in the USA and Canada (FAO, 2021). The majority (>80%) of the total recorded commercial catches of sablefish consist of landings from Alaska and British Columbia (Sonu, 2014). For Canada and the USA, the largest export markets for sablefish are Japan and the UK, while consumer demands are growing in Korea and Hong Kong (Sonu, 2014, DFO, 2021b). Because of its high market value, the sablefish is one of the most valuable fish species caught on North America's west coast (Hartley *et al.*, 2020, DFO, 2021b). However, both USA and Canadian sablefish population levels are relatively low compared to historic levels (pre-21st century) and harvests have been declining for over a decade (Kotlarov, 2018, DFO, 2021b). Therefore, conservation measures (e.g., keeping catch allowances moderate) are an important management strategy to ensure the sustainability of the fishery (DFO, 2021b).

1.3.4. Aquaculture

Because of the decline in wild stocks (Kotlarov, 2018, DFO, 2021b), increasing consumer demands from emerging markets (Sonu, 2014) and a high market value (Hartley *et al.*, 2020, DFO, 2021b), sablefish farming is an emerging industry (Goetz *et al.*, 2021). This may reduce the fishing pressure on wild sablefish populations (Arkoosh *et al.*, 2018), and in Canada, sablefish aquaculture would diversify the aquaculture sector; currently Atlantic salmon (*Salmo salar*) represents >80% of the total finfish produced in Canada (by weight) (DFO, 2021a). Further, sablefish perform well when reared in indoor facilities and grown-out in marine net pens (Gores *et al.*, 1984), can be cultured at high rearing densities (Kennedy, 1972), and have rapid growth rates (Shenker *et al.*, 1986, Sogard *et al.*, 2001). Finally, the sablefish is a candidate of interest as a 'fed trophic level' in integrated multi-trophic aquaculture (IMTA) (Reid *et al.*, 2017), for instance, for farming along with sea cucumbers (*Parastichopus californicus*) (Hannah *et al.*, 2013) and green urchins (*Strongylocentrotus droebachiensis*) (Orr *et al.*, 2014). Currently there is only one major sablefish production company (Golden Eagle Sable Fish) in British Columbia that has developed captive broodstock, and which grows sablefish commercially for domestic and international markets (Hartley *et al.*, 2020). In the USA (Goetz *et al.*, 2021) and South Korea (Kim *et al.*, 2017a-c), sablefish aquaculture is also attracting interest, but is still limited to the use of wild broodstock followed by the commercial grow-out of juveniles.

1.4. Thesis Objectives and Structure

The primary objective of this dissertation was to increase our fundamental

knowledge of the effects of two important climate-related stressors – high temperatures and hypoxia – on the cardiorespiratory physiology and immunology of fish, using the sablefish as a study model/species. Obtaining this kind of fundamental physiological data ultimately helps to improve our understanding of the consequences of climate change on fish populations and species, and has relevance to fisheries, aquaculture and conservation efforts.

Throughout this thesis, various aspects of the sablefish's physiology are explored, and each of the chapters addresses different pressing questions in the field of fish ecophysiology (many of which have been introduced above) by applying a number of experimental approaches. Specifically, **Chapter 2** describes the metabolic physiology and tolerance of sablefish to acute exposure to high temperatures and hypoxia, and explores whether metabolic parameters (AS and RMR) explain differences in upper thermal and hypoxia tolerance using data from sablefish and Atlantic salmon. These data were mainly intended to lay the groundwork for the rest of the thesis by providing basic physiological information on the sablefish, but they also add to the ongoing debate about the generality of the OCLTT hypothesis. **Chapter 3** describes the cardiorespiratory and stress responses of sablefish to acute high temperatures and hypoxia, including when these two stressors are combined, which to the best of my knowledge, had not been investigated prior to this thesis research. Further, this chapter assesses the effect of chronic hypoxia on this species' cardiorespiratory function and thermal tolerance, with the aim of gaining more insight into the presence/level of cross-tolerance and plasticity. Finally, **Chapter 4** describes the effect of chronic hypoxia on the innate and adaptive immunity of sablefish, using antigens from a common pathogenic bacteria (*Aeromonas salmonicida*) to evaluate

this species' immune response. The goal of this chapter was to elucidate the impacts of chronic hypoxia on these two branches of immunity, which in contrast to the impacts of acute hypoxia, are not well understood. In order to achieve this goal, several analytical tools were developed for the sablefish, including real-time quantitative polymerase chain reaction (qPCR) primers for innate immunity-relevant genes and a direct enzyme-linked immunosorbent assay (dELISA) to measure IgM levels in serum. See Figure 1.2 for a visual presentation/summary of these three research themes/chapters, and the relationship between them.

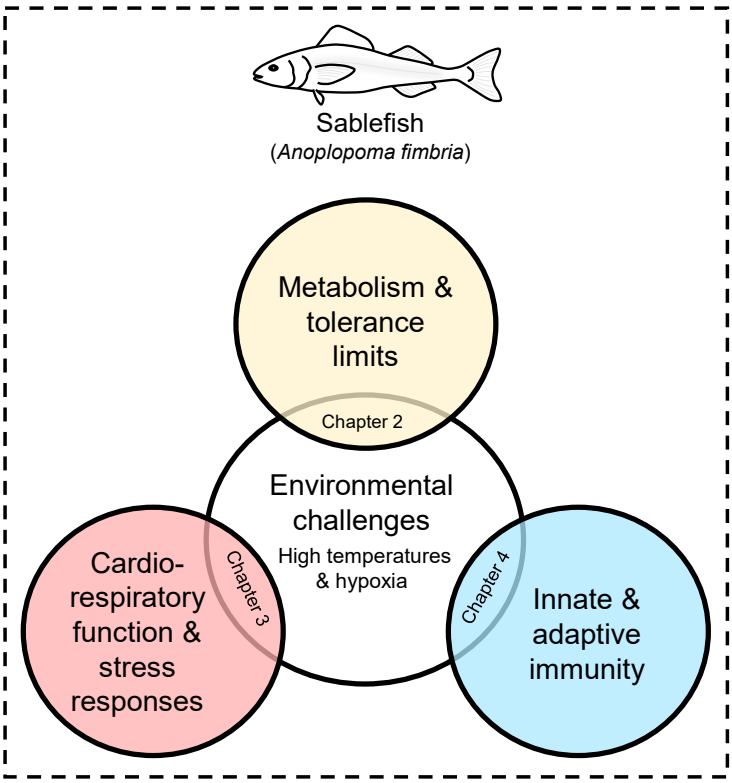

Figure 1.2. Schematic overview using a Venn diagram to show the three research themes that are covered by this thesis, and the corresponding chapters. This research centres on the sablefish, *Anoplopoma fimbria*, a teleost species with a unique life history, and with significant fisheries importance and potential in aquaculture. In each of the chapters, I investigated how various aspects of the sablefish's physiology are affected by environmental challenges; specifically, elevated temperatures and hypoxia. Chapter 2 describes the metabolic physiology and tolerance of sablefish to these two environmental stressors. Chapter 3 investigated the cardiorespiratory and stress responses of sablefish to these stressors, including when combined. Finally, Chapter 4 describes the effects of hypoxia alone on the innate and adaptive immunity of sablefish. See Section 1.4 for details.

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Chapter 2: **The Environmental Tolerances and Metabolic Physiology of Sablefish (*Anoplopoma fimbria*)**

2.1. Abstract

Given the potential impacts of global warming, such as increases in temperature and the frequency/severity of hypoxia in marine ecosystems, it is important to study the impacts of these environmental challenges on sea-cage reared aquaculture species. This study focuses on the sablefish (*Anoplopoma fimbria*), an emerging aquaculture species that has a unique ecology in the wild. For instance, adults inhabit oxygen minimum zones and cool waters at depths up to 1,500 m. Using Atlantic salmon (*Salmo salar*) (~1132 g adults) as a comparative species, I used intermittent-flow respirometry to characterize the tolerance and metabolic response of sablefish (~10 g juveniles and ~675 g adults) to acute increases in temperature (2°C h^{-1}) and decreases in water oxygen levels (~10% air saturation h^{-1}). Adult sablefish were much more hypoxia tolerant than adult salmon [O_2 level at loss of equilibrium ~5.4% *vs*. ~24.2% air saturation, respectively]. In addition, sablefish could withstand upper temperatures only slightly lower than salmon [critical thermal maximum (CT_{max}) values ~24.9°C *vs*. ~26.2°C, respectively]. Sablefish juveniles were both less hypoxia and thermally tolerant than adults [critical O_2 tension ~18.9% *vs*. ~15.8% air saturation; CT_{max} ~22.7°C *vs*. ~24.9°C, respectively]. Interestingly, many of these differences in environmental tolerance could not be explained by differences in metabolic parameters (aerobic scope or routine metabolic rate). These findings show that sablefish are tolerant of high temperatures, and very tolerant of hypoxia, traits that are advantageous for an aquaculture species in the era of climate change.

2.2. Introduction

Fish inhabit a wide array of ecosystems, in which environmental conditions such as oxygen level and temperature vary greatly. However, these environmental conditions are getting closer to, or are already exceeding, the tolerance limits of numerous fish species and/or populations (Cheung *et al.*, 2009, Booth *et al.*, 2017, Comte *et al.*, 2017). This is because freshwater and marine habitats (especially coastal areas where the majority of aquaculture activities takes place) are experiencing elevated temperatures (Masson *et al.*, 2007, Doney *et al.*, 2012, Wernberg *et al.*, 2011, IPCC, 2021) and hypoxia; the latter due to eutrophication and global warming associated with anthropogenic activities (Diaz *et al.*, 2008, Diaz *et al.*, 2009, Breitburg *et al.*, 2018, Claret *et al.*, 2018). What mechanisms determine the tolerance of fishes to these environmental conditions, and what processes will shape a species' success and survival (and ultimately its future ecological niche and distribution) are the focus of a major scientific effort (e.g., see McKenzie *et al.*, 2016).

The sablefish (*Anoplopoma fimbria*) is an interesting model for studying the environmental tolerance of marine fishes and its underlying determinants. This species is widely distributed along the North Pacific, and while juveniles live in-shore, the adults inhabit cold and deep waters (up to 1,500 m) that are often severely hypoxic (Mason *et al.*, 1983). For example, adult sablefish are known to inhabit the oxygen minimum zone off the California coast where oxygen levels range from 0.34 to 0.80 mg O_2 L^{-1} (Moser *et al.*, 1994). Furthermore, this species supports significant capture fisheries and is an emerging aquaculture species. With regards to aquaculture, although there is currently only one company growing sablefish commercially (Golden Eagle Sable Fish, Salt Spring

Island, B.C., Canada), this species holds great promise. The sablefish adapts well to aquaculture rearing (Gores *et al.*, 1984), is a good candidate as a fed trophic level for integrated multi-trophic aquaculture (Reid *et al.*, 2017), has a very high market value (DFO, 2018) and there are increasing consumer demands from emerging markets (Sonu, 2014). Nevertheless, very limited information is available on the sablefish's metabolism and environmental tolerances (see Sullivan *et al.*, 1982), and metrics such as its critical thermal maximum (CT_{max}), critical O_2 tension (P_{crit}) and O_2 level at loss of equilibrium have not been determined.

The concept of Oxygen and Capacity Limited Thermal Tolerance (OCLTT) [for reviews, see Pörtner, 2010, Pörtner *et al.*, 2017] hypothesizes that aerobic scope (AS) [the difference between standard and maximum metabolic rate (SMR, MMR)] is the primary factor that determines the upper thermal tolerance of aquatic organisms, including fishes. The OCLTT concept is supported with considerable evidence in numerous teleost species (Pörtner *et al.*, 2007, Sylvestre *et al.*, 2007, Farrell *et al.*, 2008, Farrell, 2009, Clark *et al.*, 2011, Eliason *et al.*, 2011, Anttila *et al.*, 2013, Kelly *et al.*, 2014). However, there are also experimental findings (especially those that have measured acute thermal tolerance; CT_{max}) that contradict this concept (Norin *et al.*, 2013, Gräns *et al.*, 2014, Wang *et al.*, 2014, Brijs *et al.*, 2015, Ekström *et al.*, 2016, Ern *et al.*, 2016, Lefevre, 2016). Thus, the validity and relevance of the OCLTT to fish biology is currently being hotly debated (e.g., see Clark *et al.*, 2013, Pörtner *et al.*, 2017, Jutfelt *et al.*, 2018, Pörtner *et al.*, 2018, Lefevre *et al.*, 2021).

In addition, it has been proposed that the acute hypoxia tolerance of fishes is dependent on their metabolic rate (Farrell *et al.*, 2009, Barnes *et al.*, 2011, Claireaux *et*

al., 2016). In this case, routine metabolic rate (RMR) is thought to be an important determinant of acute hypoxia tolerance, as fishes with a relatively low RMR have less difficulty in meeting their metabolic demands when exposed to a limited environmental O_2 supply. This concept is also supported by experimental findings in various fish species (McKenzie *et al.*, 2008, Mandic *et al.*, 2009, Barnes *et al.*, 2011, Fu *et al.*, 2011, Dan *et al.*, 2014), although it has not been explored as much as the OCLTT hypothesis. Parameters commonly used for comparing hypoxia tolerance are the P_{crit} (defined here as the O_2 level at which RMR starts to rapidly decline due to a switch from O_2 regulation to conformity) and the O_2 level at loss of equilibrium (LOE). Although, Wood (2018) recently questioned whether P_{crit} provides a valuable metric with regards to a fish's hypoxia tolerance and suggested alternative approaches.

In this study, I used intermittent-flow respirometry to characterize the metabolic response and tolerance of 10-12°C acclimated sablefish (~10 g juveniles and ~675 g adults) to acute incremental increases in temperature (2°C h^{-1}) or stepwise decreases in the O_2 level (~10% air saturation h^{-1}). Furthermore, experiments were concurrently performed on Atlantic salmon (*Salmo salar*) adults (~1130 g) to allow for a direct comparison of metabolic parameters and tolerance limits with those for the sablefish. Such parallel studies need to be performed if meaningful insights into inter-specific differences in tolerance limits, and their determinants, are to be obtained. Disparities in methodologies (e.g., acclimation temperatures/conditions, experimental conditions, rate of temperature increase or O_2 decrease) can significantly impact the results of such experiments. I chose the Atlantic salmon as a comparative species because it is a eurythermal fish whose environmental tolerances have been reported previously (e.g.,

Anttila *et al.*, 2013, Anttila *et al.*, 2015, Hvas *et al.*, 2017), and it is an important aquaculture species worldwide that is experiencing elevated temperatures and hypoxic conditions at cage-sites (Oppedal *et al.*, 2011, Burt *et al.*, 2012, Oldham *et al.*, 2017, Stehfest *et al.*, 2017, Gamperl *et al.*, 2021). Based on the ecology/life history of the sablefish, I hypothesized that this species would be less thermally tolerant than the Atlantic salmon, but more hypoxia tolerant. Further, I expected that differences between life-stages and species in upper temperature and hypoxia tolerance would be related to variations in metabolic parameters.

2.3. Materials & Methods

2.3.1. Animals and Holding Conditions

Juvenile sablefish (~0.5 g) were acquired from the NOAA Manchester Research Station (Port Orchard, WA, USA) and transported to the Department of Ocean Sciences at Memorial University of Newfoundland (MUN; St. John's, NL, Canada) by air. Upon arrival, the fish were initially held at the Cold-Ocean and Deep-Sea Research Facility (CDRF, MUN) for quarantine. Following veterinary clearance, the fish were transferred to the Dr. Joe Brown Aquatic Research Building (JBARB, MUN) and kept in 500 L tanks supplied with seawater at 10-11°C and with 24 h light. These fish were eventually transferred to 3,000 L tanks at the same temperature, but on a 12 h light:12 h dark photoperiod. Atlantic salmon smolts were obtained from Northern Harvest Smolt Ltd. (Stephenville, NL, Canada) and held in 3,000 L seawater tanks at 12°C and on a 12 h light:12 h dark photoperiod. Sablefish and Atlantic salmon were fed a commercial cod/haddock diet (Skretting Europa: 55% protein, 15% fat, 2% carbohydrate, 3%

calcium, 2% phosphorous) and salmonid diet (Skretting Optiline: 38% protein, 33% fat, 2% carbohydrate, 2% calcium, 1% phosphorous), respectively, at a ration of 2.5-5.0% (juveniles) and 0.75-1.00% (adults) of body mass day^{-1}. However, all fish were fasted for one day prior to experiments. All experimental procedures were performed at the Department of Ocean Sciences at MUN, and were approved by MUN's Animal Care Committee (protocol #16-92-KG).

2.3.2. Experiments with Juvenile Sablefish

The tolerance limits and metabolic physiology of juvenile sablefish were investigated in two separate experiments: an acute upper thermal (CT_{max}) challenge, and an acute hypoxic (P_{crit}) challenge. Each experiment was 2 days long, included 4 fish, and was repeated 4 times (total n=16 fish per experiment). On the first day, 4 fish were anaesthetized with 0.15 g L^{-1} tricaine methanesulphonate (MS-222; AquaLife TMS, Syndel Laboratories Ltd, Nanaimo, BC, Canada) and body mass was recorded. The MS-222 dose was similar to that used in prior studies with juvenile sablefish (e.g., Schenker *et al.*, 1986, Friesen *et al.*, 2013). The fish were then placed into 400 mL cylindrical glass respirometers (9.2 cm in diameter×6.1 cm high) (Figure S2.1), and allowed to settle for ≥5 h. Mass-specific O_2 consumption ($\dot{M}O_2$) (mg O_2 kg^{-1} h^{-1}) was continuously measured overnight using 15 min cycles, each composed of 'flushing' (8 min), 'wait' (2 min) and 'recirculation' periods (5 min). Typically, $\dot{M}O_2$ decreased quickly in the first 2.5 h and remained stable overnight. These overnight measurements were used to determine the fish's standard metabolic rate (SMR) and routine metabolic rate (RMR), which were calculated as the mean of the lowest 10% of all recorded values,

and the mean of all values recorded during the last 4 h of the night, respectively. The water in the respirometers was constantly replaced by aerated water from an ambient tank, and kept at 12°C and 90-100% air saturation unless mentioned otherwise.

On the following day, in the case of the acute upper thermal challenge, $\dot{M}O_2$ was continuously measured while the temperature of the water was increased by 2°C h^{-1}. The CT_{max} was determined as the temperature at which the fish lost equilibrium. In the case of the acute hypoxia challenge, $\dot{M}O_2$ was measured at each of the following O_2 levels: 100%, 60%, 50%, 40%, 30%, 25%, 20% and 15% air saturation. The O_2 level was decreased to these levels in a step-wise manner every 60 min until the O_2 level at which the fish lost equilibrium (LOE) was reached. To regulate the O_2 level in the respirometers and surrounding water bath, I used the same custom-built solenoid valve system that has been described by Motyka *et al.* (2017).

In both experiments, fish were recovered after LOE by rapidly cooling the water to 12°C or by increasing the O_2 level to 100% air saturation. Metabolic rate was measured with intermittent-flow respirometry using a computer running AutoResp software 2.2.2 (Loligo Systems, Viborg, Denmark), and the same equipment and procedures as described by Zanuzzo *et al.* (2015) with the following slight modifications. Because $\dot{M}O_2$ varied strongly with temperature and O_2 level, the durations of the 'flushing' and 'recirculation' periods were adjusted throughout the CT_{max} test. In this way, a R^2>0.90 for each measurement was achieved, while ensuring that the O_2 level in the chambers did not drop below 90% air saturation.

2.3.3. Experiments with Adult Sablefish and Atlantic Salmon

Similar to the experiments with juveniles, the tolerance limits and metabolic response of adult sablefish and Atlantic salmon were investigated in two experiments; either an acute upper thermal or a hypoxic challenge. Each experiment was 2-3 days long and was repeated 9-13 times for each species. On the first day, one fish from each species was anaesthetized with 0.1 g L^{-1} MS-222, and body mass and fork length were recorded. The fish were then placed into cylindrical plexiglass respirometers that were submerged in a 'water table', and allowed to settle for ≥24 h. Because the two species in this study had significantly different body masses (Table 2.1), two different sizes of respirometer were used (14.1 L respirometers for sablefish [17.2 cm in diameter×61.0 cm long] and 19.8 L respirometers for salmon [20.3 cm in diameter×61.0 cm long]) (Figure S2.1). The position of the respirometers (i.e., at the front or back of the water table) for each experiment was randomized. The O_2 level in the water was regulated by a control system (OXY-REG, Loligo Systems) that monitored the O_2 level with a galvanic O_2 probe (MINI-DO), and that made adjustments to the water's O_2 level by either releasing O_2 or N_2 into the seawater reservoir that supplied the water table.

After the initial 24 h period, in the case of the acute upper thermal challenge, $\dot{M}O_2$ was continuously measured for 24 h to determine SMR and RMR, followed by a thermal challenge on day 3 (using the same procedure as described for the juveniles). For the overnight $\dot{M}O_2$ measurements, 20 min cycles were used, whereby the length of the 'flushing', 'wait' and 'recirculation' periods varied slightly (due to variations in body size and $\dot{M}O_2$) but were approximately 12 min, 2-3 min, and 5-6 min. In the case of the acute hypoxia challenge, $\dot{M}O_2$ was measured twice at each of the following O_2 levels: 90-100%,

80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% and 5% air saturation. The O_2 level was decreased by 10% h^{-1} until 10% air saturation was reached, after which the O_2 level was lowered to 5% over an hour and maintained at this level of air saturation until LOE occurred. Fish were acclimated to the new O_2 level for a minimum of 10 min before $\dot{M}O_2$ measurements were made. The O_2 level at LOE was recorded, as well as the time until LOE at 5% air saturation (in sablefish). If possible, more than two $\dot{M}O_2$ measurements were made at 5% air saturation. $\dot{M}O_2$ measurements were made using the same equipment and control systems as used for the juvenile sablefish.

In both experiments, fish were euthanized in 0.2 g L^{-1} MS-222 after LOE occurred. Because one fish from each species was tested in parallel, and CT_{max} and the O_2 level at LOE varied between individuals and species (Table 2.2), the fish that reached its tolerance limit first was euthanized immediately by injecting 0.2 g L^{-1} MS-222 into its respirometer while in the 'recirculation' mode. This allowed me to continue measurements on the remaining fish while ensuring that policies for the ethical treatment of animals were followed. Once the remaining fish reached its LOE, it was immediately taken out of the respirometer and euthanized. Finally, the ventricular and bulbar masses of each fish were measured, and expressed as a percentage of body mass [relative ventricular mass (RVM) and bulbar mass (RBM), respectively].

2.3.4. Data Analyses

To account for the well-known effect of body size on metabolic rate, all $\dot{M}O_2$ values (mg O_2 kg^{-1} h^{-1}) were adjusted using an exponent of 0.80 to obtain allometrically scaled values (i.e., in mg O_2 $kg^{-0.80}$ h^{-1}). I am aware that scaling exponents can vary

depending on many factors such as species, lifestyle, hierarchical level and the metabolic rate parameter being measured (e.g., SMR *vs.* RMR *vs.* MMR *vs.* AS). However I chose the scaling exponent of 0.80 because this value agrees with several studies that report SMR and RMR data on >90 teleost species (Clarke *et al.*, 1999, Killen *et al.*, 2007, Killen *et al.*, 2010, Norin *et al.*, 2018). Further: 1) to my knowledge no scaling exponent for Atlantic salmon metabolic parameters has been reported, and this value is in the middle of the range of scaling factors that have been reported for other salmonids (sockeye salmon: 0.87-0.88; steelhead trout: 0.73) (Brett *et al.*, 1973, Trudel *et al.*, 2005); and 2) although Sullivan *et al.* (1982) reported an allometric scaling exponent of 0.72 for sablefish RMR, there were several serious shortcomings in their analysis, and I was not confident using their data or the exponents that could be calculated in this study based solely on two size classes of fish (which ranged from 0.76 for SMR to 0.80 for AS_T).

The P_{crit} was determined for each individual fish using a method similar to Monteiro *et al.* (2013) by: 1) calculating the mean of the RMR measurements during the range of regulation; 2) calculating the linear regression for the $\dot{M}O_2$ values that were dependent on water O_2 content (i.e., after the fish became an oxyconformer); and 3) calculating the O_2 level at the intersection between the RMR and the calculated linear regression (see Figure S2.2). I used RMR rather than the fish's SMR to determine P_{crit} because this is the most common approach in studies of P_{crit}, it provides a P_{crit} value that is more relevant than a P_{crit} based on the SMR (e.g., see Rogers *et al.*, 2016, Wood, 2018), and this method has been used in aquaculture-based studies where it is referred to as the limiting oxygen saturation (LOS, Barnes *et al.*, 2011, Remen *et al.*, 2013). Further, fishes likely exhibit a routine level of $\dot{M}O_2$ the majority of the time, whereas even under

experimental conditions that restrict the fish's movement and remove stimulation, $\dot{M}O_2$ values representative of SMR are infrequently measured.

Aerobic metabolic scope was calculated by subtracting the SMR for each fish at its acclimation temperature from its MMR during the incremental temperature increase; the latter value determined as the highest $\dot{M}O_2$ value measured during the thermal challenge (irrespective of the temperature at which it was measured). I recognize that this way of measuring aerobic scope (which I will define as AS_T) is different from the traditional method where SMR and MMR are measured at the same temperature, and MMR is determined by exercising fish to their critical swimming speed (U_{crit}) (e.g., see Brett, 1964). However, in an acute upper thermal challenge, the fish's ability to elevate its metabolism as temperature increases is likely to be the most relevant parameter to look at with respect to how 'metabolic capacity' is related to a fish's thermal tolerance. In addition, previous studies (see Gollock *et al.*, 2006 *vs.* Petersen *et al.*, 2010, Powell *et al.*, 2016) have shown that AS_T in adult cod is within 10-20% of their aerobic scope as measured by performing a U_{crit} test (i.e., their AS). These two measures are also more comparable/similar than when MMR is measured by chasing fish to exhaustion (another commonly used method of determining AS) *vs.* performing a U_{crit} test (Reidy *et al.*, 1995, Norin *et al.*, 2016). Finally, it has recently been suggested that the relationship between acclimation temperature and AS is similar when obtained by giving the fish a chase or U_{crit} test *vs.* giving them an incremental temperature increase at each acclimation temperature and measuring their thermal metabolic scope (TMS; Paschke *et al.*, 2018). Thus, I feel that AS_T is a valuable metric for examining how the capacity of fishes to elevate their metabolic rate is related to their acute thermal tolerance, and that it provides

comparable information to that obtained by calculating AS based on exhaustive exercise protocols.

In this study, the critical temperature (T_{crit}) was also estimated for each group (i.e., juvenile sablefish, adult sablefish, and adult salmon) and defined as the temperature where MMR was reached before each fish lost equilibrium.

2.3.5. Statistical Analyses

Outliers were identified as values with a standardized residual of <-3 or >3 and omitted from the dataset (<1% of the total number of values). Assumptions of normality and homoscedasticity were subsequently tested using the Shapiro-Wilk and Fligner-Killeen tests, respectively (Shapiro *et al.*, 1965, Fligner *et al.*, 1976). Differences in morphometric, metabolic and tolerance parameters (Tables 2.1-2.2) between life-stages (juvenile *vs.* adult sablefish) and species (sablefish *vs.* salmon adults) were identified using Student's *t*-tests or Mann-Whitney U tests (i.e., non-parametric tests) if the above assumptions failed. The effect of temperature or O_2 level on $\dot{M}O_2$ (Figure 2.1) within each group (i.e., juvenile sablefish, adult sablefish, adult salmon) was determined using one-way ANOVAs, and the data were transformed in the case of non-normality and heterogeneity. If the effect of temperature or O_2 level was significant, differences between the starting temperature (12°C) and higher temperatures, or between the starting O_2 level (90-100% air saturation) and lower O_2 levels, were identified using Dunnett's post-hoc comparisons. All statistical analyses were performed using R v3.4.0 (R Core Team, 2019) and RStudio software, and with $P<0.05$ as the level of significance. All values reported in the text, figures and tables are means±standard error of the mean (s.e.m.).

2.4. Results

$\dot{M}O_2$ data recorded during the acute thermal and hypoxia challenges for 5 out of 32 juvenile sablefish, 3 out of 22 adult sablefish, and 1 out of 20 adult Atlantic salmon, were not used in the final analysis. These fish struggled (i.e., did not settle and were agitated), which resulted in 2-to-3-fold elevations in $\dot{M}O_2$ compared to the previous $\dot{M}O_2$ measurement, even though the temperature or O_2 level had changed by only 1°C or 10% air saturation. However, the body and cardiac morphometrics, SMR and RMR for these fish were included in the data set.

2.4.1. Body and Cardiac Morphometrics

The adult sablefish and Atlantic salmon used in these experiments differed significantly in body mass ($P<0.0001$), fork length ($P<0.0001$) and condition factor ($P=0.006$); all these parameters being higher for the salmon. In contrast, the sablefish had a significantly higher RVM than the Atlantic salmon (0.086±0.002% *vs.* 0.066±0.002%, respectively; $P<0.0001$) (Table 2.1). While no difference in RBM existed (~0.020% for both species), the ratio between bulbar and ventricular masses was lower in sablefish as compared to the salmon (0.243±0.008 *vs*. 0.289±0.008; $P=0.0002$).

2.4.2. Standard and Routine Metabolic Rates

The SMR (57.6±3.4 mg O_2 $kg^{-0.80}$ h^{-1}) and RMR (81.0±4.6 mg O_2 $kg^{-0.80}$ h^{-1}) of adult sablefish were significantly lower as compared to both the juvenile sablefish (by ~14-15%) and the adult salmon (by ~22-24%) (Table 2.2).

2.4.3. Acute Upper Thermal Tolerance and Metabolic Parameters

The $\dot{M}O_2$ of all groups was significantly higher by 15-16°C as compared to that measured at the fish's acclimation temperature (12°C). The acute upper thermal tolerance (CT_{max}) of the adult sablefish was approx. 2.2°C higher than that of juveniles (24.9±0.2°C *vs.* 22.7±0.3, respectively; $P<0.0001$), but approx. 1.3°C lower than that of the Atlantic salmon (26.2±0.5°C, respectively; $P=0.018$) (Table 2.2, Figure 2.1A), and this pattern was also reflected in their T_{crit} values (juvenile sablefish: 21.8±0.3°C *vs.* adult sablefish: 22.7±0.4°C *vs.* adult salmon: 23.7±0.5°C). Despite these differences in CT_{max}, AS_T values for all three groups were very similar (juvenile sablefish: 183.2±15.8 mg O_2 $kg^{-0.80}$ h^{-1}; adult sablefish: 176.9±7.0 mg O_2 $kg^{-0.80}$ h^{-1}; adult salmon: 188.5±11.3 mg O_2 $kg^{-0.80}$ h^{-1}) (Table 2.2). This was largely because life-stage or species differences in SMR offset any differences in maximum metabolic rate (MMR); the MMR of both the juvenile sablefish and adult salmon being ~11-13% higher than what was recorded for the adult sablefish.

2.4.4. Acute Hypoxia Tolerance and Metabolic Parameters

The acute hypoxia tolerance of adult sablefish was greater than that of juveniles as indicated by the significantly lower P_{crit} values (15.8±1.5% *vs.* 18.9±0.4% air saturation, respectively; $P=0.037$) and the significantly lower O_2 level at LOE (5.4±0.5% *vs.* 8.3±0.4% air saturation, respectively; $P=0.0002$) (Table 2.2, Figure 2.1B). While the mean values for RMR during the period of O_2 regulation were similar for the two groups (64.9±7.5 *vs.* 75.5±2.2 mg O_2 $kg^{-0.80}$ h^{-1}, respectively; $P=0.229$), there was a difference in the nature of the $\dot{M}O_2$ response to decreases in the water O_2 level (Figure 2.1B). While the $\dot{M}O_2$ of adult sablefish changed very little between 40% and 20% air saturation, $\dot{M}O_2$ in

Table 2.1. Body and cardiac morphometrics for adult sablefish (*A. fimbria*) and Atlantic salmon (*S. salar*). *A significant difference (P<0.05) between adult sablefish and Atlantic salmon. [1]Fulton's K, calculated as 100×[body mass/length^3]. [2]Relative ventricular mass. [3]Relative bulbar mass. [3]Ratio between bulbar mass and ventricular mass. Values are means±s.e.m.

	Sablefish	**Atlantic salmon**
Body morphometrics	*n=22*	*n=20*
Body mass (g)	675±19	1132±54*
Fork length (cm)	39.1±0.4	45.0±0.6*
Condition factor[1]	1.11±0.02	1.19±0.02*
Cardiac morphometrics	*n=21*	*n=19*
RVM[2] (%)	0.086±0.002	0.066±0.002*
RBM[3] (%)	0.021±0.001	0.019±0.001
BM/VM[3]	0.243±0.008	0.289±0.008*

Table 2.2. Metabolic parameters, and acute upper thermal and hypoxia tolerance, in juvenile and adult sablefish (*A. fimbria*) and adult Atlantic salmon (*S. salar*). [+]A significant difference (P<0.05) between juvenile and adult sablefish. *A significant difference (P<0.05) between adult sablefish and Atlantic salmon. [1]Standard metabolic rate. [2]Routine metabolic rate. [3]Maximum metabolic rate; measured as the highest metabolic rate for each fish independent of the temperature at which it was recorded. [4]Aerobic scope, calculated as the difference between SMR measured at the fish's acclimation temperature and the MMR recorded for each fish. [5]Critical temperature, the temperature at which the highest metabolic rate was measured, before fish started to lose equilibrium. [6]Critical thermal maximum. [7]Critical oxygen level, P_{crit}. P_{crit} could not be identified for three juvenile and one adult sablefish, thus, sample size was reduced to n=10 and n=8, respectively. P_{crit} could also not be identified with confidence for any Atlantic salmon individuals tested. [8]Loss of equilibrium. [9]Time until fish lost equilibrium while kept at an O_2 level of 5% air saturation. For this parameter, the range of values recorded is shown in the brackets. Values are means±s.e.m.

	Sablefish		**Atlantic salmon**
	Juvenile (~10 g)	**Adult (~675 g)**	**Adult (~1132 g)**
Metabolic parameters	*n=32*	*n=13*	*n=11*
SMR (mg O_2 $kg^{-0.80}$ h^{-1})[1]	67.4±2.5[+]	57.6±3.4	75.3±3.7*
RMR (mg O_2 $kg^{-0.80}$ h^{-1})[2]	94.5±3.7[+]	81.0±4.6	104.4±3.8*
Acute upper thermal tolerance	*n=14*	*n=10*	*n=10*
MMR (mg O_2 $kg^{-0.80}$ h^{-1})[3]	260.3±15.4	234.3±5.9	265.0±11.5*
AS_T (mg O_2 $kg^{-0.80}$ h^{-1})[4]	183.2±15.8	176.9±7.0	188.5±11.3
T_{crit} (°C)[5]	21.8±0.3	22.7±0.4	23.7±0.5
CT_{max} (°C)[6]	22.7±0.3[+]	24.9±0.2	26.2±0.5*
Acute hypoxia tolerance	*n=13*	*n=9*	*n=9*
RMR during regulation (mg O_2 $kg^{-0.80}$ h^{-1})	75.5±2.2	64.9±7.5	79.7±6.6
P_{crit} (% air sat.)[7]	18.9±0.4 (10)[+]	15.8±1.5 (8)	N/A
O_2 level at LOE[8] (% air sat.)	8.3±0.4[+]	5.4±0.5	24.2±1.8*
Time until LOE at 5% air sat. (min)[9]	N/A	45±11 (12-106)	N/A

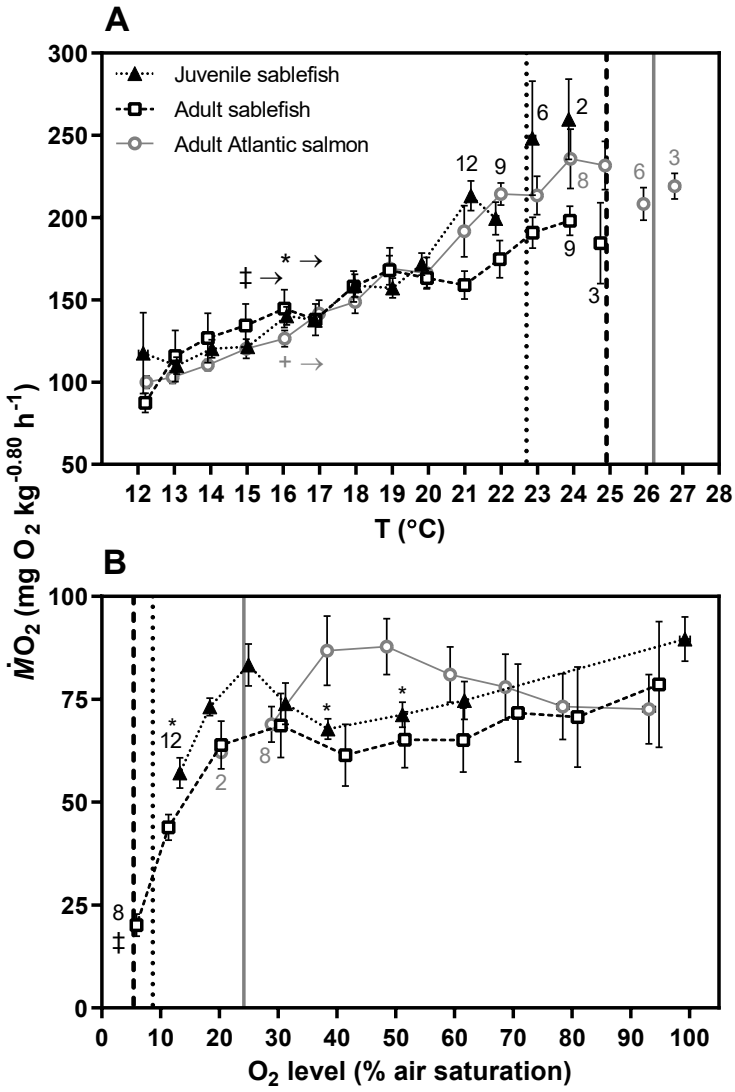

Figure 2.1. Mass-specific O_2 consumption ($\dot{M}O_2$, mg O_2 $kg^{-0.80}$ h^{-1}) of juvenile and adult sablefish (*A. fimbria*) and adult Atlantic salmon (*S. salar*), as affected by increases in water temperature (°C) (A) and reductions in water O_2 level (% air saturation) (B). $\dot{M}O_2$ was measured using intermittent-flow respirometry. Vertical lines indicate the average CT_{max} (**A**) or O_2 level at loss of equilibrium (LOE) (**B**), and numbers indicate reduced sample sizes due to LOE. When the sample size became <8 fish, data points were no longer connected with a line. Data are means±s.e.m. See Table 2.2 for sample sizes, and values for SMR, RMR, MMR, CT_{max}, T_{crit}, P_{crit}, and O_2 level at LOE based on these data. Significant differences (P<0.05) between the $\dot{M}O_2$ at the starting temperature (12°C) *vs.* higher temperatures, or at the starting O_2 level (90-100% air saturation) *vs.* lower O_2 levels, are indicated with a * for juvenile sablefish, a ‡ for adult sablefish, and $^{+}$ for Atlantic salmon. The horizontal arrows (**A**) indicate that there is a significant difference at all following temperatures.

the juveniles at 25% air saturation increased by approx. 23% (compared to that at 40% air saturation) before declining. This was associated with an increase in swimming activity of the juvenile sablefish.

P_{crit} could not be identified with confidence for any of the Atlantic salmon tested using the present protocol, and could therefore, not be compared with that of sablefish. This was because none of the Atlantic salmon in this study showed a clear reduction in $\dot{M}O_2$ at low O_2 levels (O_2 conformity) and instead, maintained a relatively stable $\dot{M}O_2$ until LOE (see Figure S2.2). Indeed, $\dot{M}O_2$ did not significantly decline with the lowering of water O_2 levels in Atlantic salmon (Figure 2.1B). The O_2 level at LOE for the sablefish was much lower than that of the Atlantic salmon (5.4±0.5% *vs.* 24.2±1.8% air saturation, respectively; P=0.0004) (Table 2.2, Figure 2.1B). Furthermore, sablefish adults were able to maintain equilibrium at 5% air saturation for a considerable amount of time (on average 45 min; range 12-106 min).

2.5. Discussion

In this study, I showed that the sablefish is quite tolerant of elevated temperatures and very hypoxia tolerant. These are advantageous traits in aquaculture, given that marine ecosystems are projected to experience higher temperatures (IPCC, 2021) and more frequent and severe hypoxic conditions as a result of climate change (Diaz *et al.*, 2008, Diaz *et al.*, 2009, Breitburg *et al.*, 2018). In fact, O_2 levels along the entire British Columbia (Canada) coast (where many aquaculture cage-sites are located) are already declining due to the persistent upwelling of deep, oxygen-poor, water (Crawford *et al*., 2013).

2.5.1. Routine Metabolic Rate

The present measurements for RMR in ~0.68 kg sablefish at 12°C (~65-81 mg O_2 $kg^{-0.80}$ h^{-1}; Table 2.2) are within the range of values reported by Sullivan *et al.* (1982) for ≥0.7 kg sablefish (~75 mg O_2 $kg^{-0.80}$ h^{-1}). Further, the RMR value reported here for the Atlantic salmon (~1.1 kg; ~80-104 mg O_2 $kg^{-0.80}$ h^{-1}) at 12°C is comparable to that measured in ≥0.15 kg post-smolt salmon at 10-14°C by Deitch *et al.* (2006) (~63 mg O_2 $kg^{-0.80}$ h^{-1}), Lucas (1994) (~84 mg O_2 $kg^{-0.80}$ h^{-1}) and Barnes *et al.* (2011) (~101 mg O_2 $kg^{-0.80}$ h^{-1}).

2.5.2. Acute Upper Thermal Tolerance

The CT_{max} of adult Atlantic salmon in this study (~26.2°C) is in line with values reported by several other authors for this species (~24.5-30°C) (Anttila *et al.*, 2013, Anttila *et al.*, 2015, Bowden *et al.*, 2018), even though these studies used smaller fish (~25-75 g) (see below) and a much faster (and less ecologically relevant) rate of heating (0.1-0.3°C min^{-1}) (e.g., Anttila *et al.*, 2013, Anttila *et al.*, 2015). The CT_{max} of adult sablefish was only ~1.3°C lower than that of the Atlantic salmon, even though adult sablefish live in deep and cold waters where they would typically not encounter high temperatures (Mason *et al.*, 1983). Further, their CT_{max} was approximately 2°C higher than that of adult Arctic char (*Salvelinus alpinus*), which has a Holarctic distribution and a CT_{max} of ~23°C (Penney *et al.*, 2014). This relatively high upper thermal tolerance, while unexpected, is a beneficial trait for sablefish adults kept in sea-cages. High temperatures can be experienced during the summer months, and in the Georgia Strait (British Columbia, Canada) temperature at all depths is increasing at a rate of ~0.024°C

per year (Masson *et al.*, 2007).

This study also showed that juvenile sablefish have a lower CT_{max} (by ~2.2°C) than that of adult sablefish. This is surprising given that juveniles in the wild are more likely to encounter high temperatures in their in-shore habitat, and smaller fishes generally have higher CT_{max} values [coho salmon (*Oncorhynchus kisutch*), Clark *et al.*, 2012; leopard coral grouper (*Plectropomus leopardus*), Messmer *et al.*, 2017; Delta smelt (*Hypomesus transpacificus*), Komoroske *et al.*, 2014; and coral reef damselfishes, Clark *et al.*, 2017]. The reason for this is not clear (e.g., see below), but these data suggest that juvenile sablefish may be more susceptible to high sea-cage temperatures.

In this study, variations in acute upper thermal tolerance (i.e., mean CT_{max} values ranging from ~22.7 to 26.2°C) could not be explained by differences in AS_T. The AS_T of all three groups was approximately 180 mg O_2 $kg^{-0.80}$ h^{-1} (Table 2.2), and no significant correlations were evident within the groups between CT_{max} and AS_T (see Figure S2.3). Although the present experiments were primarily designed to measure the sablefish's metabolism and environmental tolerances, and were not specifically meant to systematically test the OCLTT hypothesis, these data suggest that AS_T is not a determinant of acute thermal tolerance in these fishes (even if rates of temperature increase are ecologically relevant). This finding is supported by a large number of other studies (e.g., Norin *et al.*, 2013, Gräns *et al.*, 2014, Wang *et al.*, 2014, Brijs *et al.*, 2015, Ekström *et al.*, 2016, Ern *et al.*, 2016, Lefevre, 2016). While these studies do not preclude AS_T or aerobic scope (AS) from playing an important role in fish thermal tolerance when evaluated over longer time scales (i.e., days to months, etc.), they contribute to the ongoing debate about the relevance of the OCLTT concept to fish thermal

biology/ecology. Recently, this concept has been challenged by a large group of scientists in the field (see Jutfelt *et al.*, 2018), and recent data from Hvas *et al.* (2017) further question the relevance of the OCLTT concept. These authors showed that the CT_{max} of juvenile Atlantic salmon correlated positively with acclimation temperature, and that >20% of fish acclimated to 23°C died, despite AS being highest at 23°C and similar to that measured in fish acclimated to 15 and 18°C.

Mechanisms that determine upper thermal tolerance in fishes are constantly being investigated and debated [e.g., see Jutfelt *et al.*, 2018 *vs.* Pörtner *et al.*, 2017, 2018]. It is possible, for example, that some of the differences in upper thermal tolerance (CT_{max}) between adult sablefish and salmon were determined by differences in cardiac function and the temperature sensitivity of their cardiovascular systems. In many species, CT_{max} is preceded by cardiac collapse (i.e., cardiac arrhythmias followed by a sudden fall in cardiac output) (Farrell, 2002, Gollock *et al.*, 2006, Clark *et al.*, 2008), and Anttila *et al.* (2015) recently showed that maximum heart rate and the temperature at which the heart goes arrhythmic are sensitive to acclimation temperature and highly plastic. Perhaps, cardiac collapse occurred at a lower temperature in sablefish than in Atlantic salmon, as Atlantic salmon have a coronary circulation that supplies O_2-rich blood to the ventricular myocardium, whereas the sablefish do not (pers. observation). Anttila *et al.* (2013) showed that CT_{max} was positively correlated with the RVM of Atlantic salmon families, and suggested that heart size may be an important determinant of thermal tolerance. However, it is clear that heart size and blood oxygen carrying capacity did not determine the difference in CT_{max} between the adult salmon and sablefish. The RVM of sablefish was 30% larger than in the Atlantic salmon (Table 2.1), and haematocrit (the percentage

of the blood comprised of erythrocytes) and blood [haemoglobin] in normoxic adult sablefish acclimated to 10-11°C are 22.5±1.0% and 7.5±0.3 g dL^{-1} (Leeuwis *et al.*, 2021). Both these latter values are lower than for the Atlantic salmon stocks that are used for experiments at Memorial University (Deitch *et al.*, 2006, Harter *et al.*, 2019).

2.5.3. Acute Hypoxia Tolerance

The present findings clearly show that the sablefish is a highly hypoxia tolerant species, and are in agreement with Sullivan *et al.* (1982). These authors showed that the RMR of unfed sablefish did not fall appreciably between 8 and 1 mg O_2 L^{-1} (~15% air saturation). This conclusion is also supported when these data are compared with that collected for other fish species. For example, the P_{crit} for juvenile and adult sablefish (~18.9% and ~15.8% air saturation, respectively) is much lower than the P_{crit} of hypoxia intolerant species such as Dover sole (*Solea solea*) (~49.3% air saturation) (McKenzie *et al.*, 2008) and lumpfish (54.4% air saturation) (*Cyclopterus lumpus*) (Ern *et al.*, 2016) at similar temperatures. Moreover, the P_{crit} for sablefish is similar to the P_{crit} reported for relatively hypoxia tolerant species like goldfish (*Carassius auratus*) (~14.8% air saturation) (Fu *et al.*, 2011) and several sculpins species of the Cottidae family (~16% air saturation) (Mandic *et al.*, 2009). However, it is not as low as that of extremely hypoxia tolerant cyprinid species (~7.8% air saturation) (Fu *et al.*, 2013). The O_2 level at LOE for sablefish juveniles and adults (~8.3% and ~5.4% air saturation, respectively) is also well below the lowest O_2 level that can be endured by Atlantic cod (~20.8% air saturation) (Petersen *et al.*, 2010), a relatively hypoxia intolerant marine species. It is important to note here that the O_2 level at LOE for adult sablefish is likely to be even lower than the

value reported in this study. This is because the experimental set-up did not allow me to lower the O_2 level beyond 5% air saturation, and adult sablefish were able to tolerate this low O_2 level for 45±11 min (Table 2.2).

I was not able to confidently determine the P_{crit} for adult Atlantic salmon in this study, as none of the individuals tested showed a reduction in metabolic rate at low O_2 levels that would indicate a switch from O_2 regulation to conformity (e.g., see Figure S2.2). This was surprising given that other studies have successfully determined the P_{crit} for Atlantic salmon. For instance, Stevens *et al.* (1998) reported a P_{crit} of ~38% air saturation for ~0.04 kg pre-smolts, Barnes *et al.* (2011) showed that the P_{crit} for ~0.15 kg post-smolts was ~42% air saturation, and Remen *et al.* (2013) found that P_{crit} was ~39% air saturation for 0.3-0.6 kg fish. However, Barnes *et al.* (2011) also reported that only 56% of the salmon (15 out of 27) tested showed a transition from O_2 regulation to conformity, and thus, P_{crit} could not be calculated for the remaining 44% of their fish. Apparently, some Atlantic salmon individuals in their study population had the ability to reduce their metabolic rate at low O_2 levels, whereas others could not. It is possible that the 9 fish included in the present study were also unable to lower their $\dot{M}O_2$ as the water O_2 level fell. However, the methodology used in the present study might be another reason why the P_{crit} could not be clearly identified for Atlantic salmon. Stevens *et al.* (1998) and Barnes *et al.* (2011) used closed respirometry, rather than intermittent-flow respirometry as employed here. With closed respirometry, the O_2 level in the tank or respirometer is gradually/continuously lowered by the animal's respiration, and thus, allows for many metabolic rate measurements to be made at a range of O_2 levels. In contrast, in the present study, the O_2 level was decreased in a controlled, step-wise,

manner (by reductions of ~5-10% air saturation), and only two $\dot{M}O_2$ measurements were made per fish at each step.

With regards to the O_2 level at LOE determined for the Atlantic salmon in this study (24.2±1.8% air saturation), it is difficult to compare this finding with the literature. There are very few measurements of the O_2 level at LOE in Atlantic salmon exposed to acute hypoxia. Anttila *et al.* (2013) showed that salmon smolts could tolerate 10% air saturation for 23 to 120 min (depending on the family), and Wood *et al.* (2017) reported that the O_2 at LOE for salmon was ~12.5% saturation. However, there are several important differences between these studies and this current study. Anttila *et al.* (2013) conducted their study at 4°C, and both studies lowered O_2 levels at a much faster rate (Anttila *et al.*, 2013: 1.5% min^{-1} until 10% air saturation; Wood *et al.*, 2017: 4-5% per minute until 45% air saturation, then 0.3% min^{-1}) than in this study. Because of the temperature dependency of $\dot{M}O_2$ and hypoxia tolerance in salmon (e.g., see Remen *et al.*, 2013), and that rapidly decreasing water O_2 levels reduces exposure time to low oxygen conditions, these methodological differences could have allowed the salmon in the above studies to achieve lower P_{crit} and LOE values than reported here.

Overall, the high acute hypoxia tolerance of sablefish is not surprising. Adults of this species are known to colonize the oxygen minimum zone off the coast of California, where they can encounter O_2 levels of 0.34-0.80 mg L^{-1} (Moser *et al.*, 1994). Further, recent work in our lab shows that they can survive weeks of exposure to 20% air saturation (Gerber *et al.*, 2019). However, it is currently not clear what mechanisms might confer their significant hypoxia tolerance. It is unlikely that this enhanced hypoxia tolerance was associated with their RVM, as a value of 0.086% is not large for fishes

(Farrell *et al.*, 2017). Their haematocrit (~22%; Leeuwis *et al.*, 2021) is not high, and approx. midway between that measured in salmon (~30%) (Deitch *et al.*, 2006; Harter *et al.*, 2019) and inactive species such as flatfish (~15%) (Wood *et al.*, 1979, Turner *et al.*, 1983). The RMR of adult sablefish was only ~20% lower than that of the salmon used in these experiments (Table 2.2). Further, it does not appear that the sablefish's capacity for anaerobic metabolism can explain their tolerance of low oxygen conditions. Plasma lactate was barely detectable (i.e., <0.1 mM) in normoxia-acclimated adult sablefish, those acutely exposed to 40% air saturation at 12°C, or adult sablefish chronically acclimated to 40% and 20% air saturation at 12°C, and only reached 5 mM when sablefish were acutely exposed to 40% air saturation and then given an acute thermal challenge to CT_{max} (Leeuwis *et al.*, 2021 and Table S2.1). In contrast, plasma lactate levels are ~0.8 mM in normoxic rainbow trout at 13°C, and at this temperature they increase rapidly after the fish are exposed to ~30% air saturation (levels 5 and 7 mM after 30 and 60 min, respectively) (Omlin *et al.*, 2010).

At O_2 levels beyond the P_{crit}, the metabolic rate of both sablefish juveniles and adults decreased (by ~36% and ~74%, respectively) as compared to that measured under normoxia. One explanation for this decrease in oxygen consumption is that this species uses adaptive responses to reduce its O_2 demand to survive periods of acute hypoxia, which could include metabolic depression (Richards, 2009). This may involve mechanisms such as behavioural quiescence (Speers-Roesch *et al.*, 2018, Reeve *et al.*, 2022), the reduced perfusion of organs/organ systems (such as the gastrointestinal tract) not essential for survival (Seth *et al.*, 2011), and the downregulation of protein synthesis and other energetically expensive anabolic processes (Lewis *et al.*, 2007, Lewis *et al.*,

2010). It is also possible that their hypoxia tolerance is, at least in part, related to unique aspects of their red blood cell physiology. Sablefish erythrocytes lack a β-adrenergic responsive Na^+/H^+ exchanger (β-NHE), and the onset of the Root effect (i.e., the drop in Hb-O_2 carrying capacity due to a pH decrease) occurs at a much lower red blood cell intracellular pH (~6.9) than in other teleost fish; rainbow trout (*Oncorhynchus mykiss*) (~7.3), common carp (*Cyprinus carpio*) (~7.1) and copper rockfish (*Sebastes caurinus*) (~7.5) (Rummer *et al.*, 2010). The copper rockfish is a species that belongs to the same taxonomic order (Scorpaeniformes) as the sablefish and inhabits a similar, but shallower habitat, where it would normally not encounter hypoxic conditions. The magnitude of the Root effect (i.e., the percentage reduction of Hb-O_2 saturation) is also considerably lower in sablefish (~35%) than in copper rockfish and rainbow trout (~50-60% reduction), and more closely resembles that of the very hypoxia tolerant common carp (~20%) (Salama *et al.*, 1988). Thus, a reduced Root effect and a low onset pH might protect O_2 transport in sablefish, and this unique physiological attribute may in part explain the ability of this species to tolerate acute hypoxia and survive chronic exposure to low O_2 levels (i.e., as low as 20% air saturation; Gerber *et al.*, 2019).

The P_{crit} and LOE of juvenile sablefish were slightly higher than measured in adult fish (~18.9% *vs.* ~15.6% and ~8.3% *vs.* ~5.4% air saturation, respectively). This difference in hypoxia tolerance is not likely due to the size difference between the two groups (Nilsson *et al.*, 2008), but to the higher RMR in juvenile fish (~94.5 *vs.* ~81.0 mg O_2 $kg^{-0.80}$ h^{-1}; P=0.043) and/or the difference in behaviour as water O_2 levels were lowered. For example, the activity of juvenile sablefish increased as the water O_2 level approached 30% air saturation, whereas the adults remained calm (pers. observation); a

finding supported by the increase in $\dot{M}O_2$ of the juveniles prior reaching to their P_{crit} (Figure 2.1B), and that explains why there was a strong positive relationship between P_{crit} and RMR during the O_2 regulation period in adult sablefish, but not juveniles (Figure S2.4). The behavioural response to acute hypoxia involves a trade-off between increasing swimming activity to enhance the probability of finding more oxygenated water, and decreasing swimming activity to lower oxygen requirements (Killen *et al.*, 2012), and it appears that juvenile sablefish are more proactive than their adult conspecifics. Proactive fish have been shown by van Raaij *et al.* (1996) to be less tolerant than fish that remain calm as water oxygen levels are lowered. This difference in behavioural response may not be unexpected, however, as juvenile sablefish inhabit in-shore regions that are likely to be highly oxygenated, whereas adults lead a bathydemersal existence and encounter very low oxygen levels in this habitat.

2.5.4. Summary

In this study, the tolerances and physiological (metabolic) responses of adult and juvenile sablefish to an acute temperature increase and acute hypoxia were examined. Based on a comparison of CT_{max}, P_{crit} and LOE values for this species with the literature, and the data I collected on Atlantic salmon, this research shows that the adult sablefish is quite tolerant of high temperatures (CT_{max} ~25°C), and that both juvenile and adult sablefish are very tolerant of acute hypoxia (P_{crit} and LOE values of <20% and <9% air saturation, respectively). These data suggest that the sablefish should have no problem dealing with changes in these two environmental conditions at British Columbia (Canada) sea-cage sites; although ongoing research on the combined effects of exposure to high

temperatures and hypoxia, and the physiological impacts of chronic hypoxia, will also be critical for the aquaculture industry.

Based on the inability to determine a P_{crit} value for the salmon in the current study, and the lack of a relationship between RMR and P_{crit} for juvenile sablefish (likely due to hyperactivity as the water O_2 level decreased), I am in agreement with Wood (2018) that LOE and other metrics are better suited for examining the hypoxia tolerance of fishes. This research also adds to the ongoing debate about the relevance of the OCLTT concept to fish biology/ecology/conservation [i.e., see Jutfelt *et al.*, 2018 *vs.* Pörtner *et al.*, 2017, 2018] as the tolerances of the sablefish and salmon to high temperatures in this study were not related to AS_T.

The sablefish has a unique life history and ecology, and it has already been shown that it has novel physiological attributes as compared to other teleost species (e.g., see Rummer *et al.*, 2010). At present, however, it is unclear what specific mechanisms confer the sablefish's tolerance to the high temperatures and low oxygen conditions that were used in this study. Therefore, performing further experiments that assess the cardiovascular function, stress physiology and biochemistry of the sablefish when challenged with hypoxia and elevated temperatures, is important to provide more clarity.

2.6. References

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Table S2.1. Plasma lactate levels in sablefish acclimated to normoxia (~100% air sat.) or chronic hypoxia (6 months at ~40% air sat. followed by 3-4 weeks at ~20% air sat.). These are unpublished data that were collected from sablefish used in Gerber et al. (2019). Plasma samples were taken from sablefish that were euthanized (by a blow to the head) right after netting from the tank, and were immediately frozen in liquid N_2, stored at -80°C and analysed as described by Leeuwis et al. (2021). In brief, samples were first deproteinized with 6% (v/v) perchloric acid, then measured spectrophotometrically at 340 nm using the production of NADH/NADPH by lactate dehydrogenase. There was no significant difference (P>0.05) between the acclimation groups (assessed by a Student's t-test). Values are means±s.e.m. and sample size is n=9 for each group.

	Acclimation treatment	
	Normoxia-acclimated	Hypoxia-acclimated
Lactate (mmol L^{-1} plasma)	0.044±0.012	0.044±0.008

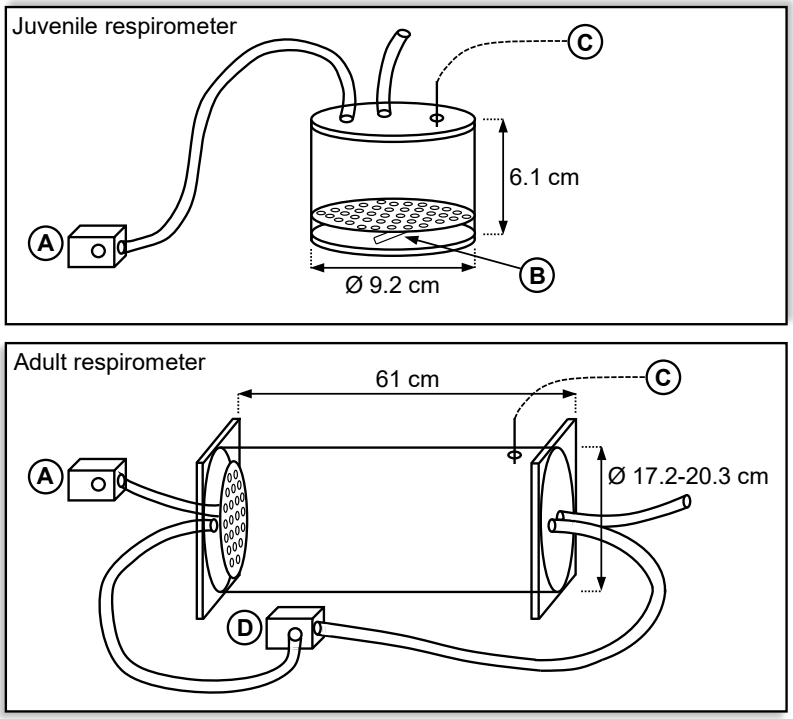

Figure S2.1. Schematic overview of the cylindrical respirometers used in experiments with juvenile and adult fish to measure mass-specific O_2 consumption ($\dot{M}O_2$) with intermittent-flow respirometry. Top panel - Glass respirometer used for juvenile sablefish (9.2 cm in diameter×6.1 cm high, 400 mL). **Bottom panel** - Plexiglass respirometer used for adult sablefish (17.2 cm in diameter×61.0 cm long, 14.1 L) and Atlantic salmon (20.3 cm in diameter×61.0 cm long, 19.8 L). In both respirometry systems, a flush pump (**A**) supplied fresh oxygenated water from the water table to the respirometer during the 'flushing' period of the measurement cycle. In the juvenile respirometer, a stir bar (**B**) in the bottom (separated from the fish by a perforated cover) was used to mix the water inside the respirometer during the 'recirculation' (closed) period. In the adult respirometer, a recirculation pump (**D**) mixed the water during the 'recirculation' period. Fibre-optic O_2 probes (**C**) were used to record O_2 levels inside both of the respirometers.

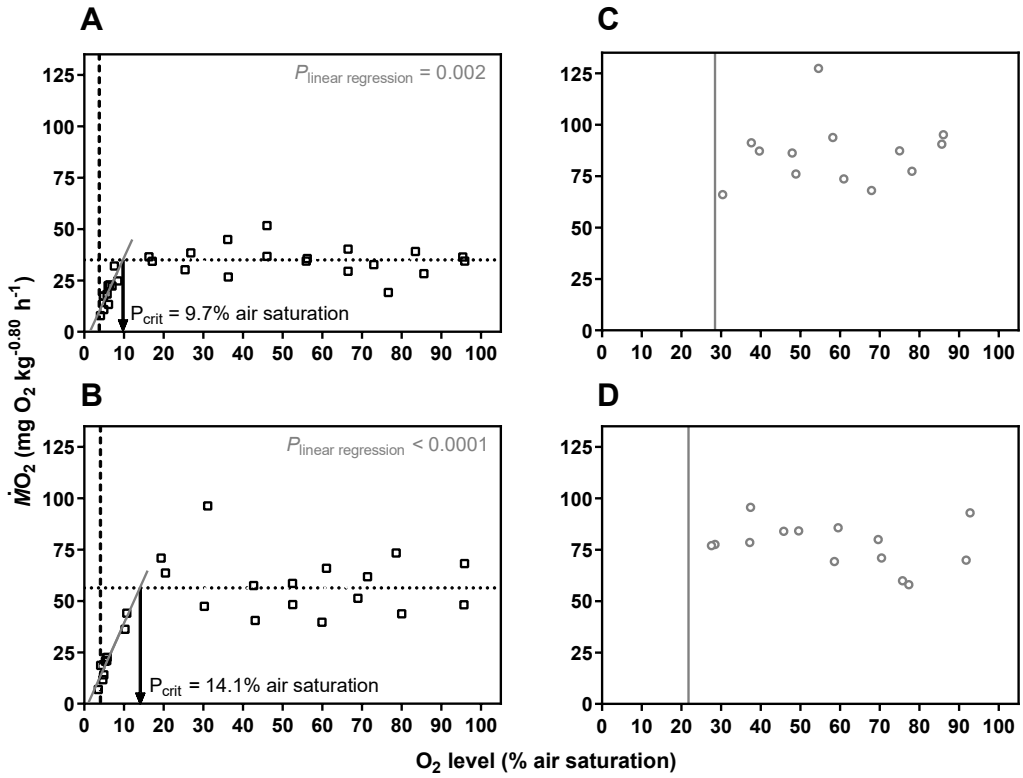

Figure S2.2. Representative examples of the mass-specific O_2 consumption ($\dot{M}O_2$, mg O_2 $kg^{-0.80}$ h^{-1}) in two adult sablefish (*A. fimbria*) (A-B) and Atlantic salmon (*S. salar*) (C-D) relative to water O_2 level (% air saturation). These data illustrate that sablefish showed a clear reduction of $\dot{M}O_2$ at low O_2 levels (i.e., a period of O_2 conformity), whereas Atlantic salmon did not. Without a clear period of conformity, it was not possible to calculate P_{crit} for this group. Vertical lines indicate the O_2 level at LOE for the individual fish shown. For sablefish, P_{crit} was determined as the O_2 level at the intersection (vertical arrow) of the average $\dot{M}O_2$ during the period of O_2 regulation (horizontal line), and the linear regression for $\dot{M}O_2$ during the period of O_2 conformity (grey solid line).

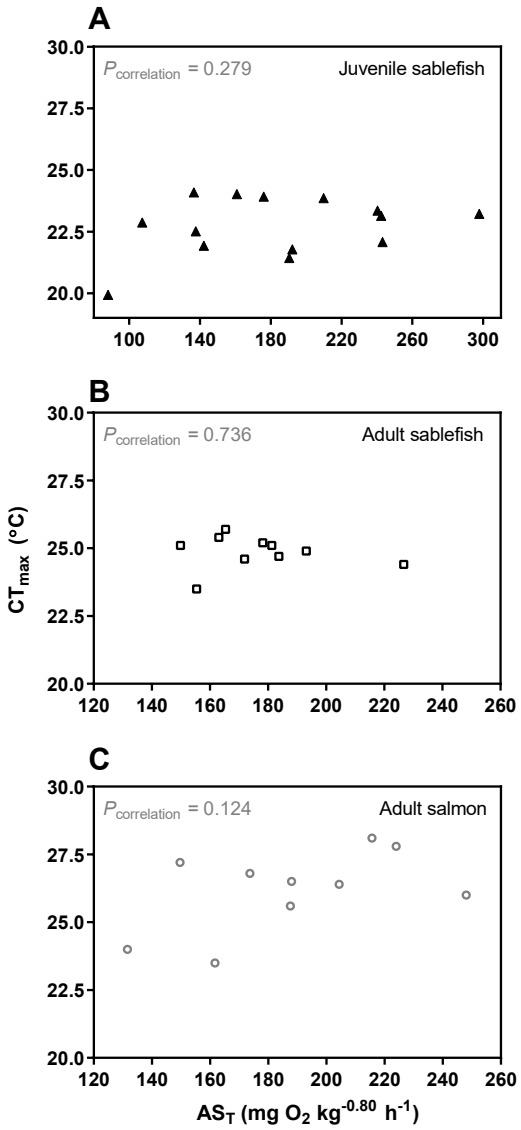

Figure S2.3. Lack of significant correlation between temperature-induced metabolic scope (AS_T) (mg O_2 $kg^{-0.80}$ h^{-1}) and CT_{max} (°C) in juvenile sablefish (*A. fimbria*) (A), adult sablefish (B) and adult Atlantic salmon (*S. salar*) (C). Pearson *r* correlation coefficients were 0.311 (**A**), -0.123 (**B**) and 0.519 (**C**); *P*-values for the correlations in each group are shown in upper left corner of each panel.

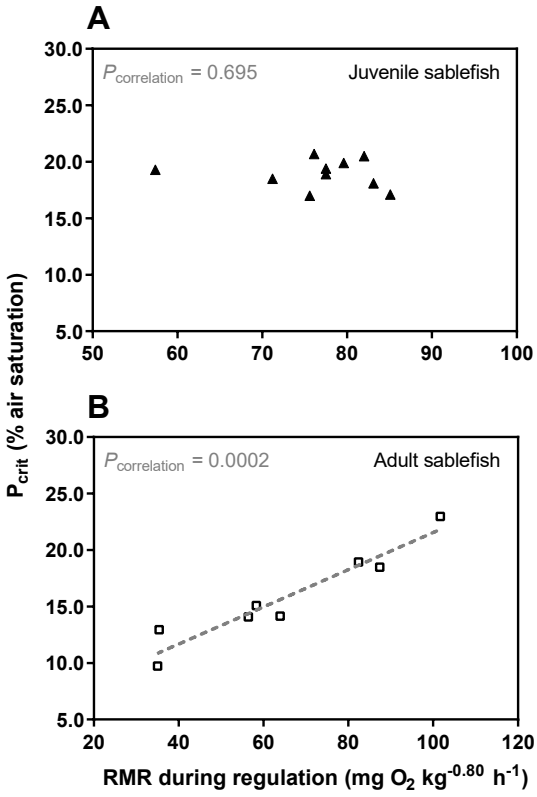

Figure S2.4. Correlations between routine metabolic rate (RMR) during oxygen regulation and P_{crit} (% air saturation) in sablefish (*A. fimbria*) juveniles (A) and adults (B). Pearson *r* correlation coefficients were -0.142 (**A**) and 0.957 (**B**); *P*-values for the correlations in each group are shown in upper left corner of each panel. For adult sablefish, the equation for the linear regression is y=0.165x+5.090.

Chapter 3: **The Effect of Chronic Hypoxia on the Thermal Tolerance and Cardiorespiratory Physiology of Sablefish (*Anoplopoma fimbria*)**

3.1. Abstract

Studies of heart function and metabolism have been used to predict the impact of global warming on fish survival and distribution, and their susceptibility to acute and chronic temperature increases. Yet, despite the fact that hypoxia and high temperatures often co-occur, only one study has examined the effects of hypoxia on fish thermal tolerance, and the consequences of hypoxia for fish cardiac responses to acute warming have not been investigated. In this study, I report that sablefish (*Anoplopoma fimbria*) did not increase heart rate or cardiac output when warmed while hypoxic, and that this response was associated with reductions in maximum O_2 consumption and thermal tolerance (CT_{max}) of 48% and approximately 3°C, respectively. Further, acclimation to hypoxia for four to six months did not substantially alter the sablefish's temperature-dependent physiological responses or improve its CT_{max}. These results provide novel, and compelling, evidence that hypoxia can impair the cardiac and metabolic responses to increased temperatures in fish, and suggest that some coastal species may be more vulnerable to climate change-related heat waves than previously thought. Further, they support research showing that cross-tolerance and physiological plasticity in fishes following hypoxia acclimation are limited.

3.2. Introduction

More frequent and extreme warming events (i.e., heat waves) are a consequence of climate change (IPCC, 2014, Frölicher *et al.*, 2018, Oliver *et al.*, 2018). The ability of fishes to survive exposure to high temperatures depends to a large extent on the capacity of the heart to deliver more O_2 to the tissues to meet elevated metabolic demands (i.e., oxygen consumption, $\dot{M}O_2$) (Pörtner, 2010, Pörtner *et al.*, 2017). For example, heart rate (f_H) and cardiac output ($\dot{Q}$; the amount of blood pumped per minute) increase with temperature, and cardiac collapse (Wang *et al.*, 2007, Farrell *et al.*, 2009, Eliason *et al.*, 2011, Eliason *et al.*, 2017, Farrell *et al.*, 2017) and neural impairment (Andreassen *et al.*, 2020) appear to be key factors in determining the upper thermal limit of fishes. The occurrence and severity of hypoxia are also increasing with climate change (IPCC, 2014, Breitburg *et al.*, 2018), and hypoxia often coincides with high temperatures in coastal environments (Kemp *et al.*, 2005, Breitburg *et al.*, 2018), including at aquaculture cage-sites (Burt *et al.*, 2012, Stehfest *et al.*, 2017, Burke *et al.*, 2020). This could severely limit fish thermal tolerance (Ern *et al.*, 2016). This is because low O_2 levels result in a regulated decrease in f_H [a response known as bradycardia (Farrell, 2007)] that, theoretically, may prevent necessary increases in cardiac function (f_H and $\dot{Q}$) when fishes are exposed to high temperatures. The effect of hypoxia on thermal tolerance has only been examined in two fish species (Ern *et al.* 2016), and this condition's impact on temperature-dependent cardiorespiratory responses is unknown. Further, despite evidence that acclimation (long-term exposure) to hypoxia modifies the cardiorespiratory response to warming at high O_2 levels (i.e. under normoxic conditions) considerably (Motyka *et al.*, 2017), there is conflicting information about whether hypoxic acclimation improves

thermal tolerance, and vice versa [i.e. that there is 'cross-tolerance' between these two oxygen-limiting conditions (Burleson *et al.*, 2011, Anttila *et al.*, 2015, McBryan *et al.*, 2016, Motyka *et al.*, 2017, Levesque *et al.*, 2019, McDonnell *et al.*, 2019)]. Specifically, the hypothesis that hypoxia acclimation increases upper thermal tolerance when O_2 levels are low has not been experimentally tested. These are important questions given the need to understand how physiological plasticity is related to a fish's tolerance to environmental change, and have implications for fish survival and potential shifts in species' distribution with global warming.

In this study, I conducted two experiments on sablefish (*Anoplopoma fimbria*) with the goal of providing insights into the potential consequences of hypoxia on the cardiorespiratory response and susceptibility of fishes to acute warming events. The sablefish is a widely distributed species across the North Pacific, that lives in coastal waters as juveniles but successfully inhabits oxygen minimum zones (OMZs; areas with O_2 levels of less than 2 mg L^{-1}) as adults (Mason *et al.*, 1983, Doya *et al.*, 2017). Given that this species encounters, and tolerates (Leeuwis *et al.*, 2019), a wide range of temperatures and O_2 levels, it is an ideal model in which to test the above questions/hypotheses with respect to the relationship between acute hypoxia, adaptation to hypoxia and thermal tolerance. In the first experiment, I acclimated sablefish to either 100% (normoxia) or 40% air saturation (hypoxia) at 10°C for 4-6 months. This is a moderate hypoxic level for the sablefish at this temperature, as it is well above the critical and lethal O_2 thresholds (15.8% and 5.4% air sat., respectively) for this species (Leeuwis *et al.*, 2019), but also low enough to potentially constrain O_2 delivery and trigger a plastic response. Then, I performed surgery to allow for *in vivo* cardiovascular measurements

and repeated blood sampling (see below), and recovered both acclimation groups under normoxia for 2 days before exposing them to acute hypoxia (a decrease to 40% air sat. over 2 h) followed by warming (at 2°C h^{-1}) until their critical thermal maximum (CT_{max}; the temperature at which the fish could no longer maintain equilibrium). Throughout the experiment, I recorded $\dot{Q}$, and analysed blood O_2-carrying capacity ([haemoglobin] and haematocrit), stress hormone and glucose levels, and lactate (an index of anaerobic metabolism) (Figure S3.1). I also measured $\dot{M}O_2$ using intermittent-flow respirometry, and used $\dot{M}O_2/\dot{Q}$ (the amount of O_2 consumed per millilitre of blood pumped) to estimate O_2 extraction by the tissues based on rearranging the Fick equation. It should be noted that during warming, 40% air saturation likely becomes a more severe hypoxia level for sablefish, given that a fish's critical O_2 threshold (P_{crit}) increases with temperature in other species (Remen *et al.*, 2013, Ern *et al.*, 2016). Subsequently, I performed a follow-up experiment involving the same cardiorespiratory measurements, but where warming occurred under normoxia, to better understand the responses observed under hypoxia.

3.3. Materials & Methods

3.3.1. Experimental Animals and Hypoxia Acclimation

Juvenile sablefish (1 g) were acquired from NOAA's Manchester Research Station (Port Orchard, WA, USA) and transported to Memorial University of Newfoundland (MUN; St. John's, NL, Canada) by air. After clearance from quarantine at the Cold-Ocean and Deep-Sea Research Facility (CDRF), the fish were reared for ~1 year at the Dr. Joe Brown Aquatic Research Building (JBARB) in tanks supplied with 10°C seawater with

an O_2 level of ~100% air saturation (157 mmHg; 9.3 mg L^{-1}), and a 12 h light:12 h dark photoperiod. Procedures used to acclimate the sablefish to hypoxia (~40% air saturation; 63 mmHg; 3.7 mg L^{-1}) have been described previously (Gerber *et al.*, 2019). The average initial masses of the normoxia- and hypoxia-acclimated sablefish at the start of hypoxia acclimation were 855±36 and 843±34 g, respectively. The hypoxic acclimation period was 4-6 months, depending on when the fish were used in the experiments. Water P_{O_2} and temperature in the hypoxic and normoxic tanks during this period were 42.4±0.1% air saturation and 10.2±0.0°C, and 106.2±0.5% air saturation and 10.3±0.0°C, respectively. Water O_2 levels were checked daily, and water 'quality' was measured weekly throughout the 6-month experiment and remained similar between the normoxic and hypoxic tanks; with pH ranging from 7.9-8.2, NH_3 levels of <0.006 ppm and NH_3/NH_4^+ levels of ≤0.50 ppm. The latter parameter is especially important to measure given that exposure to total ammonia levels of ≥1.25 ppm may elevate plasma cortisol and glucose levels in sablefish (Kim *et al.*, 2017).

The fish were fed a marine fish diet (Skretting Europa: 55% protein, 15% fat, 2% carbohydrate, 3% calcium, 2% phosphorous) at 0.65% of their body weight day^{-1}, and on days when the hypoxia-acclimated fish did not consume their full ration, the normoxia-acclimated fish were fed the same amount. This ensured that there were no nutritional or morphometric differences between the two experimental groups [given the suppressive effect of chronic hypoxia on appetite in other fish species; e.g., see Pichavant *et al.* (2001), Magnoni *et al.* (2018)]. The fish used for the thermal challenge experiments were taken from the tank before feeding (i.e., they were fasted for 24 h prior to surgery).

Over the course of the experiment, average feed intake for both acclimation groups was 0.4% body weight day^{-1}. The weight (approx. 1,320 g), length, condition factor, cardiac and splenic masses of the normoxia- and hypoxia-acclimated sablefish at the time of the experiments were similar (see Table S3.4).

3.3.2. Surgery and Recovery

Sablefish were initially anaesthetized by immersion in seawater containing 0.1 g L^{-1} tricaine methanesulphonate (MS-222; AquaLife TMS, Syndel Laboratories Ltd, Nanaimo, BC, Canada) until ventilatory movements ceased. After weight and fork length were recorded, the fish were transferred to a surgery table where their gills were continuously irrigated with chilled (4°C) and oxygenated seawater containing a maintenance dose of MS-222 (0.05 g L^{-1}). To measure *in vivo* cardiac function, a Transonic® flow probe (2.0-2.5 S) was placed around the ventral aorta (Figure S3.1), following the procedures described for Atlantic cod (Gollock *et al.*, 2006), but with some modifications for sablefish. Sablefish have a thin fold of skin that connects the ventral margin of their operculum to the body wall, and this was cut to allow access to the opercular cavity. The operculum and gills were then lifted, secured in place with umbilical tape, and a small (<10 mm) incision was made approx. 15-20 mm anterior to the ventral portion of the 4^{th} gill arch. The ventral aorta was isolated using blunt dissection, a flow probe (2.0 or 2.5 S) was placed around the ventral aorta, and correct flow probe placement was confirmed by connecting the probe to a flow meter (Model TS-420, Transonic® Systems Inc., Ithaca, NY). The flow probe cable was then secured to the skin with 2-0/3-0 silk sutures at three sites: below the incision at the edge of the

opercular cavity, anterior to the pectoral fin, and next to the dorsal fin. To allow for repeated blood sampling, a polyethylene cannula (PE 50; Intramedic®, Becton Dickinson and Co., NJ, USA) filled with heparinized 0.9% saline (100 U mL^{-1}) was subsequently inserted into the afferent branchial artery of the second or third gill arch (Figure S3.1). The cannula was then tied to the gill arch with 3-0 silk and sutured to the fish at positions close to where the flow probe lead was secured. Finally, the opercular skin fold was re-attached to the ventral side of the fish using 2-0 silk suture and interrupted stitches.

Fish were recovered in aerated anaesthetic-free seawater at 12°C. When ventilation commenced, the fish were individually placed into 19.8 L cylindrical respirometers (20.3 cm in diameter×61.0 cm long) for 42-46 h to recover from surgery under normoxic conditions (Figure S3.1). The respirometers were submerged in a water table supplied with seawater from a temperature- and P_{O_2}-controlled ~600 L reservoir, which initially kept water in the respirometers at 12°C and approximately 100% air saturation. The reservoir temperature was maintained by a custom built heater/chiller (Technical Services, MUN) and titanium coils that received water from circulating water baths (Fisher Scientific, Ottawa, Canada). Water O_2 levels in the reservoir were regulated by a computer running WitroxCTRL software (Loligo Systems, Viborg, Denmark) that was interfaced with a fibre-optic O_2 meter, an O_2 probe (PreSens, Regensburg, Germany) and a DAQ-4 module (Loligo Systems). This system controlled two solenoid valves which released air or N_2 gas into the reservoir as required. The water table was surrounded with black plastic sheeting to minimize external stimuli during observations or blood collection. During recovery, the cannulas were flushed twice a day with

heparinized saline (100 U mL^{-1}) to prevent blood clotting within them. Surgery was always performed on one fish from each acclimation group (the respirometer they were placed into randomized), so that these two fish could be tested in parallel.

Sablefish were allowed to recover from surgery for 2 days in the initial experiment, as previous authors have shown that 1-3 days of recovery in the laboratory was adequate for blood parameters in Atlantic salmon to normalize after invasive surgery (i.e., hepatic portal vein cannulation) (Eliason *et al.*, 2007), and hypoxia-acclimated Atlantic cod and steelhead trout recovered for 2 days in normoxia still showed striking effects of hypoxic acclimation on their physiology (Petersen *et al.*, 2010a-b, Motyka *et al.*, 2017). Blood parameters were indeed back to baseline in sablefish after the 2 day recovery, based on the low glucose (~30 mg dL^{-1}), lactate (~0.1 mg dL^{-1}) and catecholamine levels (≤1 nM) levels (Figure 3.4D-E, G-H). However, cortisol was still elevated (~140 ng mL^{-1}; Figure 3.4F) as compared to previously reported resting levels in this species (≤60 ng mL^{-1}) (Olla *et al.*, 1998, Davis *et al.*, 2001, Lupes *et al.*, 2006). Further, I monitored the recovery of cardiac function and $\dot{M}O_2$ at 24 hours post-surgery, and found that these parameters were not significantly different from values measured on the following day ($0.37<P<0.99$ depending on the cardiorespiratory parameter; data not shown).

3.3.3. Hypoxic Warming Challenge

On the experimental day, the flow probe leads were connected to the Transonic® flow meter, after which the fish were left to settle for 2 h before performing any measurements. To investigate the cardiorespiratory response to warming under acute

hypoxia, the water P_{O_2} was first gradually decreased over the course of 2 h to 40% air saturation. Subsequently, the temperature was increased at 2°C h^{-1} until the fish reached its CT_{max}. This rate of heating in CT_{max} tests is widely used in the field of fish physiology to assess acute thermal tolerance, and is considered to be ecologically relevant [e.g., Ern *et al.*, (2016)]. Cardiac function and $\dot{M}O_2$ were measured at the following points during the protocol: (i) under normoxia (100% air saturation) at 12°C; (ii) under hypoxia (40% air saturation) at 12°C; and (iii) at every 2°C increase thereafter. Cardiac function and $\dot{M}O_2$ were always recorded simultaneously and measured twice at each time point. The temperature at which the heartbeat became arrhythmic/irregular ($T_{arrhythmia}$) was also recorded. Blood (approx. 0.7 mL) was sampled from the cannula immediately after the cardiac function and $\dot{M}O_2$ measurements were taken at four points during the experiment: (i) under normoxia at 12°C; (ii) under hypoxia at 12°C; (iii) under hypoxia at 18°C; (iv) under hypoxia at CT_{max}. At each sampling, this volume was replaced by an equal volume of 0.9% saline to help maintain the fish's blood volume. The entire protocol took approximately 11 h to complete. Upon reaching their CT_{max}, fish were euthanized as quickly as possible inside their respirometer (by injecting MS-222 into their respirometer through a tube while in 'recirculation' mode: final concentration 0.3 g L^{-1}) (Leeuwis *et al.*, 2019). This was important as the CT_{max} of the paired fish often differed. After removing the fish from their respirometer, correct placement of the flow probe was verified. Then, the heart and spleen were dissected out, rinsed in saline, blotted dry and weighted to determine the relative atrial, ventricular, bulbus and splenic masses [(tissue mass/body mass)×100].

3.3.4. Measurements of Cardiorespiratory Function

Cardiac function was recorded at 20 Hz by interfacing the flow meter with an MP100A-CE data acquisition system and a laptop running AcqKnowledge software (BIOPAC Systems Inc., Goleta, CA, USA). I calculated f_H (in beats min^{-1}) manually by determining the time required for 20 systolic peaks in the blood flow recording. This time period was also used to measure $\dot{Q}$ (in mL min^{-1}), which was expressed relative to the fish's body mass (in mL min^{-1} kg^{-1}). I calculated V_S (in mL $beat^{-1}$ kg^{-1}) as $\dot{Q}/f_H$. Sometimes, small peaks were seen in the recordings (e.g., Figure S3.2). These were not counted as systolic peaks, as they resulted from the Transonic® flow probes picking up ventilatory movements. However, it is interesting that ventilation appeared to remain regular after the heart went arrhythmic (see Figure S3.2D, H), and this is consistent with recent data suggesting that arrhythmias at high temperatures are not caused by nervous dysfunction, but by ionic disturbance at the level of the ventricular myocytes (Haverinen *et al.*, 2020).

Before the experiments began, the Transonic® flow probes were tested for temperature sensitivity and accuracy using Transonic® calibration tubing, as follows. The tubing was immersed in a seawater filled plexiglass box and fitted to peristaltic tubing (Masterflex 16 L/S, Tygon E-LFL) through which sablefish blood (haematocrit ~5%) was pumped at three flow rates (15, 30, and 45 mL min^{-1}) and at five temperatures (12, 15, 18, 21, 24°C). To prevent signal reflection (background noise) the plexiglass box was lined with foam (1 cm thick), and acoustic gel was used to ensure good signal transmission from the tubing to the flow probe. Although the probes overestimated blood flow by 11-13%, none of the probes displayed significant temperature sensitivity. *In vivo* $\dot{Q}$

measurements were corrected for this error.

$\dot{M}O_2$ (in mg O_2 min^{-1} kg^{-1}) was measured with the automated intermittent-flow respirometry system described previously (Leeuwis *et al.*, 2019, Zanuzzo *et al.*, 2015). The durations of the 'flushing' and 'recirculation' periods were adjusted throughout the protocol, to ensure a R^2>0.90 for each measurement, and to avoid a decline in P_{O_2} inside the respirometers of more than approximately 5% air saturation. The respirometers were cleaned regularly to prevent background bacterial respiration. This background was considered negligible based on overnight measurements and experiments using respirometers without fish. In these tests, P_{O_2} declined by less than 1% air saturation during the measurement period. $\dot{M}O_2$ per unit of $\dot{Q}$ ($\dot{M}O_2/\dot{Q}$; mg O_2 L^{-1} blood) was used as a measure of tissue O_2 extraction, by rearranging the Fick equation:

$$\dot{M}O_2 = \dot{Q} \times (C_aO_2 – C_vO_2),$$

where C_aO_2 and C_vO_2 are the O_2 content of the arterial and venous blood, respectively. I am aware that $\dot{M}O_2/\dot{Q}$ is an indirect measure of O_2 extraction and has limitations [i.e., the equation does not account for potential O_2 uptake through cutaneous respiration, see Farrell *et al.* (2014)], and that for a direct assessment C_aO_2 and C_vO_2 would need to be measured. However, this approach is used by various research groups [see Claësson *et al.* (2016), Motyka *et al.* (2017), Joyce *et al.* (2018), Harter *et al.* (2019)], and I measured blood haemoglobin content so that changes in blood O_2-carrying capacity could be accounted for in the interpretation of the data. Blood O_2 content was not estimated from

haemoglobin content, given that the combined effects of temperature, pH and CO_2 on Hb-O_2 affinity and maximum saturation of sablefish blood are still unknown. For each fish, the routine and maximum $\dot{M}O_2$ were determined as the average of the $\dot{M}O_2$ measurements at 42-46 h post-surgery, and as the highest $\dot{M}O_2$ recorded during exposure to hypoxic warming, respectively. The scope for $\dot{M}O_2$ was calculated as maximum–routine $\dot{M}O_2$. I acknowledge that aerobic scope is most commonly determined using swimming-flumes and/or chase protocols, but the use of 'temperature-induced' aerobic scope (AS_T) is appropriate for this thermal tolerance study and provides equivalent data to these traditional methods [e.g. Paschke *et al.* (2018), Leeuwis *et al.* (2019), Norin *et al.* (2019)]. Resting, and maximum values, and values for the scope of cardiac parameters (f_H, $\dot{Q}$, V_S) and $\dot{M}O_2/\dot{Q}$ were determined in the same way as for $\dot{M}O_2$.

3.3.5. Blood Sampling and Analyses

The amount of blood withdrawn from each individual (~2.8 mL) during the hypoxic warming challenge was small (~5%) relative to the sablefish's estimated blood volume (~52 mL based on a ~1.3 kg body mass and a typical teleost blood volume of 30-40 mL kg^{-1}; Olson, 1992). This is unlikely to cause adverse physiological effects (Lawrence *et al.*, 2020). Still, to verify that blood sampling did not confound the results for thermal tolerance (for instance, by negatively impacting blood O_2 carrying capacity), five fish from each acclimation group were included from which no blood was collected during the experiment. These fish had sham-cannulations, or did not have blood withdrawn as their cannula was plugged. There was no significant difference between the CT_{max} of fish with/without blood sampling, regardless of acclimation condition (P=0.48;

Figure S3.3). Therefore, the cardiorespiratory and thermal tolerance data (presented in Figures 3.1-3.3) from the groups with and without blood sampling were pooled for further analysis in this study.

The collected blood samples were immediately aliquoted for the analysis of various haematological parameters. Blood was first drawn into microhaematocrit tubes and these were centrifuged at 10,000 ×*g* for 5 min to determine Hct (%). An aliquot of 50 µL of blood was collected for the measurement of blood Hb concentration. Then, the remaining blood was centrifuged for 1 min at 10,000 ×*g* in a mini-centrifuge (catalogue #05-090-128, Fisher Scientific) and 300 µL of plasma was pipetted into a 1.5 mL brown (opaque) Eppendorf tube containing 15 µL of 0.2 M EDTA and 15 µL of 0.15 M glutathione for later measurement of circulating catecholamine levels. The rest of the plasma was divided into 50 µL aliquots for the measurement of cortisol, lactate and glucose. The RBC pellet was used for the analysis of RBC protein content. All samples were immediately frozen in liquid N_2 and stored at -80°C.

All blood analyses were done in duplicate. Hb concentration (mg mL^{-1}) was determined using the cyanomethaemoglobin method (Drabkin's reagent, D5941, Sigma Aldrich, Oakville, Canada), whereby the absorbance was measured at 540 nm using a plate reader (SpectraMax 5, Molecular Devices, San Jose, USA) connected to a computer running SoftMax Pro software. Hb concentration was calculated from standard curves generated using bovine Hb (Sigma, H2500) that were run on the same plates. Mean cellular Hb concentration (MCHC, in mg mL^{-1}) was calculated as
Hb concentration/Hct×100. ELISA kits were used to measure plasma levels of cortisol (Neogen Life Sciences, 402710, Lexington, KY, USA) and adrenaline and noradrenaline

(Abnova KA1877, Taipei, Taiwan), following the manufacturer's instructions. The catecholamine samples were analysed within 2 months of storage at -80°C. Plasma samples for lactate and glucose were first deproteinized with 6% (v/v) perchloric acid, then measured spectrophotometrically at 340 nm using the production of NADH/NADPH by lactate dehydrogenase (Sigma L2500), and by glucose-6-phoshate dehydrogenase with hexose kinase (Sigma G8404 and H4502), respectively. Lactate and glucose concentrations were calculated in reference to standard curves (Sigma L6402 and G5767, respectively). The RBC protein level was determined using a Coomassie (Bradford) assay kit (Thermo Scientific 23200) according to the manufacturer's instructions.

3.3.6. Additional Experiment

The primary aim of this experiment was to verify that sablefish have the capacity to enhance f_H and $\dot{Q}$ when warmed under normoxia, like other teleosts (Eliason *et al.*, 2017, Farrell *et al.*, 2017). The sablefish used for this experiment were acquired from Golden Eagle Sable Fish (Vancouver Island, BC, Canada) as young juveniles. These fish were transported to MUN using the same procedures, and reared for ~1.5 years at the JBARB at the same temperature, O_2 level and light regime, and fed the same diet and ration, as the sablefish used in the initial experiment. The sablefish were also of a comparable age (less than two months older), and had similar body morphometrics (e.g., the same body mass), although length, condition factor, and atrial and ventricular mass differed (see Table S3.4). The surgical procedures in the additional experiment were exactly the same as in the initial experiment, except that fish were not cannulated. This research was focused on measuring the fish's cardiac response and the presence/absence

of blood sampling did not influence thermal tolerance (Figure S3.3). Furthermore, surgical recovery was only for 1 day (22-23 h) instead of 2 days (42-46 h), because it was shown in the initial experiment that 1 day was adequate for the cardiorespiratory variables to stabilize (see Section 3.3.2). Measurements of cardiac function and $\dot{M}O_2$, and all other procedures, were performed as described for the initial experiment. To maintain normoxia (P_{O_2} at ~100% air saturation) throughout the thermal challenge, including the 'recirculation' periods of $\dot{M}O_2$ measurements, O_2 was bubbled into the water as required using the control system (OXY-REG, Loligo Systems) that was described previously (Leeuwis *et al.*, 2019).

Apart from the main aim of the additional experiment, the results were also used to assess whether there was any confounding effect of surgery (incl. anaesthesia and flow probe implantation) on the metabolic physiology and thermal tolerance of sablefish. This was done by comparing the CT_{max} and metabolic parameters of fish from the additional experiment, with values from a previous normoxic warming trial that did not involve any surgical procedures (Leeuwis *et al.*, 2019) (Table S3.5). There were no significant differences; which suggests that surgery did not affect the overall performance of sablefish during the warming challenge. This is in contrast to data for Atlantic cod, which showed significant reductions in maximum $\dot{M}O_2$ and aerobic scope (by ~30% and ~57%, respectively) during a hypoxic critical swimming speed test following similar surgeries as in this study (Petersen *et al.*, 2010b).

The sablefish in the additional experiment had a ~30% lower f_H and ~30% higher V_S at rest as compared to those in the initial experiment, which can be explained by their

~20-30% greater relative atrial and ventricular masses (Tables S3.3-3.4). A larger atrium and ventricle would allow the heart to pump a greater volume of blood per beat, and thus, would require a lower f_H to meet routine O_2 demands. Because the two groups of sablefish were similar in age and rearing conditions, but originated from different suppliers, this variation may be genetically-driven. Importantly, there was no significant difference in resting $\dot{Q}$ between the populations (P=0.189, Table S3.3), as the differences in f_H and V_S offset each other. Because $\dot{Q}$ is the most relevant/important parameter with regard to cardiac function, I argue that the two groups of sablefish remain sufficiently comparable. However, this is still a limitation of the study, and I acknowledge that it would have been preferred that all sablefish had originated from the same population.

3.3.7. Data Statistical Analyses

All statistical analyses were performed using Rstudio v1.2.5033 with R v3.6.2 (R Core Team, 2020) unless mentioned otherwise. Cardiorespiratory and haematological data from the initial experiment shown in Figures 3.1, 3.4 and S3.4 were analysed using a general linear mixed model (lmer function), which included fish as a random factor, and acute condition (hypoxia and warming), acclimation condition and their interaction, as fixed effects. This mixed model was graphically assessed for violations of the assumptions of normality and homogeneity using quantile-quantile plots and residual-fit plots, respectively. There were no obvious/influential outliers in the data set, and there were no violations of the assumptions of normality and homogeneity, making transformations and non-parametric tests unnecessary. Main effects were assessed using ANOVAs (anova function) with type III sums of squares. If the model indicated a

significant fixed effect, then differences between the categories among that effect were analysed using post-hoc least-squared means comparisons, with the fdr method (Bonferroni-based) for multiplicity *P*-value adjustment (emmeans and pairs functions). If there was a significant interaction between the main effects of the model (condition×acclimation), then conditions were compared within each acclimation group, and acclimation groups were compared at each condition. Cardiorespiratory data from the additional experiment shown in Figure 3.1 were analysed in the same way, except that the model did not include a factor for the effect of acclimation. For the cardiorespiratory, thermal tolerance and morphometric parameters shown in Figures 3.2 and S3.3, and Tables S3.3-S3.5, comparisons were made using two-sided Student's *t*-tests or 2-way ANOVAs, and these analyses were done in GraphPad Prism 8. Statistical significance was set at $P<0.05$ and all data are shown as means±s.e.m.

3.4. Results

3.4.1. Cardiorespiratory Response to Warming when Hypoxic

In normoxia-acclimated sablefish, acute hypoxia resulted in a significant decrease in f_H (from 41.3±2.2 to 33.4±1.8 beats min^{-1}, $P<0.01$), and surprisingly, f_H declined even further during warming (to 25.3±1.3 beats min^{-1}, $P<0.0001$) (Figures 3.1A and S3.2; Tables S3.1-S3.2). Stroke volume (V_S; the amount of blood pumped per heartbeat) increased slightly with temperature (Figure 3.1E), but this was not sufficient to compensate for the decrease in f_H. Thus, $\dot{Q}$ fell during hypoxia (from 25.5±2.2 to 21.1±1.6 mL min^{-1} kg^{-1}, $P<0.001$) and remained at this level until it declined further at 22°C (i.e., as fish approached their CT_{max}) (Figure 3.1C); this response resulting in a

negative scope for $\dot{Q}$ (Figure 3.2A and Table S3.3). Hypoxia-acclimated sablefish did not experience bradycardia when exposed to acute hypoxia at 12°C (Figures 3.1A and S3.2; Tables S3.1-S3.2), and this likely contributed to their higher aerobic scope (AS_T; maximum–routine $\dot{M}O_2$) when warmed (P<0.05; Figure 3.2B and Table S3.3). Nonetheless, f_H and $\dot{Q}$ also failed to increase with temperature in this group (Figure 3.1A, C), and hypoxia acclimation did not change the temperature at which cardiac arrhythmias began ($T_{arrhythmia}$, ~21.6°C) or CT_{max} (~22.2°C) (P=0.078 and P=0.124, respectively) (Figure 3.2D).

There was an unusual relationship between $\dot{M}O_2$ and $\dot{Q}$ in sablefish exposed to hypoxic warming (Figure 3.3). Normally, $\dot{M}O_2$ and $\dot{Q}$ are positively correlated in fishes [e.g., Motyka *et al.* (2017)]. However, I show that as $\dot{M}O_2$ increased, $\dot{Q}$ either declined (in normoxia-acclimated fish) or remained the same (in hypoxia-acclimated fish) (Figure 3.1C, G), and that there was no significant (negative or positive) correlation between $\dot{M}O_2$ and $\dot{Q}$ in the normoxia- and hypoxia-acclimated groups (P=0.064 and P=0.884, respectively; Figure 3.3). To raise $\dot{M}O_2$ during hypoxic warming without increasing $\dot{Q}$, both groups relied solely, and to the same extent, on enhanced $\dot{M}O_2/\dot{Q}$, which increased from approximately 45-55 to 115-135 mg O_2 L^{-1} blood pumped (P<0.0001; Figure 3.1I and Table S3.3). This was partially mediated by increases in blood haemoglobin levels (by 25%; Figure 3.4A, B), which occurred despite cell swelling that resulted in a decrease in the mean cellular haemoglobin concentration (MCHC; Figure 3.4C) and RBC protein levels (Figure S3.4). However, it is clear that the enhancement of $\dot{M}O_2/\dot{Q}$ was primarily due to augmented O_2 uptake by the tissues. Further, while hypoxia-acclimated sablefish had significantly lower plasma levels of adrenaline at CT_{max}, other stress hormone

(cortisol and noradrenaline) and glucose levels increased during warming to a similar degree in both groups (Figure 3.4F-H). The capacity for anaerobic metabolism was considerable in this species (plasma lactate increased from <0.2 to ~45 mg dL^{-1}) and was initiated at 18°C; indicating that the sablefish experienced insufficient O_2 delivery well before its CT_{max}. However, this parameter was also not affected by hypoxia acclimation (Figure 3.4D).

3.4.2. Cardiorespiratory Response to Warming when Normoxic

The finding that f_H and cardiac function were severely constrained during warming when the sablefish was hypoxic is unprecedented. Therefore, I performed an additional experiment with normoxia-acclimated sablefish to verify that this species has the capacity to elevate f_H and $\dot{Q}$ when warmed under normoxia, as is typical for other fishes (Eliason *et al.*, 2017, Farrell *et al.*, 2017). Indeed, sablefish were able to increase f_H and $\dot{Q}$ by 2-fold (up to 59.1±3.5 beats min^{-1} and 39.7±4.3 mL $beat^{-1}$ kg^{-1}, respectively, P<0.0001) (Figures 3.1-3.2 and S3.2, Tables S3.1 and S3.3). Further, $\dot{Q}$ and $\dot{M}O_2$ were positively correlated (P<0.001; Figure 3.3), which is consistent with the relationship that is normally observed for fishes [e.g., Motyka *et al.* (2017)]. These findings suggest that acute hypoxia prevented the sablefish's normal cardiac response to warming, and that this limited their AS_T and thermal tolerance. For example, CT_{max} was ~3°C lower in fish exposed to acute hypoxia when compared with those tested under normoxia (Figure 3.2D), and this reduction in CT_{max} was associated with a much lower AS_T (~59 *vs.* ~174 mg O_2 h^{-1} kg^{-1}) (Figure 3.2B); although values for $\dot{M}O_2/\dot{Q}$ were similar (Figures 3.1I, J and 3.2C; Table S3.3).

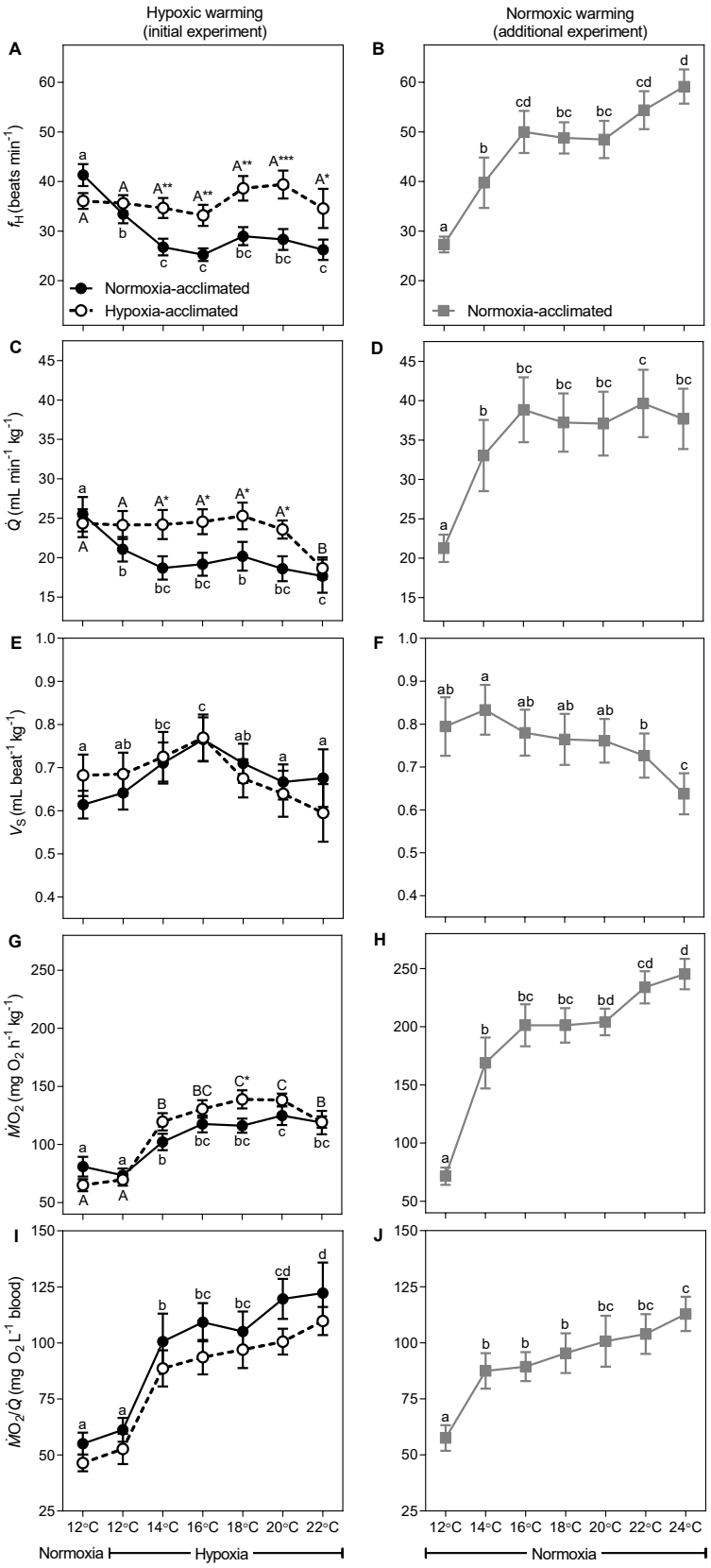
Hypoxic warming
(initial experiment)
Normoxic warming
(additional experiment)
A
B
C
D
E
F
G
H
I
J
f_H (beats min^{-1})
$\dot{Q}$ (mL min^{-1} kg^{-1})
V_S (mL beat^{-1} kg^{-1})
$\dot{M}O_2$ (mg O_2 h^{-1} kg^{-1})
$\dot{M}O_2/\dot{Q}$ (mg O_2 L^{-1} blood)
Normoxia-acclimated
Hypoxia-acclimated
12°C 12°C 14°C 16°C 18°C 20°C 22°C
Normoxia
Hypoxia
12°C 14°C 16°C 18°C 20°C 22°C 24°C
Normoxia

Figure 3.1. Cardiorespiratory responses of normoxia- and hypoxia-acclimated sablefish exposed to hypoxic or normoxic warming. Shown are (**A-B**) heart rate (f_H), (**C-D**) cardiac output ($\dot{Q}$), (**E-F**) stroke volume (V_S), (**G-H**) O_2 consumption ($\dot{M}O_2$) and (**I-J**) O_2 extraction ($\dot{M}O_2/\dot{Q}$). Hypoxic warming, left panels; normoxic warming, right panels. Symbols without a letter in common are significantly different across the sampling points/temperatures (P<0.05). In the case of a significant acclimation effect and/or acclimation×sampling interaction, lower and uppercase letters indicate differences within the normoxia- and hypoxia-acclimated groups, respectively. Asterisks indicate significant differences between acclimation groups at a particular sampling point (*P<0.05; **P< 0.01; ***P<0.001). Values are means±s.e.m. with n=14-15 per group for the initial experiment (except at 22°C where n=9-10 per group, because 4-6 fish had already reached their CT_{max}), and with n=9 for the additional experiment (except at 24°C where n=8, because one fish had already reached its CT_{max}).

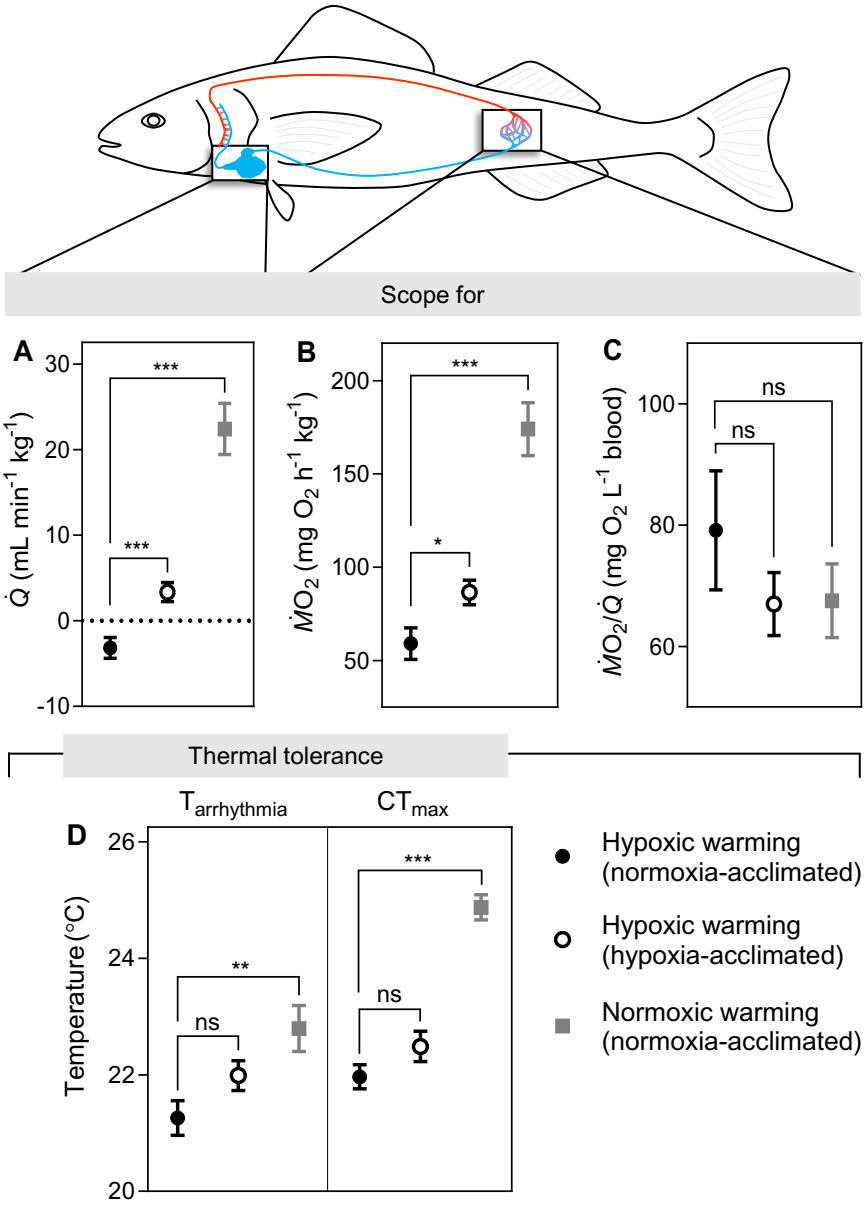

Figure 3.2. The capacity of normoxia- and hypoxia-acclimated sablefish to increase cardiorespiratory function (i.e., heart function and oxygen consumption/extraction by the tissues) when exposed to hypoxic and normoxic warming, and parameters related to thermal tolerance. Values for scope were calculated as maximum–resting values for (**A**) cardiac output ($\dot{Q}$), (**B**) O_2 consumption ($\dot{M}O_2$) and (**C**) O_2 extraction ($\dot{M}O_2/\dot{Q}$). (**D**) Parameters for thermal tolerance are the onset temperature of cardiac arrhythmias ($T_{arrhythmia}$) and the critical thermal maximum (CT_{max}). The normoxia-acclimated fish tested under hypoxia are compared to the other two groups (n.s.=P>0.05; *P<0.05; **P<0.01; ***P<0.001). Values are means±s.e.m. with n=14-15 and n=9 per group for hypoxic and normoxic warming, respectively.

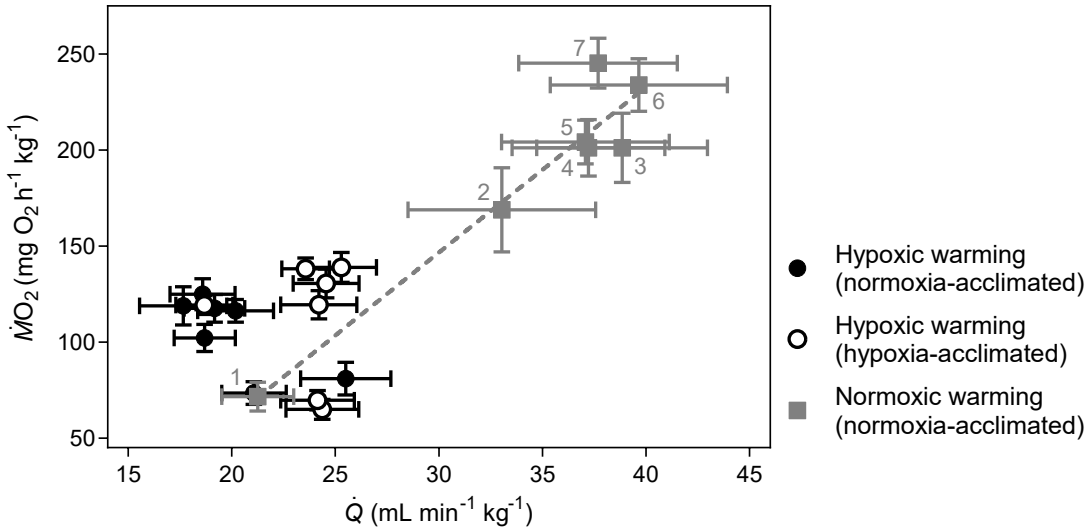

Figure 3.3. The relationship between $\dot{M}O_2$ and $\dot{Q}$ in normoxia- and hypoxia-acclimated sablefish exposed to hypoxic and normoxic warming. No significant linear regression could be fitted to the data for the normoxia and hypoxia acclimation groups tested under hypoxia (P=0.064 and P=0.884, respectively), whereas a significant linear regression was fitted to the data for the fish tested under normoxia (P<0.001, r^2=0.918, y=8.6x–112.0). The symbols for the latter group are labelled with grey numbers, which indicate the following conditions: 1, 12°C; 2, 14°C; 3, 16°C; 4, 18°C; 5, 20°C; 6, 22°C; 7, 24°C at normoxia. Values are means±s.e.m. with n=14-15 per group for the hypoxic warming experiment (except at 22°C where n=9-10 per group, because 4-6 fish had already reached their CT_{max}), and with n=9 for the normoxic warming experiment (except at 24°C where n=8, because one fish had already reached its CT_{max}).

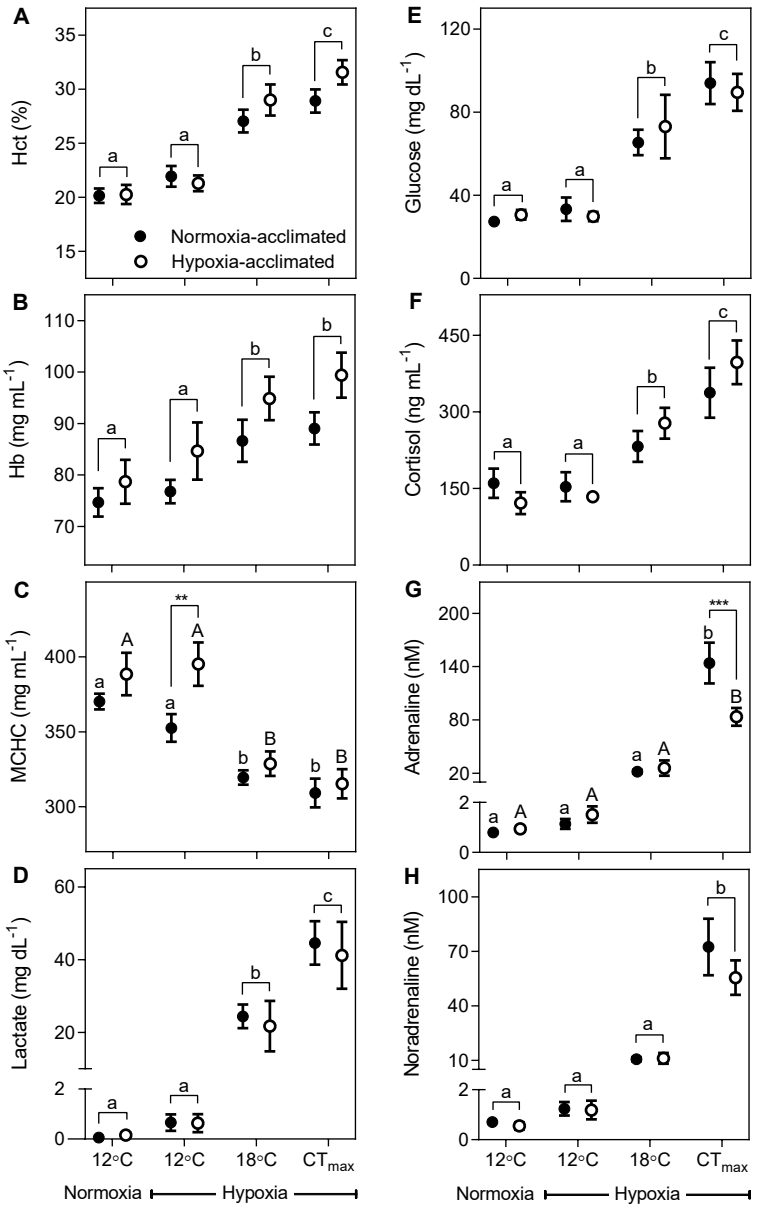

Figure 3.4. Haematological changes in normoxia- and hypoxia-acclimated sablefish when exposed to hypoxic warming. Shown are blood/plasma levels of (**A**) haematocrit (Hct), (**B**) haemoglobin (Hb), (**C**) mean cellular haemoglobin concentration (MCHC), (**D**) lactate, (**E**) glucose, (**F**) cortisol, (**G**) adrenaline and (**H**) noradrenaline. CT_{max}, critical thermal maximum. Symbols without a letter in common are significantly different across the sampling points/temperatures (P<0.05). In the case of a significant acclimation effect and/or acclimation×sampling interaction, lower and uppercase letters indicate differences within the normoxia- and hypoxia-acclimated groups, respectively. Asterisks indicate significant differences between acclimation groups at a particular sampling point (**P<0.01; ***P<0.001). Values are means±s.e.m. with n=9-10 per group.

3.5. Discussion

The results of this study (i) provide additional evidence that cardiac function is linked with temperature-induced aerobic scope and thermal tolerance (Figure 3.2), and further emphasize the role of the heart in delivering O_2 to the tissues to meet the fish's metabolic demands when exposed to increased temperatures (Wang *et al.*, 2007, Farrell *et al.*, 2009, Pörtner, 2010, Eliason *et al.*, 2011, Eliason *et al.*, 2017, Farrell *et al.*, 2017, Pörtner *et al.*, 2017); and (ii) show that hypoxia strongly limits the capacity of the cardiorespiratory system to respond to an acute thermal challenge. However, this study also offers other valuable physiological insights, raises several questions, and has major ecological implications.

3.5.1. Possible Reasons Why f_H Did Not Increase During Hypoxic Warming

A key question, which future studies will need to explore, is why hypoxic sablefish were unable to/did not increase f_H when exposed to rising temperatures (Figure 3.1A and Table S3.3). One of the potential explanations is that adenosine (which is produced by the heart muscle when O_2 supply is limited) depressed f_H (Sundin *et al.*, 1999, Stecyk *et al.*, 2007). However, because hypoxic bradycardia in fishes is induced by an increase in cholinergic nervous tone (Farrell, 2007, Farrell *et al.*, 2017) on the heart, it is most likely that the sablefish was unable to remove (or reduce) this nervous tone as temperature rose and that this prevented increases in f_H. Thus, it appears that while cholinergic inhibition of the heart (i.e., which prevents f_H from getting too high, and delays the onset of arrhythmias) may be beneficial for upper temperature tolerance in species such as rainbow trout (*Oncorhynchus mykiss*) while normoxic (Gilbert *et al.*,

2019), it may reduce the thermal tolerance of at least some fishes during hypoxia. This is not only because f_H does not increase in hypoxic sablefish with temperature, but also that V_S increased only slightly or did not change (Figure 3.1E), and this prevented $\dot{Q}$ from increasing with temperature. This finding is in contrast to Keen *et al*. (2012) who used the pharmacological agent zatebradine to prevent f_H increases in normoxic rainbow trout during a CT_{max} test, and showed that increases in V_S completely compensated for the inability to elevate f_H. This difference between studies is likely due to the direct negative effects that low O_2 levels have on the contractility of the fish heart (Tikkanen *et al.*, 2017, Carnevale *et al.*, 2020).

3.5.2. New Perspectives on Fish Cardiorespiratory Physiology and Thermal Tolerance

Currently, the literature on fish cardiorespiratory physiology and thermal tolerance suggests that increases in f_H (and thus $\dot{Q}$) are primarily responsible for increases in $\dot{M}O_2$ with temperature, and key to a fish's ability to tolerate warming events (Wang *et al.*, 2007, Farrell *et al.*, 2009, Pörtner, 2010, Eliason *et al.*, 2011, Eliason *et al.*, 2017, Farrell *et al.*, 2017, Pörtner *et al.*, 2017). Whereas, the importance of enhancing O_2 extraction is seldom acknowledged/reported. One reason for this may be that salmonid species are often used as a model for the teleost fish's response to increasing temperatures. For example, the increase in $\dot{M}O_2$ in rainbow trout with temperature is due to a 154% enhancement of $\dot{Q}$ but only a 16% increase in $\dot{M}O_2/\dot{Q}$ [see Figures 2 and 4 in Motyka *et al*. (2017)]. Similarly, Eliason *et al*. (2013) reported that O_2 extraction contributed little to the swimming and metabolic performance of sockeye salmon (*Oncorhynchus nerka*) at

high temperatures. By contrast, the rise in the sablefish's $\dot{M}O_2$ during normoxic warming was due to comparable increases in $\dot{Q}$ and $\dot{M}O_2/\dot{Q}$ (i.e., both by approx. 2-fold; Figure 3.1 and Table S3.3), and when $\dot{Q}$ did not increase under hypoxia, sablefish relied solely on enhanced $\dot{M}O_2/\dot{Q}$. Therefore, this finding fundamentally changes our understanding of fish cardiorespiratory function and the drivers of O_2 consumption at high temperatures. This important physiological adaptation for enhancing $\dot{M}O_2$ in the sablefish [and probably other fishes, e.g., European eel (Claësson *et al.*, 2016)] *vs.* salmonids (trout, char, salmon) may be related to differences in their maximum f_H and scope for increases in f_H, and thus $\dot{Q}$. Salmonids acclimated to 10°C, and warmed to their CT_{max}, have a maximum f_H and scope for f_H of approx. 120-130 and 70-80 beats min^{-1}, respectively (Penney *et al.*, 2014, Motyka *et al.*, 2017), whereas these values in sablefish are approx. 64 and 37 beats min^{-1} (Figure 3.1A and Table S3.3). What mechanisms allow for the large enhancement in O_2 extraction in the sablefish (and possibly other fishes) is not known. However, the sablefish represents one of the five teleostean groups where red blood cells appear to lack the β-adrenergic Na^+/H^+ exchanger (β-NHE) that protects against intracellular pH reductions (Rummer *et al.*, 2010), and this may allow for enhanced Root effect-mediated O_2 offloading from haemoglobin at the tissues. Interestingly, some red cell swelling during hypoxic warming was observed in this study (Figures 3.4C and S3.4), although this could have been due to passive/osmotic water influx and increased membrane fluidity/permeability, and is not necessarily indicative of β-NHE (Nikinmaa, 1992). Furthermore, splenic release of immature/young erythrocytes (which typically have a lower haemoglobin concentration; e.g., see Speckner *et al.*, 1989, Koldkjær *et al.*, 2004) may have occurred and, in part, contributed to the decline in MCHC. Recently, research

has highlighted the potential role of plasma-accessible carbonic anhydrase (PaCA) in tissue O_2 extraction in fishes, as this enzyme can acidify the blood as it passes through the tissues, and result in enhanced offloading of O_2 from haemoglobin (Harter *et al.*, 2019). This mechanism may also play a role in facilitating O_2 extraction in sablefish.

3.5.3. No Cross-Tolerance and Limited Plasticity

Motyka *et al*. (2017) showed that acclimation of steelhead trout to hypoxia (40% air saturation) does not affect this species' temperature-induced aerobic scope (AS_T) or CT_{max} when tested under normoxic conditions, and this largely agrees with the data that are reported here for the sablefish. Collectively, these data support the majority of research showing that there is no or very little 'cross-tolerance' between these two O_2-limiting conditions (i.e., acclimation to hypoxia does not enhance tolerance to high temperatures, and vice versa) (Burleson *et al.*, 2011, Anttila *et al.*, 2015, Motyka *et al.*, 2017, Levesque *et al.*, 2019, McDonnell *et al.*, 2019). Further, the data from these two experiments fit very well with the 'plastic floors and concrete ceilings' hypothesis (Sandblom *et al.*, 2016) which was originally formulated based on the relationship between acclimation temperature and a fish's thermal tolerance. The hypoxia-intolerant rainbow trout in Motyka *et al*. (2017) could increase tissue O_2 extraction ($\dot{M}O_2/\dot{Q}$) by 74% when acclimated to hypoxia, given that this parameter contributes little to AS_T in normoxia-acclimated fish warmed to their CT_{max}. By contrast, the sablefish [which has adapted to the OMZ conditions that they live in as adults (Mason *et al.*, 1983, Doya *et al.*, 2017)] has no plasticity to enhance $\dot{M}O_2/\dot{Q}$ further through hypoxic acclimation because it is already a major contributor (i.e., it increased by 117%) to this fish's AS_T (Figures

3.1-3.2 and Table S3.3). This suggests that hypoxia-tolerant species may have no, or limited, scope for enhancing their cardiorespiratory capacity upon exposure to oxygen-limiting conditions because it has already been fully exploited as a result of their evolutionary adaptation to environmental hypoxia.

3.5.4. Concluding Remarks and Ecological Implications of the Research

By explicitly testing the effect of an acute warming event on cardiac function when fish are already experiencing moderate hypoxia, I was able to show that prior exposure to acute hypoxia prevents the sablefish from increasing f_H when subsequently exposed to high temperatures (Figure 3.1). These data suggest that the 'physiological chain of command' (in this case nervous control of f_H) may be detrimental under certain environmental conditions, or when they are experienced in a particular order (e.g., hypoxia before acute warming). In other words, the depression of f_H by hypoxia appears to dominate over the requirement for increasing f_H during warming, and this severely limits the fish's cardiac response to the latter stressor, and ultimately, appears to constrain thermal tolerance (Figure 3.2); a finding that has recently been confirmed in the Atlantic salmon (*Salmo salar*) (Gamperl *et al*., in prep.). Further, these results indicate that the importance of O_2 extraction in determining the thermal tolerance of fishes has been underappreciated, and raise important questions (and testable hypotheses) about: the control of heart rate (function) when high temperatures and hypoxia occur simultaneously; and the role of PaCA in O_2 extraction in fishes under varying conditions.

Importantly, these findings also confirm the findings of Ern *et al*. (2016) that hypoxia limits fish acute thermal tolerance (Figure 3.2), and draw attention to the possible

role of hypoxia in determining whether fish species/populations survive in the current era of climate change. Coastal areas are experiencing more severe and frequent heat waves (IPCC, 2014, Frölicher *et al.*, 2018, Oliver *et al.*, 2018), and periods of hypoxia (IPCC, 2014, Breitburg *et al.*, 2018), and it is very likely that fish species in some of these areas will suffer a greater loss of biomass than the 5-17% estimated by Lotze *et al*. (2019) for higher trophic level organisms in the oceans. This is because many fisheries and marine ecosystem models do not incorporate such periodic events [e.g., Bell *et al.* (2013), Lotze *et al.* (2019)], and the interactive effects of temperature and hypoxia on organisms in coastal ecosystems are only starting to be addressed (Roman *et al.*, 2019, Howard *et al.*, 2020). Thus, climate change-induced declines in fish populations may be more severe than predicted to date. In addition, there is little evidence that acclimation to hypoxia substantially improves the tolerance of fishes to high temperatures (i.e., there is no cross-tolerance at the whole animal level) (Burleson *et al.*, 2011, Anttila *et al.*, 2015, Motyka *et al.*, 2017, McDonnell *et al.*, 2019). Although there are instances where the capability for plasticity in fishes is considerable [e.g., Anttila *et al.* (2014)], it appears that many species of fish (and other ectothermic animals) have limited plasticity to respond to these climate-related challenges [present study, and Muñoz *et al.* (2015), Sandblom *et al.* (2016), Motyka *et al.* (2017), Kielland *et al.* (2019)]. Finally, while transgenerational plasticity has been shown to improve some aspects of fish physiology (e.g., aerobic scope) following acclimation to hypoxia or high temperatures, it is unlikely that this will translate into an increased ability to tolerate higher temperatures (Donelson *et al.*, 2012, Muñoz *et al.*, 2015, Kielland *et al.*, 2019, White *et al.*, 2020). Thus, it is uncertain whether cross-tolerance, developmental plasticity or adaptation will allow coastal fish

populations to persist across their current ranges as marine heat waves and hypoxic zones become more common, and extreme, with climate change.

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Table S3.1. Results of the general linear mixed model used to test the main effects of acclimation and test condition on cardiorespiratory variables. Provided are the degrees of freedom (df), F-statistic and *P*-values for the initial and additional experiments. Accl=acclimation; Cond=test condition (i.e., acute hypoxia, warming); Accl×Cond=interaction between the main effects; f_H=heart rate; $\dot{Q}$=cardiac output, V_S=stroke volume; $\dot{M}O_2$=O_2 consumption; $\dot{M}O_2/\dot{Q}$=O_2 extraction.

	Main effect	**Df**	**F**	***P***
Initial experiment				
f_H	Accl	1	8.995	0.0058
	Cond	6	7.465	<0.0001
	Accl×Cond	6	5.647	<0.0001
$\dot{Q}$	Accl	1	2.868	0.1019
	Cond	6	11.146	<0.0001
	Accl×Cond	6	4.747	0.0002
V_S	Accl	1	0.005	0.9426
	Cond	6	6.028	<0.0001
	Accl×Cond	6	1.2415	0.2882
$\dot{M}O_2$	Accl	1	1.020	0.3215
	Cond	6	43.127	<0.0001
	Accl×Cond	6	3.102	0.0068
$\dot{M}O_2/\dot{Q}$	Accl	1	1.972	0.1716
	Cond	6	51.719	<0.0001
	Accl×Cond	6	0.429	0.8587
Additional experiment				
f_H	Cond	6	11.56	<0.0001
$\dot{Q}$	Cond	6	11.681	<0.0001
V_S	Cond	6	7.7897	<0.0001
$\dot{M}O_2$	Cond	6	19.944	<0.0001
$\dot{M}O_2/\dot{Q}$	Cond	6	11.348	<0.0001

Table S3.2. Results of the post-hoc comparisons for sablefish cardiac parameters in the initial experiment, between test conditions within an acclimation group. Provided are the *t*-statistic and *P*-values. nor=normoxia; hyp=hypoxia; f_H=heart rate; $\dot{Q}$=cardiac output; V_S=stroke volume.

		f_H				$\dot{Q}$				V_S	
		Normoxia-acclimated		Hypoxia-acclimated		Normoxia-acclimated		Hypoxia-acclimated			
Comparison		***T***	***P***	***T***	***P***	***t***	***P***	***t***	***P***	***t***	***P***
12°C, nor	12°C, hyp	-3.395	0.0026	-0.200	0.8458	-4.058	0.0003	-0.204	0.9294	0.600	0.6787
	14°C, hyp	6.262	<0.0001	0.596	0.7242	6.238	<0.0001	0.145	0.9294	-2.773	0.0219
	16°C, hyp	6.923	<0.0001	1.216	0.4740	5.806	<0.0001	-0.165	0.9294	-4.676	0.0001
	18°C, hyp	5.316	<0.0001	-1.056	0.5441	4.873	<0.0001	-0.816	0.7932	-1.743	0.1592
	20°C, hyp	5.600	<0.0001	-1.376	0.4489	6.325	<0.0001	0.707	0.8330	-0.198	0.8856
	22°C, hyp	5.715	<0.0001	0.903	0.5940	6.645	<0.0001	4.258	0.0003	0.076	0.9397
12°C, hyp	14°C, hyp	2.867	0.0124	0.397	0.8285	2.181	0.0645	-0.059	0.9527	-2.173	0.0730
	16°C, hyp	3.528	0.0019	1.017	0.5441	1.748	0.1545	-0.369	0.9294	-4.076	0.0004
	18°C, hyp	1.921	0.1081	-1.255	0.4740	0.815	0.4857	-1.021	0.7886	-1.143	0.3823
	20°C, hyp	2.205	0.0608	-1.575	0.3519	2.268	0.0578	0.502	0.8625	0.402	0.8029
	22°C, hyp	2.823	0.0125	0.723	0.7060	3.200	0.0050	4.074	0.0003	0.601	0.6787
14°C, hyp	16°C, hyp	0.661	0.5945	0.620	0.7242	-0.433	0.6990	-0.310	0.9294	-1.902	0.1240
	18°C, hyp	-0.946	0.4543	-1.652	0.3519	-1.366	0.2516	-0.961	0.7886	1.030	0.4262
	20°C, hyp	-0.662	0.5945	-1.972	0.2647	0.087	0.9310	0.562	0.8625	2.575	0.0329
	22°C, hyp	0.381	0.7777	0.365	0.8285	1.348	0.2516	4.127	0.0003	2.503	0.0351
16°C, hyp	18°C, hyp	-1.607	0.1927	-2.272	0.2345	-0.933	0.4353	-0.652	0.8330	2.933	0.0163
	20°C, hyp	-1.323	0.3034	-2.592	0.2200	0.520	0.6677	0.871	0.7932	4.478	0.0002
	22°C, hyp	-0.182	0.8558	-0.195	0.8458	1.715	0.1545	4.406	0.0002	4.167	0.0004
18°C, hyp	20°C, hyp	0.284	0.8157	-0.320	0.8285	1.452	0.2398	1.523	0.3895	1.545	0.201
	22°C, hyp	1.187	0.3556	1.857	0.2742	2.508	0.0347	4.993	<0.0001	1.601	0.1951
20°C, hyp	22°C, hyp	0.945	0.4543	2.145	0.2345	1.274	0.2684	3.621	0.0014	0.249	0.8856

Table S3.3. Cardiorespiratory parameters and thermal tolerance in sablefish exposed to hypoxic warming (initial experiment) or normoxic warming (additional experiment). For each parameter, Student's *t*-tests were used to assess statistical differences ($P<0.05$): (i) between acclimation groups within the initial experiment (significant differences denoted with $^{+}$); and (ii) between normoxic acclimation groups in each experiment (significant differences denoted with *). *T*-statistic and *P*-values are provided for each comparison. Values are means±s.e.m., and sample sizes are n=14-15 and n=9 for the initial and additional experiments, respectively. f_H=heart rate; $\dot{Q}$=cardiac output, V_S=stroke volume; $\dot{M}O_2$=O_2 consumption; $\dot{M}O_2/\dot{Q}$=O_2 extraction; $T_{arrhythmia}$=temperature at which the heart went arrhythmic; CT_{max}=temperature at which the fish lost equilibrium.

		Hypoxic warming			**Normoxic warming**	
		Normoxia-acclimated	**Hypoxia-acclimated**		**Normoxia-acclimated**	
f_H (beats min^{-1})	Rest	41.31±2.23	36.09±1.59	t=1.883; P=0.071	27.30±1.60*	t=4.450; P=0.0002
	Maximum	36.81±1.83	45.39±2.36^{+}	t=2.902; P=0.007	64.42±3.35*	t=7.908; P<0.0001
	Scope	-4.50±2.15	9.29±2.79^{+}	t=3.948; P=0.0005	37.12±3.83*	t=10.27; P<0.0001
$\dot{Q}$ (mL min^{-1} kg^{-1})	Rest	25.50±2.18	24.37±1.76	t=0.402; P=0.691	21.25±1.74	t=1.357; P=0.189
	Maximum	22.33±1.67	27.71±1.81^{+}	t=2.191; P=0.037	43.64±4.20*	t=5.506; P<0.0001
	Scope	-3.17±1.21	3.12±1.10^{+}	t=3.930; P=0.0005	22.40±2.99*	t=9.206; P<0.0001
V_S (mL $beat^{-1}$ kg^{-1})	Rest	0.61±0.03	0.68±0.05	t=1.191; P=0.244	0.79±0.07*	t=2.699; P=0.013
	Maximum	0.81±0.05	0.82±0.05	t=0.126; P=0.901	0.87±0.06	t=0.709; P=0.486
$\dot{M}O_2$ (mg O_2 h^{-1} kg^{-1})	Routine	77.4±6.7	64.6±3.8	t=1.640; P=0.113	91.6±11.9	t=1.120; P=0.275
	Maximum	136.6±6.9	151.1±6.1	t=1.569; P=0.128	265.6±8.5*	t=11.64; P<0.0001
	Scope	59.1±8.4	86.5±6.6^{+}	t=2.533; P=0.017	174.0±14.2*	t=7.435; P<0.0001
$\dot{M}O_2/\dot{Q}$ (mg O_2 L^{-1})	Rest	55.05±4.91	46.46±3.73	t=1.379; P=0.179	57.54±5.75	t=0.321; P=0.751
	Maximum	134.20±11.31	113.45±5.66	t=1.604; P=0.120	125.07±8.74	t=0.565; P=0.578
	Scope	79.15±9.81	66.99±5.18	t=1.073; P=0.293	67.53±6.07	t=0.854; P=0.402
$T_{arrhythmia}$ (°C)		21.3±0.3	22.0±0.3	t=1.830; P=0.078	22.8±0.4*	t=3.120; P=0.005
CT_{max} (°C)		22.0±0.2	22.5±0.3	t=1.587; P=0.124	24.9±0.2*	t=9.269; P<0.0001

Table S3.4. Body, cardiac and splenic morphometrics for sablefish in the initial and additional experiment. Condition factor (Fulton's K) is calculated as 100×[body mass/length^3]. Cardiac and splenic mass are expressed as a percentage of body mass. For each parameter, Student's *t*-tests were used to identify statistical differences ($P<0.05$): (i) between acclimation groups within the initial experiment (no significant differences found); and (ii) between normoxic acclimation groups from the two experiments (significant differences denoted with *). *T*-statistic and *P*-values are provided for each comparison. Values are means±s.e.m., and sample size is $n=15$ for the initial experiment (except for spleen mass where $n=9$), and $n=9$ for the additional experiment.

	Initial experiment			**Additional experiment**	
	Normoxia-acclimated	**Hypoxia-acclimated**		**Normoxia-acclimated**	
Body mass (g)	1303±34	1339±34	$t=0.554$; $P=0.463$	1414±48	$t=3.647$; $P=0.069$
Fork length (cm)	49.9±0.6	51.0±0.5	$t=2.249$; $P=0.145$	54.7±0.9*	$t=21.98$; $P=0.0001$
Condition factor	1.05±0.03	1.01±0.02	$t=1.612$; $P=0.215$	0.87±0.03*	$t=17.10$; $P=0.0004$
Atrium mass (%)	0.023±0.001	0.026±0.001	$t=2.880$; $P=0.101$	0.031±0.001*	$t=17.80$; $P=0.0004$
Ventricle mass (%)	0.078±0.002	0.077±0.002	$t=0.013$; $P=0.911$	0.092±0.004*	$t=12.66$; $P=0.002$
Bulbus mass (%)	0.020±0.001	0.020±0.001	$t=0.292$; $P=0.593$	0.022±0.001	$t=1.852$; $P=0.187$
Spleen mass (%)	0.064±0.004	0.062±0.003	$t=0.127$; $P=0.726$	0.053±0.004	$t=3.422$; $P=0.083$

Table S3.5. Comparison between metabolic parameters and the thermal tolerance of sablefish in the additional experiment and a prior study (Leeuwis *et al.*, 2019), in order to assess the effect of surgery. Body mass of fish in the prior study in our lab was ~607 g. Statistical differences were identified using Student's *t*-tests (α=0.05); no significant differences were found. For each comparison, *t*-statistic and *P*-value are provided. Values are means±s.e.m., and sample sizes are *n*=9 for the additional experiment, and *n*=10-13 for the prior study. [1]Leeuwis *et al.* (2019).

		Additional experiment **Surgery**	Reference study[1] **No surgery**	
$\dot{M}O_2$ (mg O_2 h^{-1} kg^{-1})	Standard	67.2±5.9	63.6±3.6	t=0.542; P=0.594
	Routine	91.6±11.9	89.4±4.9	t=0.189; P=0.852
	Maximum	265.6±8.5	258.5±6.7	t=0.660; P=0.518
	Scope	174.0±14.2	170.5±10.0	t=0.208; P=0.838
CT_{max} (°C)		24.9±0.2	24.9±0.2	t=0.046; P=0.964

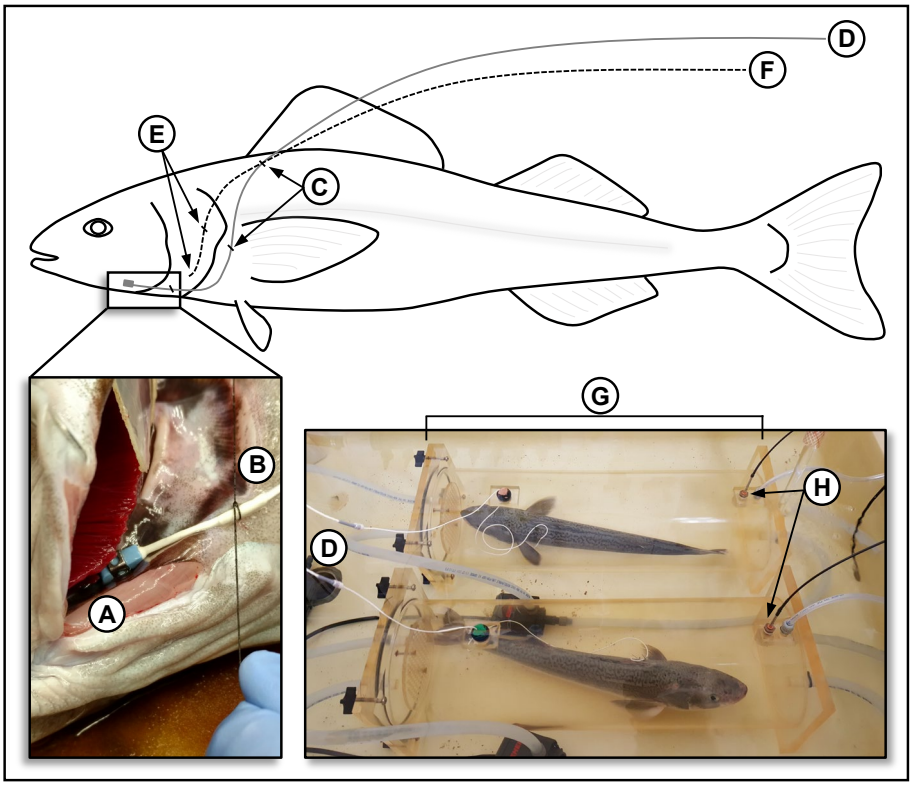

Figure S3.1. Overview of the surgical procedures and experimental setup used to investigate cardiorespiratory function in sablefish. A Transonic® flow probe was implanted around the ventral aorta, which was accessed by a small incision in the skin inside the opercular cavity (**A**). The probe lead was attached with sutures on the edge of the opercular cavity (**B**) and at two locations on the fish's body (**C**), and connected to a flow meter to allow for recordings (**D**). In the initial experiment, a cannula was also inserted into a gill afferent artery (**E**), and secured with sutures, from which blood samples were withdrawn (**F**). The fish were recovered for 1-2 days in respirometers (**G**). These were fitted with fibre-optic O_2 probes (**H**) that enabled O_2 consumption measurements.

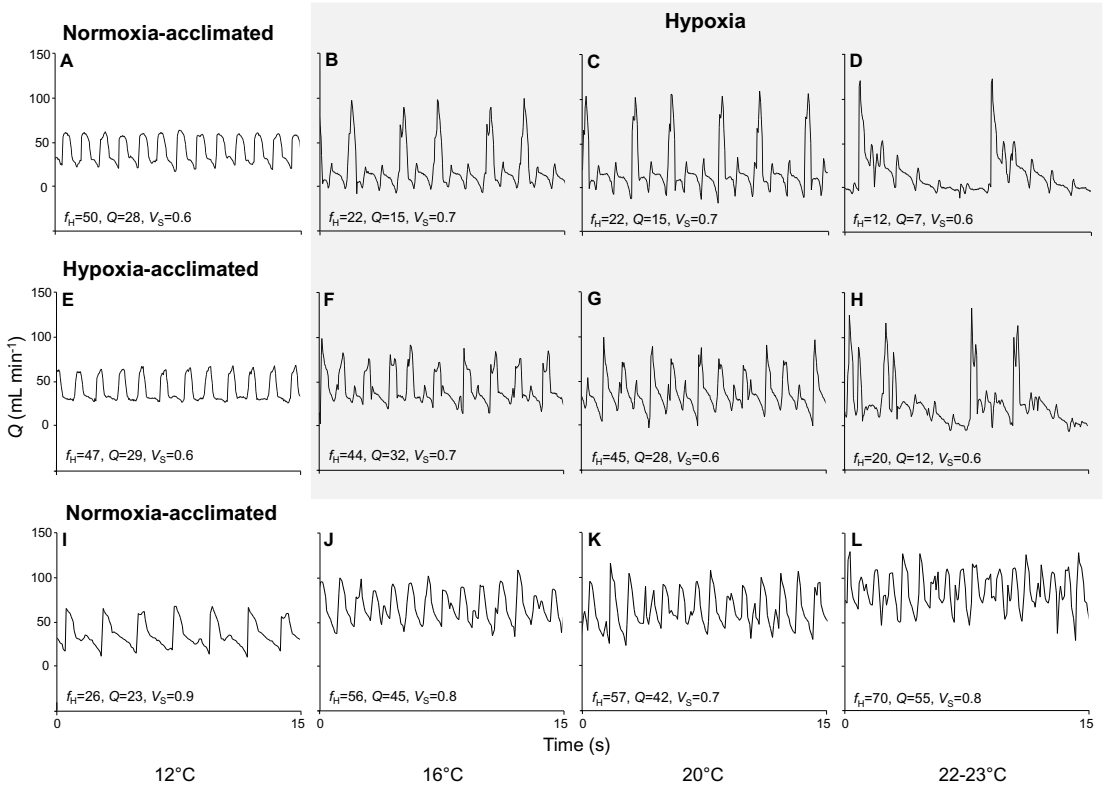

Figure S3.2. *In vivo* recordings of ventral aortic blood flow in sablefish as affected by acclimation and test conditions. Shown are recordings for a normoxia- and a hypoxia-acclimated sablefish exposed to acute hypoxia followed by warming to their CT_{max} (22-23°C) (**A-D** and **E-H**, respectively), and for a normoxia-acclimated fish warmed to its CT_{max} (~25°C) under normoxic conditions (although only data to 22-23°C are shown) (**I-L**). These traces illustrate differences in cardiac output ($\dot{Q}$, mL min^{-1} kg^{-1}), heart rate (f_H, beats min^{-1}) and stroke volume (V_S, mL beat^{-1} kg^{-1}) responses amongst the three experimental groups over the course of the experiment. The *x*- and *y*-axes represent elapsed time (sec) and blood flow (mL min^{-1}), respectively.

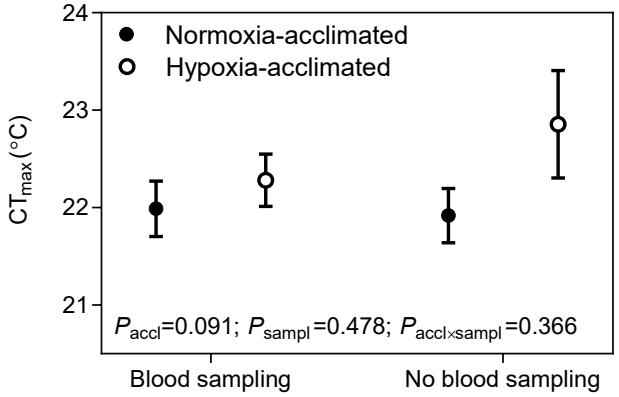

Figure S3.3. CT_{max} in normoxia- and hypoxia-acclimated sablefish exposed to hypoxic warming, with and without blood sampling. Blood sampling did not have a significant effect on the CT_{max} of these fish. The effects of blood sampling (sampl), acclimation (accl), and their interaction, were analysed using a 2-way ANOVA, and the *P*-values are provided. Values are means±s.e.m., where *n*=9-10 for fish with blood sampling and *n*=5 without blood sampling.

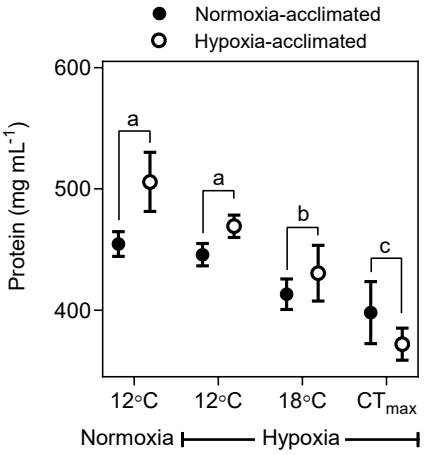

Figure S3.4. The protein level of red blood cells during hypoxic warming in normoxia- and hypoxia-acclimated sablefish. The red blood cell (RBC) protein level (mg mL^{-1}) declined significantly during hypoxic warming, but hypoxic acclimation had no significant effect. The effects of acute exposure condition, acclimation condition, and their interaction, were analysed using a general linear mixed model. Differences among experimental conditions were assessed with post-hoc contrasts using least-squares means, and are denoted with different lowercase letters. Values are means±s.e.m. with *n*=9-10 fish per acclimation group.

Chapter 4: **The Effect of Chronic Hypoxia on the Innate and Adaptive Immune Responses of Sablefish (*Anoplopoma fimbria*) to Formalin-Killed *Aeromonas salmonicida***

4.1. Abstract

The occurrence and severity of hypoxic zones are increasing worldwide due to climate change. However, it is not well understood how this will impact the immune function of fish, and their ability to respond to pathogens. In the present study, I examined the effect of chronic hypoxia on the innate and adaptive immune responses of sablefish (*Anoplopoma fimbria*) to formalin-killed *Aeromonas salmonicida* (the causative agent of furunculosis). I acclimated sablefish to 40% or 100% air saturation for 6 weeks, then measured innate immunity-related haematological parameters and the transcript expression levels of 16 known bacteria-responsive genes in the spleen using qPCR analyses (with *de novo* designed primers) prior to and 24 hours after intraperitoneal injection with the bacterin or saline. Respiratory burst, lysozyme concentration and alternative complement activity in the blood, and the expression levels of 16 transcripts in the spleen, were generally elevated in response to the bacterin, but were not substantially affected by hypoxia. I also measured adaptive immunity-related haematological parameters at 0, 6 and 10 weeks post-intraperitoneal injection. Using a newly developed ELISA (antibody specificity verified with Western blotting), I found that hypoxia completely inhibited the increase in total serum immunoglobulin (IgM) levels at 10 weeks after bacterin injection. However, FACS analysis on circulating leukocytes showed that the relative abundance of IgM^+ cells at this time point was actually higher in hypoxia-acclimated fish; the cell morphology of these IgM^+ cells consistent with B lymphocytes based on fluorescent confocal imaging. Overall, these findings demonstrate that chronic hypoxia modifies the adaptive, but not substantially the innate, immune response in sablefish, and provide potential insights into mechanisms underlying this effect.

4.2. Introduction

Over the past 50 years, oxygen (O_2) levels have declined in marine waters worldwide, and hypoxic zones (O_2 content <2 mg L^{-1}) are becoming more extensive and severe due to global warming and eutrophication (Gilbert *et al.*, 2005, Kemp *et al.*, 2005, Diaz *et al.*, 2008, Rabalais *et al.*, 2010, Breitburg *et al.*, 2018, Claret *et al.*, 2018). Oxygen is essential to the survival of animals dependent on aerobic respiration, and is a key environmental factor that influences the physiology and distribution of water-breathing animals, including fishes (Diaz *et al.*, 2009, Stramma *et al.*, 2012, Zhu *et al.*, 2013, Howard *et al.*, 2020, Sampaio *et al.*, 2021). Exposure to low O_2 levels can substantially alter, and adversely impact, immune function in fish, and has been associated with disease outbreaks both in wild and farmed species (Mellergaard *et al.*, 1995, Esteve *et al.*, 2017, Abdel-Tawwab *et al.*, 2019). Short-term (acute) exposure to hypoxia (i.e., hours to days) frequently has an immunosuppressive effect on innate and adaptive immunity components in fish, and this may coincide with increased disease susceptibility (infection and/or mortality rates) (Walters *et al.*, 1980, Boleza *et al.*, 2001, Yada *et al.*, 2002, Evans *et al.*, 2003, Welker *et al.*, 2007, Bowden, 2008, Rodríguez *et al.*, 2016). For instance, following a 2-hour exposure to severe hypoxia (<2 mg L^{-1}; <25% air sat.), channel catfish (*Ictalurus punctatus*) suffered higher mortality rates (36% *vs.* 12%) from a subsequent challenge with *Edwardsiella ictaluri* (i.e., the bacterial pathogen that causes enteric septicemia) compared to the normoxic control (~6.1 mg L^{-1}; ~76% air sat.), and this was concomitant with lower haemolytic complement and bactericidal activities, and a reduced antibody response (Welker *et al.*, 2007). Overall, the current literature agrees that acute hypoxia negatively affects the host immune system in fishes (Yada *et al.*, 2002, Bowden,

2008, Abdel-Tawwab *et al.*, 2019). This effect may be primarily mediated by the stress response, and direct endocrine immunosuppression associated with elevated cortisol levels (Yada *et al.*, 2002, Welker *et al.*, 2007).

In contrast, the impact of long-term (chronic) hypoxia (i.e., weeks to months) on fish immunity and resistance to pathogens remains unclear, given the ambiguity of research findings (Cecchini *et al.*, 2002, Andersen *et al.*, 2010, Niklasson *et al.*, 2011, Kvamme *et al.*, 2013, Abdel-Tawwab *et al.*, 2014, 2015, Gallage *et al.*, 2016, Gallage *et al.*, 2017, Magnoni *et al.*, 2019, Martínez *et al.*, 2020, Zanuzzo *et al.*, 2020, Krasnov *et al.*, 2021, Schäfer *et al.*, 2021). For example, Kvamme *et al.* (2013) reported that chronic hypoxic conditions (2 months at ~52% air sat.) delayed, or suppressed, the innate immunity-related gene expression response in the head kidney of Atlantic salmon (*Salmo salar*) injected with a monovalent *Vibrio anguillarum* vaccine, as compared to the 'normoxic' group (note: the water O_2 level that this group was exposed to was only ~74% air sat.). However, Zanuzzo *et al.* (2020) found no negative impact of chronic hypoxia (3 months at ~65-75% air sat.) on this species' ability to mount a transcriptional response in the same tissue after injection with a polyvalent bacterial and viral vaccine, relative to fish from the normoxic control group (~100% air sat.). Gallage *et al.* (2016) showed that moderate hypoxia (~55% air sat. for 49 days) compromised antibody production in Nile tilapia (*Oreochromis niloticus*) after *V. anguillarum* vaccination compared to the normoxia group (~85% air sat.), but that serum lysozyme activity was unaffected. Likewise, chronic hypoxia (~4.5 mg L^{-1}; ~43% air sat.) of the same duration did not hamper lysozyme and complement activity in rainbow trout (*Oncorhynchus mykiss*) (normoxic control held at 7.9 mg L^{-1}; ~76% air sat.) (Magnoni *et al.*, 2019). The reasons

for these contradictory results may be partially related to differences in the hypoxia tolerance/sensitivity of fish species, the hypoxia exposure period (acclimation duration), the severity of hypoxia used, and the robustness of distinct systemic and tissue-specific components of innate and adaptive immunity under hypoxic conditions (Abdel-Tawwab *et al.*, 2019). Further research using different teleost and pathogen species may provide additional insights, and improve our understanding of how chronic hypoxia potentially acts as an immunomodulator. Such information is important for predicting the consequences of the spread and exacerbation of hypoxic zones on the health and survival of fish populations and/or species.

In this study, I conducted a 5-month long experiment on sablefish (*Anoplopoma fimbria*) with the aim of determining the impact(s) of long-term hypoxia on baseline innate and adaptive immune function and on the response to pathogenic bacterial antigens; with the *a priori* hypothesis that chronic hypoxia has an overall immunosuppressive effect. The sablefish was used as a model to address this question because it is a commercially important species in the North Pacific fishery and an emerging aquaculture species (Sonu, 2014, Hartley *et al.*, 2020, Goetz *et al.*, 2021). Further, in the context of hypoxia, the sablefish is known for its high tolerance to acute hypoxia (Leeuwis *et al.*, 2019) and is reported to frequently visit O_2 minimum zones (OMZs) (where it is among the most abundant macrofauna observed) (Moser *et al.*, 1994, Doya *et al.*, 2017). Formalin-killed *Aeromonas salmonicida* was selected as the bacterial antigens to evaluate the immune response, given that it is among the most prevalent pathogens in sablefish aquaculture (B. Campbell, pers. comm) where it is the causative agent of furunculosis. This is a disease that also affects a large diversity of other fish

species (Cipriano *et al.*, 2001, Sumaila *et al.*, 2005, Arkoosh *et al.*, 2018, Vasquez *et al.*, 2020, Goetz *et al.*, 2021).

Sablefish were first acclimated to either ~100% (normoxia) or ~40% air saturation (hypoxia) at 10°C for 6 weeks, which is a moderate hypoxic level for this species at this temperature, and known to induce plastic responses (Leeuwis *et al.*, 2019, Leeuwis *et al.*, 2021). Then, the effect of chronic hypoxia was assessed with regard to innate immunity, by measuring: (i) respiratory burst, alternative complement activity and lysozyme concentration in the blood; and (ii) the transcript expression of immune genes in the spleen known to be bacteria-responsive in other fish species using qPCR, prior to and 24 hours after intraperitoneal (ip) injection with *A. salmonicida* bacterin (Figure 4.1). In addition, the effect of chronic hypoxia on adaptive immunity was assessed, by measuring serum IgM levels using a novel direct ELISA, and the relative abundance of IgM^+ circulating cells using flow cytometry, prior to and 6 and 10 weeks post-injection with the bacterin. This study focuses on IgM as it is the most prevalent Ig isotype in serum among teleosts and plays a central role in the systemic adaptive immune response (Ye *et al.*, 2013, Mashoof *et al.*, 2016).

4.3. Materials & Methods

4.3.1. Experimental Animals and Hypoxia Acclimation

All procedures were approved by MUN's Institutional Animal Care Committee (protocol #16-92-KG), and followed the guidelines of the Canadian Council on Animal Care.

Juvenile sablefish (~1 g) were acquired from Golden Eagle Sable Fish (BC,

Canada) and reared at the Dr. Joe Brown Aquatic Research Building (JBARB) of MUN as described by Vasquez *et al.* (2020). From this pool of sablefish, 75 fish (272±2 g) were transferred to a 3,000 L tank supplied with seawater at 10°C and an O_2 partial pressure (P_{O_2}) of ~100% air saturation (~21 kPa; ~9.3 mg O_2 L^{-1}), and on a 12 h light:12 h dark photoperiod. These fish were reared for another ~2 months, and were hand fed a marine fish diet (Skretting Europa: 55% protein, 15% fat, 2% carbohydrate, 3% calcium, 2% phosphorous) at 1.25% of their body weight day^{-1}. Prior to the start of the experiment, the sablefish were Passive Integrated Transponder (PIT)-tagged to allow for individual identification throughout the experiment.

To start the hypoxia acclimation, the fish (400±5 g, Table 4.1) were randomly distributed between a normoxia (n=37) and a hypoxia acclimation tank (n=38), each tank 1.2 m^3 and maintained under the conditions described previously. After a 2-week period of acclimation (Figure 4.1), while the normoxia tank continued to be maintained at ~100% air saturation, the sablefish in the hypoxia tank were slowly acclimated to ~40% air saturation (~8 kPa; ~3.7 mg O_2 L^{-1}) following a similar procedure as described in Gerber *et al.* (2019) and Leeuwis *et al.* (2021). Briefly, the P_{O_2} was gradually lowered over 2 weeks (by 5-10% air saturation every 2 days) to 40% air saturation, and then maintained at this level for 16 weeks (i.e., the total duration of the acclimation period), by bubbling N_2 into the tank as controlled by a custom solenoid valve system and by reducing seawater inflow to the tank. Water quality parameters (pH, ammonia and nitrite levels) were assessed weekly and after every flow reduction, and remained the same between the normoxic and hypoxic tanks throughout the experiment (P>0.05). The pH

was 7.59±0.03 and 7.49±0.01 in the normoxic and hypoxic tanks, respectively, and the ammonia and nitrite levels did not exceed 0.009 and 0.06 ppm, respectively, in either tank.

After transfer to the experimental tanks, the ration fed to both tanks was gradually reduced over ~3 weeks to 0.60% of body weight day^{-1}, and then kept at this level for the rest of the experiment. Whenever the hypoxia-acclimated fish consumed less than this ration, the normoxia-acclimated fish were provided the same amount of feed. This feed matching was performed to ensure that the acclimation groups maintained a similar nutritional status, given the suppressive effect of long-term hypoxia on appetite observed in other fish species; e.g., see Pichavant *et al.* (2001), Burt *et al.* (2014), Abdel-Tawwab *et al.* (2014), Remen *et al.* (2016), Magnoni *et al.* (2018), Jutfelt *et al.* (2021). Indeed, there were no significant differences between the body weight and fork length of the two groups throughout the experiment (P>0.05) (Table 4.1), and both groups displayed normal growth (i.e., they doubled in weight) and condition (Fulton's K, 0.9-1.1). All fish were fasted on the day prior to sampling or injection (i.e., no feeding for at least 24 hours).

4.3.2. Bacterin Preparation

A monovalent (autogenous) bacterin mix consisting of three formalin-killed *A. salmonicida* strains [J409, J410 and J411 with GenBank accession numbers CP047374, CP047376 and CP052034, respectively (whole completed genome sequences)] isolated from infected cultured sablefish was prepared as described in Vasquez *et al.* (2020). The cell concentration of this bacterin was determined using the Bacteria Counting Kit

(Thermo Fisher Scientific, Waltham, MA, USA), whereby fluorescence was detected with a BD FACS Aria II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analysed with BD FACS Diva v7.0 software. This bacterin stock solution was stored at a concentration of 2×10^9 colony forming units (CFU) mL^{-1} at 4°C. The bacterin was diluted in phosphate buffered saline (PBS) to 10^8 CFU mL^{-1} prior to use for injections.

4.3.3. Initial Sampling and Bacterin Injection

After 6 weeks of hypoxia acclimation, 7 fish from each acclimation group were netted and euthanized with an anesthetic overdose of 0.2 g L^{-1} tricaine methanesulphonate (MS-222; Syndel Laboratories, Nanaimo, BC, Canada). After weight and fork length were recorded, blood was withdrawn by caudal puncture with heparinized syringes (50 U mL^{-1} of blood) and used for the analysis of baseline (constitutive) haematological innate immunity parameters (Figure 4.1). A 100 µL aliquot was taken for the immediate measurement of respiratory burst activity. The remainder of the blood was centrifuged at 11,000 ×*g* for 1 min at 4°C, and the plasma obtained was pipetted into Eppendorf tubes, flash frozen in liquid N_2, and stored at -80°C for later measurement of alternative complement and lysozyme activity. The spleen and liver were then dissected out with tools treated with RNaseZap® (Ambion, Thermo Fisher Scientific), rinsed in saline, blotted dry, and weighed to determine their relative masses [(tissue mass/body mass)×100%] (spleen-somatic index (SSI) and hepatosomatic index (HSI), respectively). There were no significant differences in the SSI and HSI between the groups at the initial sampling, or at the subsequent samplings (Table 4.1). The spleen was placed in a 1.5 mL RNase-free tube, flash frozen in liquid N_2, and stored at -80°C before the determination of

immune-relevant gene expression. The head kidney was not sampled given that its location could not be confidently identified in sablefish during preliminary dissections. Since the transcriptional responses of immune genes in the spleen and head kidney are typically quite comparable (e.g., see Zanuzzo *et al.*, 2020), the spleen was selected as the target organ for qPCR analysis.

After the initial (time 0) sampling, the 30-31 fish remaining in each tank were netted and anaesthetized with 0.1 g L^{-1} MS-222, and their weights and PIT-tag numbers were recorded. These fish were given a 1 µL g^{-1} (10^5 CFU g^{-1}) ip injection of the *A. salmonicida* bacterin (*n*=16-17 per tank) or PBS (control, *n*=14 per tank), and recovered from anaesthesia in a tank with 100% air saturated seawater, before being returned to their experimental tanks (Figure 4.1). The experimental tanks were temporarily divided into two compartments, to allow for the separation of fish to be sampled at 24 hours post-injection (hpi) for innate immune responses (*n*=15 per tank; incl. 8 *A. salmonicida* bacterin- and 7 PBS-injected fish) from those to be used for measurements of adaptive immunity (*n*=15-16 per tank; incl. 8-9 *A. salmonicida* bacterin- and 7 PBS-injected fish). From the latter group of fish, 1 mL of blood was withdrawn by caudal puncture just prior to injection, which was allowed to clot overnight at 4°C and then centrifuged to obtain serum. Sera samples were stored at -80°C until analysed for immunoglobulin M (IgM) level.

4.3.4. Subsequent Samplings and Boost Injection

At 24 hpi, the fish selected for innate immunity measurements were euthanized and sampled in a similar fashion as during the initial sampling (Figure 4.1). Weight, fork

length and PIT-tag number were recorded, and blood was withdrawn for the analysis of respiratory burst, alternative complement and lysozyme activity. The spleen and liver were then dissected out with RNaseZap®-treated tools, weighed, and the spleen was placed in a 1.5 mL RNase-free tube, flash frozen in liquid N_2 and subsequently stored at -80°C for the measurement of bacteria-responsive gene mRNA expression.

At 4 weeks post-injection (wpi), the fish remaining in the tanks were given a second 1 μL g^{-1} ip injection of the *A. salmonicida* bacterin (i.e., a 'boost') or PBS, with the same recovery procedure for the hypoxia tank as before (Figure 4.1). This boost was provided since the antibody response can be greater and peak more rapidly with repeated antigen exposure (Arkoosh *et al.*, 1991, Soto-Dávila *et al.*, 2020), thus ensuring optimal experimental conditions for a strong humoral adaptive immune response. At 6 and 10 wpi, blood was collected for the measurement of serum IgM level in a similar way as during the initial sampling. At 10 wpi, all fish were euthanized as described for the 0 and 24 hpi samplings, weight and fork length were recorded, and an additional 1 mL of blood was withdrawn and added to a heparinized tube (50 U mL^{-1} of blood) for analysis by flow cytometry.

4.3.5. Haematological Innate Immunity Measurements

4.3.5.1. Respiratory Burst

The production of reactive oxygen species (ROS) by sablefish blood leukocytes (i.e., respiratory burst) was measured using the chemiluminescence-based method described by Marnila *et al.* (1995), Nikoskelainen *et al.* (2004) and Pérez-Casanova *et al.* (2008), with the following modifications to allow for use with sablefish blood. First, 10

Table 4.1. Sample size and body morphometrics of sablefish at different stages of the study. Condition factor (Fulton's K) was calculated as 100×[body mass/length^3]. Spleen and liver masses are expressed as a percentage of body mass. The acclimation and injection groups at each sampling were not significantly different (assessed by Student's *t*-tests or 2-way ANOVAs). Values are means±s.e.m. and deviations from the normal sample size (*n*) are shown in parentheses beside the value.

	n	**Body mass (g)**	**Fork length (cm)**	**Condition factor (K)**	**Spleen-somatic index (SSI) (%)**	**Hepatosomatic index (HSI) (%)**
Transfer to experimental tanks						
All fish	75	399.5±5.5	33.6±0.2	1.05±0.01	-	-
0 hpi innate immunity sampling						
Normoxia-acclimated	7	600.4±18.1	39.1±0.6	1.01±0.03	0.070±0.006	1.92±0.10
Hypoxia-acclimated	7	593.1±31.8	38.7±1.0	1.02±0.04	0.063±0.004	1.82±0.09
24 hpi innate immunity sampling						
Normoxia-acclimated						
PBS-injected	7	607.4±22.4	39.4±0.6	1.00±0.02	0.064±0.004	1.58±0.08
A. salmonicida bacterin-injected	8	632.8±22.7	40.2±0.5	0.97±0.03	0.078±0.007	1.58±0.09
Hypoxia-acclimated						
PBS-injected	7	594.1±19.8	38.8±0.5	1.01±0.03	0.051±0.005	1.63±0.10
A. salmonicida bacterin-injected	8	624.9±25.2	40.1±0.7	0.97±0.03	0.069±0.005	1.57±0.09
10 wpi adaptive immunity sampling						
Normoxia-acclimated						
PBS-injected	7	757.6±56.5	43.0±1.1	0.95±0.02	-	-
A. salmonicida bacterin-injected	8	752.8±25.7	43.7±0.9	0.91±0.03	-	-
Hypoxia-acclimated						
PBS-injected	7	800.3±34.5	44.4±0.6	0.91±0.02	-	-
A. salmonicida bacterin-injected	9	694.7±29.5	42.5±0.7	0.90±0.02	-	-

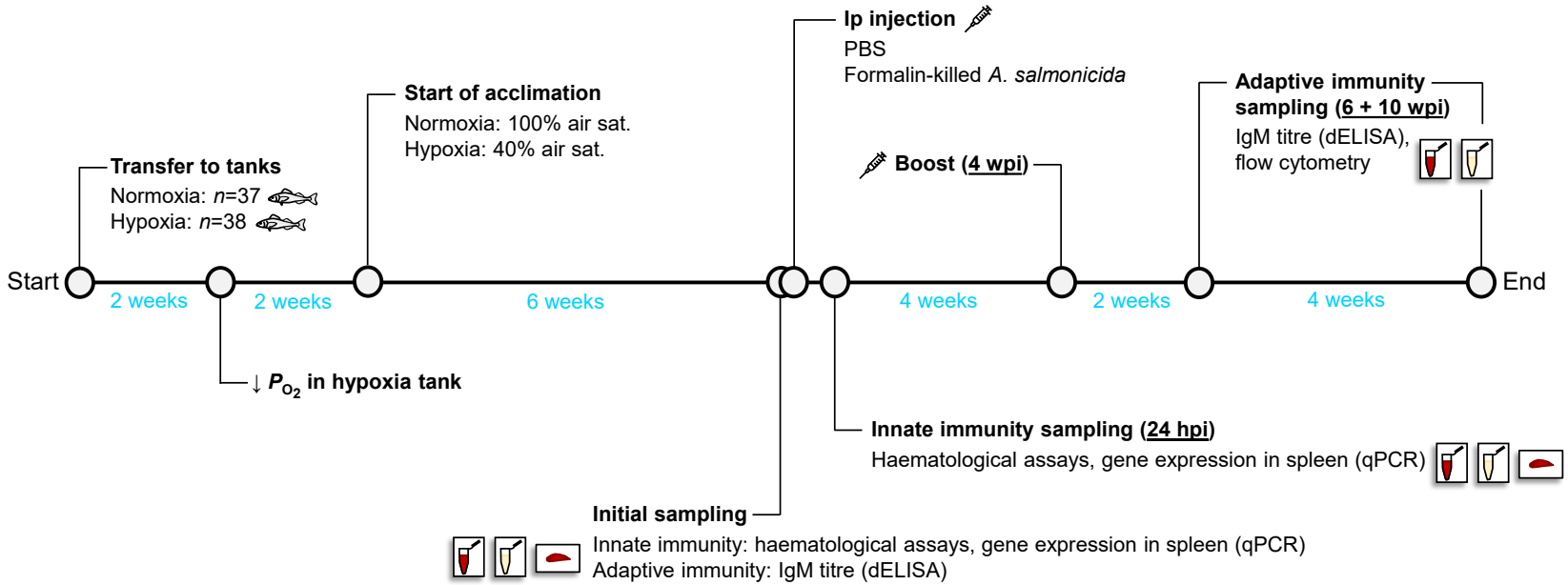

Figure 4.1. Timeline and sampling protocol for the chronic hypoxia/immunology experiment. Sablefish (400±5 g) were transferred to a normoxia (*n*=37) and a hypoxia tank (*n*=38), and acclimated to the new tanks for 2 weeks. Then, the P_{O_2} in the hypoxia tank was gradually reduced to ~40% air saturation over 2 weeks, while in the normoxia tank it was kept at ~100% air saturation, and these P_{O_2} levels were maintained for the rest of the experiment. After 6 weeks of hypoxia acclimation, 7 fish per tank were euthanized, and blood/plasma and spleen were sampled for innate immunity-related haematological parameters and constitutive gene expression, respectively. The remaining 30-31 fish in each tank were ip injected with PBS (*n*=14) or formalin-killed *A. salmonicida* (*n*=16-17), and a subset of each injection group (*n*=7-9) selected for adaptive immunity sampling also had their blood sampled non-lethally to determine initial serum IgM titres. The following day (24 hpi), the other PBS- and formalin-killed *A. salmonicida*-injected fish (*n*=15 per tank) were euthanized, and tissues were sampled to measure the same innate immunity parameters as at 0 hpi. The 15-16 fish left in each tank were given a second ip injection of PBS or formalin-killed *A. salmonicida* (boost) at 4 wpi, and at 6 and 10 wpi blood was collected to assess the serum IgM level again and to be used for flow cytometry (10 wpi only).

mM of luminol (Sigma-Aldrich) in 20 mM sodium borate was diluted 9x in PBS, and 900 µL of this mixture was added to 40 µL of each blood sample. Then, 225 µL of this solution was pipetted into each of three triplicate wells of a 96-well white Cliniplate™ (VWR 28298-610, Mississauga, ON, Canada), followed by 25 µL of 20 mg mL^{-1} *Saccharomyces cerevisiae* zymosan (Sigma-Aldrich, Z4250) in PBS. For each sample, a negative control (PBS added instead of zymosan) was included. Chemiluminescence was measured over 60 min at room temperature using a plate reader (SpectraMax® M5, Molecular Devices, San Jose, CA, USA), whereby a curve of luminescence counts per second (LCS) was obtained. Peak values were taken as the respiratory burst of each sample.

4.3.5.2. Haemolytic Activity of the Alternative Complement Pathway

The haemolytic activity of the alternative complement pathway (HA-AP) was determined using uncoated rabbit red blood cells (RaRBCs) as targets, and with the addition of Ca^{2+} chelators (EDTA/EGTA), according to the methods described by Polhill *et al.* (1978), Ferriani *et al.* (1990) and Zanuzzo *et al.* (2015), with some modifications for use with sablefish. RaRBCs were prepared from rabbit blood (Charles River, Saint-Constant, QC, Canada) by mixing the blood 1:1 (v/v) with Alsever solution (pH 6.1). Then, an equal volume of 0.1 M triethanolamine-EDTA buffer (TEA-EDTA, pH 7.4) with 0.1% gelatin (Difco) was added, followed by a 15 min incubation at 37°C. The cells were subsequently washed three times with 2 mM TEA-Mg^{2+} buffer (pH 7.4) using 10 min centrifugations at 800 ×*g* at 4°C, and stored for up to 15 days in Alsever solution at 4°C. On the assay day, 1 mL of RaRBC suspension was washed three times in 8 mM

TEA-EGTA-Mg^{2+} buffer (with 0.1% gelatin, pH 7.4) using 800 ×*g* centrifugations at 4°C, and then diluted with the same buffer to an optical density (OD) of 0.8 at a wavelength of 700 nm. Absorbance was measured using a spectrophotometer (DU 640, Beckman Coulter, Mississauga, ON, Canada). Sablefish plasma was diluted 1:60 (i.e., 10 µL of plasma with 190 µL of TEA-EGTA-Mg^{2+} buffer and 400 µL of RaRBC suspension) in cuvettes, and haemolytic activity (i.e., rate of decline in the amount of intact RaRBCs) was measured in duplicate by recording OD (at 700 nm) every 12 sec for 20 min at 20°C. The optimal sample dilution and incubation temperature were determined in advance. I also determined that heat-inactivation of the haemolytic activity of sablefish plasma starts at 40°C, and was complete after incubation for 30 min at 45°C. Samples were analysed in a randomized order, and the time (sec) required for the OD to be reduced by half (to 0.4) was calculated. The multiplicative inverse (i.e., reciprocal) of this value was then obtained as a metric for HA-AP with a direct proportional relationship.

4.3.5.3. Lysozyme Concentration

The method used to determine plasma lysozyme concentration was based on the lysis of the lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus* as described in Demers *et al.* (1997), but optimized for sablefish. Standard concentrations of 0-10 ng μL^{-1} of chicken egg white lysozyme (Sigma-Aldrich, L6876, USA) in assay buffer (66 mM KH_2PO_4, pH 6.2) were pipetted in duplicate into each 96-well plate. Plasma samples were diluted 2x in assay buffer, then heat-treated at 45°C for 30 min to inactivate the complement system; this ensured that bacterial lysis could be attributed to lysozyme activity only. I confirmed that lysozyme was not affected by exposure to 45°C

(based on comparisons with samples that were not heat-treated), but that it was entirely inactivated at 56°C (i.e., no lytic activity was observed). The samples were then pipetted in triplicate onto the plate (100 µL per well) in a randomized order, and subsequently, 100 µL of a 0.075% (w/v) suspension of *M. lysodeikticus* (Sigma-Aldrich M3770) in assay buffer were added to each well using a multichannel pipette. After mixing, absorbance was immediately measured at 450 nm at 30°C using a plate reader (SpectraMax® M5), and then measured every 5 min up to 30 min. The linear range (0-15 min) of the decline in turbidity was used for calculating the sample lysozyme activity. Values were expressed in ng μL^{-1} based on a standard curve.

4.3.6. Innate Immunity-Related Gene Expression

4.3.6.1. qPCR Primer Design

The real-time quantitative polymerase chain reaction (qPCR) analyses in the current study were designed and performed based on the Minimum Information for Publication of qPCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009). For each gene [transcripts of interest (TOIs) and endogenous controls (ECs)] that was subjected to qPCR analyses, sablefish transcripts were either identified from sequences that had been deposited into GenBank, or from sablefish transcriptome sequences which were kindly provided by Dr. Frederick Goetz (NOAA Manchester Research Station, WA, USA) and which have been deposited into the NCBI Sequence Read Archive (SRA) database under project accession no. PRJNA763935. The transcriptome was assembled *de novo* using Trinity software (Grabherr *et al.*, 2011) with an established protocol (Haas *et al.*, 2013) (see Figure S4.1 for transcriptome sequences with corresponding Trinity IDs). The

transcriptome assembly annotation was performed using a BLAST homology search against sequence databases (downloaded version January 2020) for RefSeq mRNA (nucleotide) and SwissProt (amino acid). The gene definition column of BLAST results was used to identify or mine the gene of interest. The corresponding accession no./Trinity IDs were further extracted to confirm the sequence-based sequence identification.

In addition, to validate the identity of a given transcript, determine its orientation and identify the coding sequence (CDS), a BLASTx search of the non-redundant (nr) protein sequences database in NCBI was performed using a translated nucleotide query in June 2020 (see Table S4.1 for a summary of the BLASTx search results). A local database of all confirmed transcript sequences for a given gene was compiled using Vector NTI (Vector NTI Advance® 11.5.4, Invitrogen, Life Technologies). Next, for each gene, multiple sequence alignments were performed for all the corresponding transcripts using AlignX (Vector NTI Advance® 11.5.4). These alignments were used to determine whether transcripts were identical, contained single nucleotide polymorphisms (SNPs)/sequencing errors (these areas were avoided when designing primers), or represented different gene paralogues/isoforms. In the case of the latter, the alignments were also used to identify heterologous sequences where paralogue/isoform-specific qPCR primers could be designed (see below, and Figures S4.2-S4.7).

The sequences, amplicon sizes and efficiencies for all primer pairs used in the qPCR assays are presented in Table 4.2. All primers are located in the CDS, and in the case of gene paralogues/isoforms, primers were placed in an area with ≥3 bp difference between these sequences to ensure their specific amplification. The preferred amplicon size range was 90-150 bp. Primers were designed using Primer3 (Koressaar *et al.*, 2007,

Untergasser *et al.*, 2012, Kõressaar *et al.*, 2018); although, for a few gene paralogues/isoforms, primers were hand-designed in paralogue/isoform-distinguishing areas to ensure specificity.

4.3.6.2. RNA Preparation

Spleen samples (~100 mg tissue per sample) were homogenized in 400 µL of TRIzol™ Reagent (Invitrogen, Thermo Fisher Scientific) using individual Kontes® RNase-Free Pellet Pestle Grinders attached to a cordless motor (Kimble Chase, NJ, USA). An additional 400 µL of TRIzol was added to each sample, and after mixing by pipetting, the homogenates were frozen on dry ice and stored at -80°C. Frozen homogenates were further processed by thawing on wet ice, then passing them through a QIAshredder (QIAGEN, ON, Canada) spin column following the manufacturer's instructions. Another 200 µL of TRIzol was added to each homogenate to make a total volume of ~1 mL, and subsequently, the TRIzol total RNA extractions were completed according to the manufacturer's instructions. RNA pellets were resuspended in nuclease-free water (Invitrogen, Thermo Fisher Scientific).

Each TRIzol-extracted RNA sample (45 µg) was treated with 6.8 Kunitz units of DNase I (RNase-Free DNase Set, QIAGEN) with the manufacturer's buffer (1x final concentration) at room temperature for 10 min to degrade any residual genomic DNA. DNase-treated RNA samples were then column-purified using the RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. For both the pre-cleaned and column-purified RNA samples, RNA integrity was verified by 1% agarose gel electrophoresis, and RNA purity was assessed by A260/280 and A260/230 ratios using a

NanoDrop™ One UV-Vis spectrophotometer (Thermo Scientific, USA). All column-purified RNA samples were of high integrity (i.e., tight 28S and 18S ribosomal RNA bands, with 28S being approximately twice as intense as 18S; see Figure S4.8) and purity (i.e., A260/280 and A260/230 ratios between 2.07-2.12 and 1.94-2.39, respectively).

4.3.6.3. cDNA Synthesis and qPCR Parameters

First-strand cDNA templates for qPCR were synthesized in 20 µL reactions from 1 µg of DNase I-treated, column-purified, total RNA using Random Primers (250 ng; Invitrogen, Thermo Fisher Scientific), dNTPs (0.5 mM final concentration for each dNTP; Invitrogen) and M-MLV Reverse Transcriptase (200 U; Invitrogen) with the manufacturer's First Strand Buffer (1x final concentration) and dithiothreitol (DTT; 10 mM final concentration) at 37°C for 50 min.

PCR amplifications were performed in 13 µL reactions using 1x Power SYBR® Green PCR Master Mix (Applied Biosystems, Life Technologies), 50 nM of both the forward and reverse primers, the indicated cDNA quantity (see below), and the QuantStudio™ 6 Flex Real-Time PCR system (384-well format; Applied Biosystems). The real-time analysis program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min (with fluorescence detection at the end of each 60°C step), and was followed by dissociation curve analysis. Data analysis was performed using the QuantStudio™ Real-Time PCR Software v1.3 (Applied Biosystems).

4.3.6.4. qPCR Primer Validation

All *de novo* designed primers pairs were subjected to quality control (QC) testing. Amplification efficiencies (Pfaffl, 2001) were calculated for both a cDNA pool generated from the spleen of normoxia-acclimated PBS- and *A. salmonicida* bacterin-injected fish sampled at 24 hpi (n=7 and 8, respectively). These cDNAs were synthesized for the individual 7-8 RNA samples from each of these two injection groups according to the procedure described above (Section 4.3.6.3), and then pooled. Standard curves were generated using a 5-point (or 4-point in the case of *cox2*, *il8a*, *il8b*, *ifng*, *tlr5b* and *ef1a2*) 1:3 dilution series (in duplicate) starting with cDNA representing 10 ng of input total RNA. The reported efficiencies (Table 4.2) are an average of the efficiency values for each of the cDNA pools (except for *cox2* where efficiency was only based on the cDNA pool from *A. salmonicida* bacterin-injected fish due to low expression levels in the PBS-injected fish). Primer pair testing was also performed to ensure that a single product was amplified (dissociation curve analysis), and that there was no primer-dimer present in the no-template control. PCR products were electrophoretically separated on 2% agarose gels and compared with a 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific) to verify that only the correct size fragments were amplified.

4.3.6.5. Endogenous Control Selection

Expression levels of the TOIs were normalized to transcript levels of two EC genes (also called normalizer or reference genes). These ECs were selected from six candidates: elongation factor 1-alpha 2 (*ef1a2*; GAJJ01033263), elongation factor 1-alpha 4 (*ef1a4*; TR33310_c0_g1_i8), eukaryotic translation initiation factor 3 subunit D (*etif3d*;

JO688912), polyadenylate-binding protein a (*pabpc1a*; GAJJ01013600/JO671032), polyadenylate-binding protein b (*pabpc1b*; GAJJ01026238), and 60S ribosomal protein L32 (*rpl32*; BT082131) (Table 4.2). Briefly, the fluorescence threshold cycle (C_T) values of approximately 50% of the samples in the experiment [i.e., 4 samples from each of the 6 treatment groups (24 samples in total)] were measured in triplicate for each of the candidate EC transcripts using cDNA representing 5 ng of input total RNA, and then analyzed using geNorm (Vandesompele *et al.*, 2002) in qBase$^+$ software (Biogazelle, Zwijnaarde, East Flanders, Belgium). Based on this analysis, all six candidate ECs were quite stably expressed; geNorm M values were <0.3 and average C_T values for each of the six treatment groups were comparable (Table S4.2). The EC genes *ef1a4* and *pabpc1b* were determined to be the most stably expressed transcripts (i.e., with the lowest geNorm M values; M=0.108 and 0.105, respectively), and thus, were selected as the ECs for the experimental qPCR assays. During the main qPCR studies, these EC transcripts were indeed stably expressed in all samples (Table S4.3).

4.3.6.6. Experimental qPCRs

When primer QC testing and EC selection were completed, the expression levels of 16 TOIs (Table 4.2) were measured in all spleen samples from each of the six treatment groups in the study, using qPCR. Specifically, a total of 44 samples from normoxia- and hypoxia-acclimated fish before (*n*=7 per group) and 24 hpi with PBS or formalin-killed *A. salmonicida* (*n*=7 and 8 per group, respectively) were analysed. In all PCR reactions, cDNA representing 5 ng of input RNA was used, and for each sample, the 16 TOIs and 2 ECs were tested in triplicate. On each plate, and for every transcript, a plate linker sample

(i.e., a sample that was run on all plates in a given study; a transcript/plate was deemed acceptable only if its linker C_T value was ≤0.3 of a cycle of the linker samples on all other plates in the study) and a no-template control were included. The relative quantity (RQ) of each transcript was determined using the QuantStudio™ Real-Time PCR Software v1.3 (Applied Biosystems) relative quantification study application, with normalization to both *ef1a4* and *pabpc1b* transcript levels, and with the primer amplification efficiencies incorporated. For each TOI, the sample with the lowest normalized expression (mRNA) level was set as the calibrator sample (i.e., assigned a RQ value=1.0).

4.3.7. Measurements of Adaptive Immunity

4.3.7.1. Sablefish IgM Purification

Sablefish IgM was purified and used to produce polyclonal anti-sablefish-IgM antibodies. These antibodies, in turn, were used for Western blotting, a direct enzyme-linked immunosorbent assay (dELISA), flow cytometry, and confocal microscopy (see below). Purification was performed according to Vasquez *et al.* (2020) with minor modifications. Briefly, IgM was purified from pooled sablefish serum (200 mL collected from ~2 kg sablefish) using an immobilized mannan binding protein (MBP) column kit (Pierce™ 44897, Thermo Scientific, USA) according to the manufacturer's instructions. The integrity and purity of IgM in the elution fractions were evaluated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V under reducing conditions (Sambrook *et al.*, 2001) against a marker (PageRuler Plus Prestained Protein Ladder, Thermo Scientific) followed by Coomassie staining. The protein's concentration was estimated with DirectUV (absorbance at 280 nm) using a

Table 4.2. Primers used in the qPCR studies. Of the primers designed, primer pairs for 16 immune-related genes of interest and 6 candidate endogenous controls (ECs) were validated. A subset of the two most stably expressed ECs (*ef1a4* and *pabpc1b*) was then selected, and the expression levels of the transcripts of interest were normalized against these two EC transcripts. Amplification efficiencies were calculated using a 5-point 1:3 dilution series (4-point series in the case of *cox2*, *il8a*, *il8b*, *ifng*, *tlr5b*, and *ef1a2* primers) starting with cDNA representing 10 ng of input total RNA. Efficiencies were assessed for both a sample pool from PBS- and *A. salmonicida* bacterin-injected normoxia-acclimated fish, and then averaged (except for *cox2* primers where efficiency was based on a sample pool from *A. salmonicida* bacterin-injected fish only). See Section 4.3.6 for details. Transcriptome sequences with corresponding Trinity IDs are provided in Figure S4.1. General functional descriptions of the immune genes are prostaglandin production (*cox1*, *cox2*), iron-binding (*hamp*), pro-inflammatory cytokine (*ccl19*, *il1b*, *il8*, *ifng*, *tnfa*), transcription regulatory factor (*stat1*), and bacterial recognition (*tlr5*).

Gene name (symbol)	**GenBank accession no. or Trinity ID**	**Primer sequence (5'-3')**	**Amplicon size (bp)**	**Efficiency (%)**
Genes of interest				
cyclooxygenase 1 a (*cox1a*)	GAJJ01016042	F: CATCCTCACCCACTTCCAGT R: GGAGGGCTTGGAATAAGGTC	111	86.6
cyclooxygenase 1 b (*cox1b*)	GAJJ01016043	F: GGCTCAGGCTTCACAAAGAC R: TCATCTTAACGGGTGCATCA	93	120.1
cyclooxygenase 2 (*cox2*)	GAJJ01046020/GAJJ01047049	F: TCAAGGGCTTCTGGAACATC R: CGGCGGACTATCAATCAAGT	101	133.5
C-C motif chemokine ligand 19 (*ccl19*)	BT082212	F: GGGTGAAGCCAAGCTCTTCT R: TTGACGCTCAAGCAGCAGT	96	93.0
hepcidin antimicrobial peptide a (*hampa*)	GAJJ01029649	F: TTGCTGTTGCAGTAGCCATC R: ATGTTCAGCCACTGGGTTGT	122	88.5
hepcidin antimicrobial peptide b (*hampb*)	GAJJ01004264	F: GCAGTTGCAGTGACACTCGT R: GATATCCCGCAACTGGAGTG	121	93.1
interleukin 1 beta (*il1b*)	TR28839_c0_g1_i1/c0_g2_i5	F: GAAGCACGTTGTCAACCTCA R: TTGAGCAGGTCCTCGTCTCT	102	97.2
interleukin 1 beta-like (*il1b-like*)	TR21996_c0_g1_i1/c0_g2_i3	F: GCCTCAATTCTGGAACCTCA R: CAAACGTGCTTCAGGTGAGA	122	97.7

Table 4.2. Primers used in the qPCR studies (continued).

Gene name (symbol)	GenBank accession no. or Trinity ID	Primer sequence (5'-3')	Amplicon size (bp)	Efficiency (%)
Genes of interest				
interleukin 8 a (*il8a*)	BT082167	F: GTCTGAGAAGCCTGGGAGTG R: GTGTCTTCGCAGTGGGAGTT	118	91.6
interleukin 8 b (*il8b*)	JO666101	F: CTTCAGGTGGAGTCGAGGAT R: GTTGGCCAGTCCAGCTATGA	108	91.3
interferon gamma (*ifng*)	TR26914_c0_g1_i1	F: AGGCGCCTACATCACTCAAG R: ACCCTCTTTGCCTCCATTTT	141	95.7
signal transducer and activator of transcription 1 a (*stat1a*)	TR33543_c1_g1_i14	F: GCAGGAGTTGGAGCAGAAGT R: CGACCAGAGAGTTGGAGAGG	116	97.7
signal transducer and activator of transcription 1 b (*stat1b*)	TR36335_c1_g1_i1	F: ACTCACCTGGCACAAGCTCT R: TCCACGATTCTGTCTTGCAG	108	96.1
tumor necrosis factor alpha a (*tnfaa*)	BT083097	F: TGCACCTACCACAACAGTGAC R: CTCCGTGTTGGTGATGACTG	109	91.0
toll-like receptor 5 a (*tlr5a*)	TR29278_c0_g1_i1	F: TTGCGATTTGTCAGACTCCA R: CCCGGTCGAACACTCACTAT	105	92.9
toll-like receptor 5 b (*tlr5b*)	TR29636_c0_g1_i2	F: AGCGGTTGGTTTGAATAACG R: ACCGCATTGAAGCTCAAGTT	138	105.6
ECs				
elongation factor 1-alpha 2 (*ef1a2*)	GAJJ01033263	F: TCGCTTTGCTGAGCTGCTGG R: CCACACACATGGGCTTGATGG	128	97.0
elongation factor 1-alpha 4 (*ef1a4*)	TR33310_c0_g1_i8	F: CGCATGCAAGTTCAGTGAACTC R: GCTTCTGTGGGACCAGTTTGAC	122	97.7
eukaryotic translation initiation factor 3 subunit D (*etif3d*)	JO688912	F: CACCGAGCTGAAGAACAACA R: CGAGACACGTACCCCAGTTT	96	93.6

Table 4.2. Primers used in the qPCR studies (continued).

Gene name (symbol)	GenBank accession no. or Trinity ID	Primer sequence (5'-3')	Amplicon size (bp)	Efficiency (%)
ECs				
polyadenylate-binding protein 1 a (*pabpc1a*)	GAJJ01013600/JO671032	F: GAGCGGTGTTGGAAACATCT R: AGCCATTCTCGTCACAAACC	125	102.5
polyadenylate-binding protein 1 b (*pabpc1b*)	GAJJ01026238	F: GACATGAACGGCAAGGAACT R: CTGGTCATCCGATCCTGTTT	102	97.2
60S ribosomal protein L32 (*rpl32*)	BT082131	F: CCTCACTAAGCCCAAGATCG R: CGGACCCTGTTGTCAATACC	120	87.0

Genova-Nano spectrophotometer (Jenway, UK) and measured with a bicinchoninic acid (BCA) assay (BCA protein assay kit, Pierce™, Thermo Scientific) using a plate reader (SpectraMax® M5). High quality IgM fractions were pooled and dialyzed twice against 20 mM Tris buffer (pH 8.0) at 4°C with gentle stirring using a dialysis cassette or dialysis bag [3-20 kDa molecular weight (MW) cut-off, Thermo Scientific]. Subsequently, the purified sablefish IgM was lyophilized/freeze dried (EdwarDS-Super Modulyo®, Boc Ltd, UK), resuspended in 20 mM Tris buffer (pH 8.0), and re-evaluated for integrity and concentration using 10% SDS-PAGE (Figure 4.4) and DirectUV. Finally, sablefish IgM was stored at -20°C in aliquots to protect against repeated freeze-thaw cycles. The production of chicken anti-sablefish-IgM IgY was done commercially at Somru BioScience Inc. (Charlottetown, PEI, Canada), and involved affinity purification of the antibodies from chicken eggs using the antigen, followed by biotinylation of the antibodies.

4.3.7.2. Western Blotting

Western blotting was used to validate the specificity of the polyclonal chicken anti-sablefish-IgM IgY for sablefish IgM. Purified sablefish IgM was separated by 10% SDS-PAGE as described above, and then transferred semi-dry at 20 V for 30 min to a nitrocellulose membrane (GE Healthcare, USA). The membrane was then blocked for 1 h with 2% (w/v) skim milk in PBS plus 0.05% Tween 20 (PBS-T 0.05%) at room temperature. Incubations with primary antibody (chicken anti-sablefish-IgM IgY) and secondary antibody conjugated with alkaline phosphatase (ALP) (rabbit anti-chicken-IgY IgG-ALP; Merck EMD Millipore AP162A, Sigma-Aldrich, Canada) were done overnight

at 4°C, with the membrane washed three times with PBS-T 0.05% after each incubation (washes were performed with gentle rocking at room temperature). Both antibodies were diluted 5,000x in PBS-T 0.05% with 2% skim milk (these dilutions were found to be optimal in preliminary trials). Bands were visualised by incubation with BCIP/NBT solution (VWR, Canada) followed by colorimetric imaging using the iBright CL1500 system (Invitrogen, Thermo Fisher Scientific). A negative control (containing no primary antibody) was run alongside the normal blot and no non-specific binding by the secondary antibody was detected (Figure S4.9).

4.3.7.3. Direct Enzyme-Linked Immunosorbent Assay

Vasquez *et al.* (2020) previously determined that the *A. salmonicida* A-layer strongly binds to chicken anti-sablefish-IgM IgY in a non-specific fashion, which severely confounds the results of indirect ELISAs. Therefore, instead of evaluating *A. salmonicida*-specific IgM levels in sablefish after immunostimulation, I measured total IgM levels using dELISA, according to a protocol that was developed using established procedures (Lin, 2015) and that was similar to Vasquez *et al.* (2020), but with some modifications. I determined that the matrix interference was most successfully removed by increasing sample dilution in combination with heat-treatment (Figure S4.10C). Serum samples were diluted 40x in PBS, and subsequently heat-treated at 45ºC for 30 min to inactivate the complement system. Then, samples were further diluted in PBS to 1,600x, and 5 µL of each sample was pipetted into duplicate wells of a high protein-binding polystyrene 96-well plate (Nunc MaxiSorp™, Thermo Fisher Scientific) with 95 µL of carbonate-bicarbonate coating buffer (15 mM Na_2CO_3; 35 mM $NaHCO_3$; pH 9.8).

Samples were analysed in a randomized order and immediately after heat-treatment, as repeated freeze-thaw cycles decreased IgM integrity in the sample.

For each plate, a pool of samples was diluted 250x in PBS, heat-treated, diluted further in PBS to 25,000x, and then added to duplicate wells with coating buffer at a final dilution of 500,000x to determine non-specific background. This background OD (~0.3 at 450 nm) was subtracted from each sample's OD. Each plate also included a standard curve with purified sablefish IgM that was serially diluted in coating buffer to 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 μg mL^{-1}, and a negative control with only coating buffer; both in duplicate wells (Figure 4.5A). These standard IgM concentrations were selected because they produced a wide OD range (~0.2-1.8 at 450 nm) for optimal assay sensitivity (Figure S4.10A). OD values obtained from the standard curve were fitted with a 4-parameter sigmoidal regression using the plate reader software (SoftMax Pro). This regression was then used to calculate the sample concentrations.

The plates with standards, samples, and background pool, were incubated at 4°C overnight, and then washed four times with 200 μL of PBS plus 0.1% Tween 20 (PBS-T 0.1%) using a microplate washer (BioTek™ 50TS, Thermo Fisher Scientific). Plates were blocked by incubating with 150 μL of ChonBlock™ (Chondrex Inc. 9068, Woodinville, WA, USA) per well for 1 h at 37°C, followed by washing according to the same procedure. Then, plates were incubated with 100 μL of biotinylated chicken anti-sablefish-IgM IgY per well (diluted 5,000x in PBS-T 0.05%) for 1 h at 37°C. After washing, 100 μL of streptavidin-horseradish peroxidase (HRP; BioLegend 405210, San Diego, CA, USA; diluted 2,000x in PBS-T 0.05%) was added to each well, and plates

were incubated for 1 h at 37°C. These primary antibody and enzyme conjugate dilutions were determined to be optimal as they provided the highest signal:background values (Figure S4.10B). Following washing, plates were incubated with 100 µL of TMB solution (Kementec Solutions Inc., cat. #4850, Amherst, NH, USA) per well for 30 min at room temperature in the dark. Colour development was stopped with 100 µL of 0.3 M H_2SO_4, after which the OD of each well was measured at 450 nm.

Serum IgM levels were evaluated at 0, 6, and 10 weeks post-injection (wpi) in the PBS-injected (7 fish/group) and *A. salmonicida* bacterin-injected groups (8 normoxia-acclimated and 9 hypoxia-acclimated fish).

4.3.7.4. Flow Cytometry Analysis of Whole Blood

Blood cell composition and relative white blood cell (WBC) abundance at 10 wpi were assessed by cell staining with $DiOC_6$ (3,3-dihexyloxacarbocyanine) followed by fluorescence flow cytometry as described by Inoue *et al.* (2002). Fluorescence was detected in 30,000 cells using a BD FACS Aria II flow cytometer with BD FACS Diva v7.0 software, and nozzle size and thresholds were set to 70 µm and 5000, respectively. Blood cell populations were identified by their typical location on a green fluorescence (FITC) *vs*. side scatter (SSC) dot-plot, and their relative abundance was determined for each experimental group and expressed as a percentage of the total cell count. Two separate WBC populations were identified in sablefish using this method (Inoue *et al.*, 2002) that appeared to represent lymphocytes and thrombocytes (P1), and monocytes, neutrophils and basophils (P2); although, it was not possible to distinguish between the

individual cell types grouped into these two populations. In contrast, the sablefish had two distinct red blood cell (RBC) populations with different FITC intensities (Figure 4.6A). Based on Inoue *et al.* (2002), these two FITC peaks most likely represented RBCs at different maturation stages, with immature RBCs composing a relatively small population with a higher FITC intensity. All the aforementioned blood cell types (except basophils) have been previously detected in sablefish (Schubiger *et al.*, 2021).

4.3.7.5. WBC Isolation

Sablefish WBCs were isolated from blood to allow for flow cytometry analysis and confocal microscopy imaging of IgM^+ cells (see below). WBC isolation was performed according to the method described by Smith *et al.* (2018) with the following modifications. Heparinized blood samples (0.7 mL) were mixed with cell media (4.3 mL) consisting of Leibovitz-15 (L-15, Gibco, Thermo Fisher Scientific) supplemented with 5% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco). This solution was placed on a discontinuous 34%/51% (v/v) Percoll™ gradient (GE Healthcare, USA) prepared with HBSS (Gibco) for isotonicity, and centrifuged at 400 ×*g* for 40 min at 4°C. Subsequently, the WBCs were collected from the Percoll™ gradient interface area, washed (at 600 ×*g* for 10 min at 4°C), and resuspended in cell media. Prior to flow cytometry analysis, cell viability and quantity were determined using trypan blue staining, Countess™ Cell Counting Chamber Slides and a Countess® Automated Cell Counter (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. Average viability of isolated WBCs was >80% (84±2% and 82±2% in normoxia-acclimated PBS- and *A. salmonicida* bacterin-injected fish; 84±3% and 87±1%

in hypoxia-acclimated PBS- and *A. salmonicida* bacterin-injected fish, respectively) with no significant difference between treatment groups (P>0.05). For confocal microscopy, viable cells were counted using trypan blue staining (Sigma-Aldrich) on a haemocytometer.

4.3.7.6. Relative Peripheral IgM$^+$ Cell Abundance

The relative abundance of IgM$^+$ WBCs at 10 wpi was assessed according to a leukocyte staining and flow cytometry method adapted from Rønneseth *et al.* (2015). Isolated WBCs were diluted to 5×10^6 cells mL^{-1} in cell media (L-15 with 5% FBS and 1% penicillin/streptomycin; Gibco), then 100 µL of this cell suspension (5×10^5 cells) was pipetted into a 2 mL Eppendorf tube, and washed in PBS with 1% (w/v) bovine serum albumin (BSA-PBS) at 600 ×*g* for 5 min at 4°C. The cells were initially incubated with 100 µL of biotinylated chicken anti-sablefish-IgM antibody (diluted 1,000x in BSA-PBS; this dilution found to be optimal) for 1 h at 4°C, and subsequently, with 100 µL of streptavidin-Alexa Fluor® 647 (Invitrogen S32357, Thermo Fisher Scientific; diluted 400x in BSA-PBS) for 45 min at 4°C. Each incubation was performed in the dark, and was followed by two washing steps in BSA-PBS as described above. The stained WBCs were fixed in 100 µL of PBS with 4% formaldehyde overnight at 4°C in the dark, then 200 µL of BSA-PBS was added, and fluorescence was detected from 10,000 cells using the same FACS equipment and software previously described. Intact cells were first gated out (P1) based on their position on a forward scatter (FSC) *vs.* side scatter (SSC) dot-plot (Figure 4.7A), after which IgM$^+$ cells (P2) were distinguished based on their red fluorescence (APC) (Figure 4.7B), and their relative abundance was determined as a

percentage of the total intact cell count. Three types of negative controls were run alongside the positive samples: cells that were incubated with (i) no chicken anti-sablefish-IgM IgY; (ii) no fluorescent conjugate; and (iii) polyclonal chicken isotype IgY (Abcam ab37382, USA) and a biotinylated anti-chicken-IgY antibody (both antibodies diluted 1,000x in BSA-PBS). These controls were used to identify APC positive cells, and to determine the amount of non-specific antibody and conjugate binding, and auto-fluorescence (~0.75%, ~1%, ~0.1% of cells, respectively).

4.3.7.7. Confocal Microscopy

Peripheral IgM^+ leukocytes were stained and prepared for immune fluorescence visualization with confocal microscopy according to the protocol described by Rønneseth *et al.* (2015) with modifications. Isolated WBCs in cell media (L-15 with 5% FBS and 1% penicillin/streptomycin; Gibco) were transferred to 2 mL Eppendorf tubes (5×10^6 cells/tube), and washed in BSA-PBS at 600 ×*g* for 5 min at 4°C. Cells were first incubated with 500 µL of biotinylated chicken anti-sablefish-IgM IgY (diluted 500x in BSA-PBS) for 1 h at 4°C, then with 500 µL of avidin-Texas Red (TX-R; Life Technologies, USA; diluted 200x in BSA-PBS) for 45 min at 4°C. After each incubation, cells were washed twice in BSA-PBS as described previously. The stained WBCs were fixed in 500 µL of PBS with 4% formaldehyde overnight at 4°C, followed by washing in PBS at 600 ×*g* for 5 min at 4°C. To stain nuclei, cells were incubated with 500 µL of 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich MBD0015; diluted 1,000x in PBS) for 10 min at 4°C, then washed in PBS as before. All staining incubations and fixation steps were performed in the dark. The WBCs were briefly centrifuged to concentrate the

cell suspension and resuspended in PBS, and subsequently, mounted on a glass slide with PermaFluor™ Aqueous Mounting Medium (Lab Vision, Thermo Fisher Scientific), cover slipped (Fisherbrand 12541B), and sealed with transparent nail polish. Slides were viewed using a Nikon AR1 laser scanning confocal microscope running Nikon Elements software for image acquisition. Alongside the positive samples, the same three negative controls were included as for flow cytometry analysis (see above), except that antibodies were diluted 500x. The absence of detectable red fluorescence in these negative controls (Figures 4.8D-F, S4.11F-K) confirmed the specificity of the IgM^+ cell staining. Staining and imaging were performed using WBCs from three fish on three different days.

4.3.8. Statistical Analyses

The morphological parameters shown in Table 4.1 were analysed by comparing the acclimation groups at the 0 hpi (initial) sampling using Student's *t*-tests, whereas the acclimation and injection groups at the 24 hpi and 10 wpi samplings were compared using 2-way ANOVAs and Tukey's HSD post-hoc comparisons. The haematological innate immunity parameters and expression levels of bacteria-responsive genes (Figures 4.2-4.3) were analysed in the same way, except that the PBS-injected groups at the initial *vs.* 24 hpi sampling were also compared using Student's *t*-tests. For the IgM results shown in Figure 4.5B, the PBS- and *A. salmonicida* bacterin-injected groups were analysed separately for the effects of acclimation and sampling time using 2-way ANOVAs followed by Tukey's HSD post-hoc comparisons, whereas the injection groups were compared to each other using Student's *t*-tests. I did not analyse the results in Figures 4.2-4.3 and 4.5 with 3-way ANOVAs that integrated the effects of acclimation, injection

(PBS *vs. A. salmonicida* bacterin) and time, as such models would test for various interactions (e.g., acclimation×injection×time) that were not of interest in this study, and that would reduce the sensitivity of the model. Finally, the flow cytometry results (Figures 4.6B-E and 4.7C) at 10 wpi were analysed by comparing the acclimation and injection groups using 2-way ANOVAs followed by Tukey's HSD post-hoc comparisons.

For all parameters, the model fits were graphically assessed for violations of the assumptions for normality and homogeneity using quantile-quantile and residual-fit plots, respectively. There were no influential outliers in the dataset, no violations of the model assumptions, and thus, no transformations and non-parametric tests were performed. All Student's *t*-tests were two-tailed, and all data are reported as means±s.e.m. unless specifically identified. The level of statistical significance was set at $P<0.05$. Statistical analyses were performed using Rstudio v1.4.1106 with R v4.0.5 (R Core Team, 2021), and graphs were created in GraphPad Prism 9.1.0.

4.4. Results

4.4.1. Haematological Innate Immunity Parameters

Overall, acclimation to hypoxia did not substantially affect whole blood respiratory burst (Figure 4.2A). At 24 hpi with PBS, respiratory burst was 2.2-fold higher ($P<0.05$) in the hypoxia-acclimated, but not normoxia-acclimated, sablefish, compared to the initial time point. However, this increase in respiratory burst was much less than seen in both groups at 24 hpi with formalin-killed *A. salmonicida* (the difference in normoxia- and hypoxia-acclimated sablefish was 9.2 and 2.8-fold, respectively, as compared to the PBS-injected groups; $P<0.01$ and $P<0.05$). Further, the respiratory burst following *A.*

salmonicida bacterin injection was not significantly different (P>0.05) between the acclimation groups. Chronic hypoxia also did not influence initial plasma haemolytic activity of the alternative complement pathway or lysozyme concentration, or following PBS injection (P>0.05; Figure 4.2B-C). However, complement system activity declined slightly (by 25%, P<0.05) in the hypoxia-acclimated sablefish at 24 hpi after injection with formalin-killed *A. salmonicida* (Figure 4.2B). No changes in lysozyme concentration were observed after bacterin injection (P>0.05) (Figure 4.2C).

4.4.2. Innate Immunity-Related Gene Expression

Long-term hypoxia did not significantly (P>0.05) affect the constitutive transcript expression of any of the 16 known bacteria-responsive genes evaluated in sablefish (Figure 4.3). PBS injection also had no consistent effect on the transcript expression of the 16 genes, and the effect was relatively small compared to the *A. salmonicida* bacterin-induced changes (see below). For example, while the expression of most genes in normoxia-acclimated fish remained unchanged after PBS injection, *il8a* transcript levels decreased (by 0.8-fold), whereas those for *hampb, il8b*, *stat1b*, *tlr5a* and *tlr5b* increased significantly (by 4.4, 2.1, 1.3, 1.5 and 1.8-fold, respectively) compared to their initial levels (Figure 4.3F, I-J, N, O-P). Only two significant PBS injection-associated changes in transcript expression were observed in hypoxia-acclimated fish, i.e., 1.3-fold and 2.1-fold increases for *cox1a* and *hampb*, respectively, compared to their baseline levels. Injection with formalin-killed *A. salmonicida* significantly (P<0.05) affected transcript expression levels of the majority (13 out of 16) of the genes evaluated in normoxia-acclimated sablefish at 24 hpi. Specifically, the transcript levels of *cox2*, *ccl19*,

hampa, *hampb*, *il1b* and *tlr5b* were strongly upregulated after bacterin injection (by 11, 7.1, 42, 18, 30 and 35-fold, respectively), and levels of *il8a*, *ifng*, *stat1a*, *stat1b*, *tnfaa* and *tlr5a* were moderately elevated (by 3.3, 4.2, 2.6, 3.4, 1.6 and 2.5-fold, respectively), as compared to the PBS-injected group (Figure 4.3C-G, I, K-P). Interestingly, *il1b-like* was downregulated in the bacterin-injected fish, as compared to the PBS-injected fish (by 0.5-fold; Figure 4.3H), and formalin-killed *A. salmonicida* injection did not significantly influence *cox1a* and *cox1b* transcript expression ($P>0.05$; Figure 4.3A-B).

There were only a few differences in the post-injection transcript expression levels between the normoxia- and hypoxia-acclimated sablefish. The hypoxia-acclimated sablefish had significantly higher transcript levels of *ccl19* (25±4 *vs.* 15±2 RQ, respectively; a 1.7-fold increase) and *stat1a* (5.9±0.8 *vs.* 4.1±0.4 RQ, respectively; a 1.4-fold increase) ($P<0.05$) at 24 hpi after injection with formalin-killed *A. salmonicida* as compared to the normoxia-acclimated sablefish (Figure 4.3D, M). Whereas, for *tlr5b*, the transcript levels were lower (166±21 *vs*. 257±36 RQ, respectively; a 0.6-fold decrease) ($P<0.05$) at 24 hpi after bacterin injection in the hypoxia- *vs.* normoxia-acclimated sablefish (Figure 4.3P).

4.4.3. Humoral and Cellular Adaptive Immunity

4.4.3.1. Total Serum IgM Levels

Sablefish IgM was purified from 200 mL of serum using an immobilized MBP column, and after concentration by lyophilization, a stock solution of 1.6 mg mL^{-1} IgM was obtained. Purified IgM was visualized by 10% SDS-PAGE under reducing conditions, and this revealed that this protein was comprised of both a heavy (H; ~75

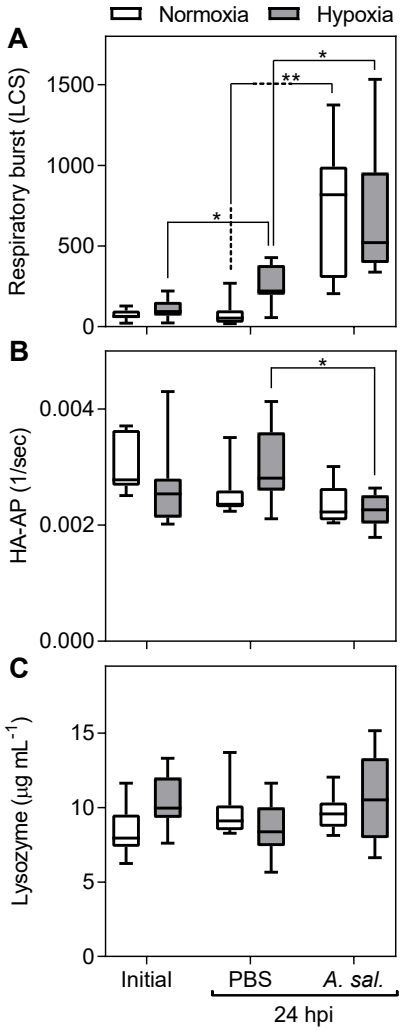

Figure 4.2. The effect of hypoxia acclimation on the sablefish's haematological innate immune response to formalin-killed *A. salmonicida*. (**A**) Blood leukocyte respiratory burst in luminescence counts per sec (LCS); (**B**) haemolytic activity of the alternative complement pathway (HA-AP, 1/sec) in the plasma; (**C**) plasma lysozyme concentration (µg mL^{-1}). Sablefish were acclimated to normoxia or hypoxia (100% and 40% air saturation, respectively) and sampling occurred before (initial) and at 24 hours post-injection (hpi) with PBS or formalin-killed *A. salmonicida* (*A. sal.*). Boxes and whiskers indicate the 25^{th}-75^{th} and 10^{th}-90^{th} percentiles, respectively, with the median value marked by a horizontal line. Effects of acclimation, injection and time were assessed using 2-way ANOVAs followed by Tukey's HSD post-hoc comparisons, or Student's *t*-tests. Significant differences between groups are indicated with asterisks (*=P<0.05; **=P<0.01). Sample sizes are n=7 for the initial and PBS-injected groups, and n=8 for the *A. sal.*-injected groups.

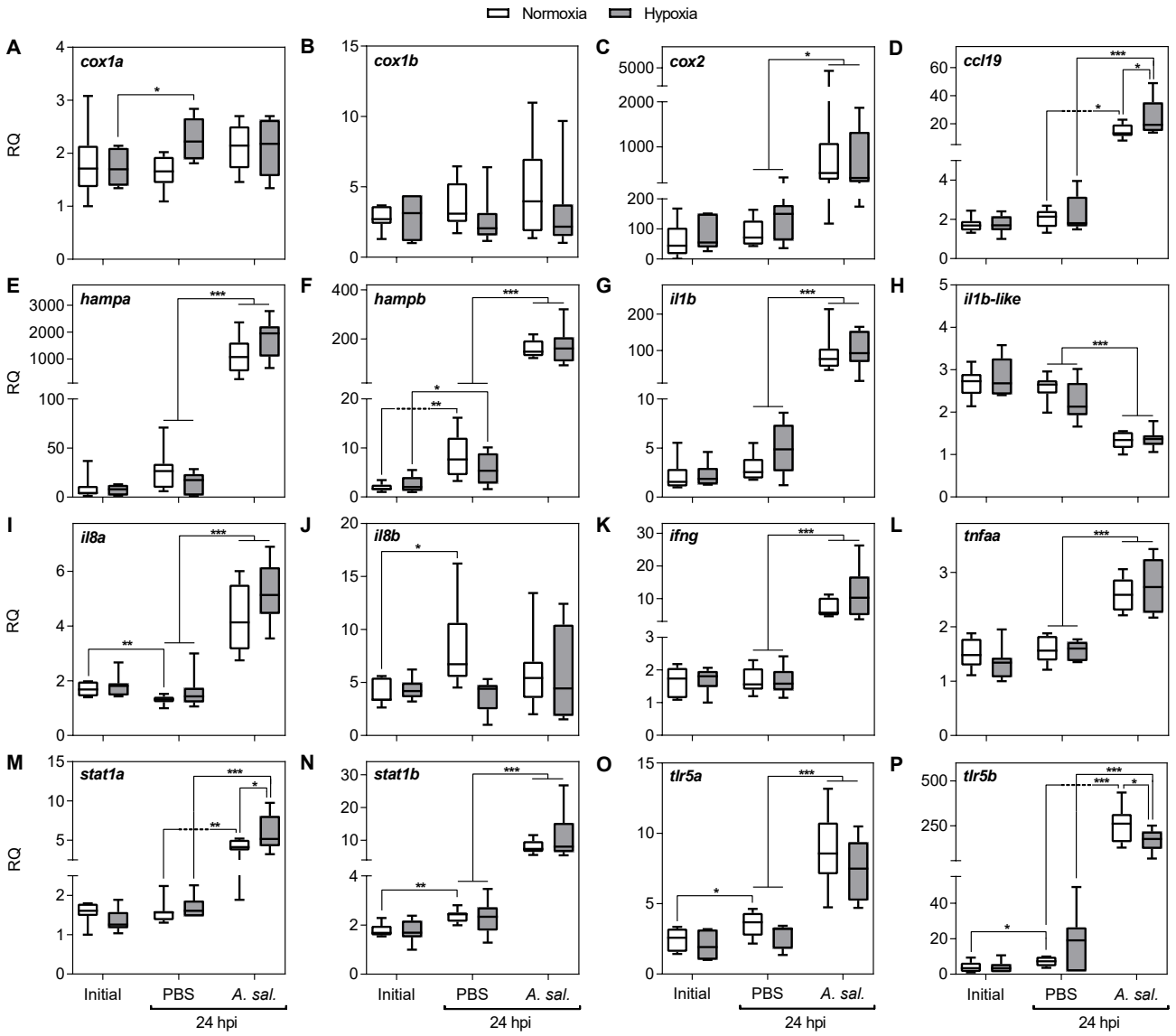
Normoxia
Hypoxia
A
cox1a
B
cox1b
C
cox2
D
ccl19
E
hampa
F
hampb
G
il1b
H
il1b-like
I
il8a
J
il8b
K
ifng
L
tnfaa
M
stat1a
N
stat1b
O
tlr5a
P
tlr5b
RQ
Initial
PBS
A. sal.
24 hpi

Figure 4.3. The effect of chronic hypoxia on the transcription of known bacteria-responsive genes in the spleen of sablefish. Sablefish were acclimated to normoxia or hypoxia (100% and 40% air saturation, respectively) and sampled before (initial) and at 24 hours post-injection (hpi) with PBS or formalin-killed *A. salmonicida* (*A. sal.*). Transcript levels are shown for 16 genes typically involved in the antibacterial response: (**A**) *cox1a*; (**B**) *cox1b*; (**C**) *cox2*; (**D**) *ccl19*; (**E**) *hampa*; (**F**) *hampb*; (**G**) *il1b*; (**H**) *il1b-like*; (**I**) *il8a*; (**J**) *il8b*; (**K**) *ifng*; (**L**) *tnfaa*; (**M**) *stat1a*; (**N**) *stat1b*; (**O**) *tlr5a*; (**P**) *tlr5b*. Expression levels are presented as relative quantity (RQ) values, which were determined as the transcript levels for the TOIs normalized to both *ef1a4* and *pabpc1b* transcript levels, and calibrated to the individual with the lowest expression for each TOI. Boxes and whiskers indicate the 25^{th}-75^{th} and 10^{th}-90^{th} percentiles, respectively, with the median value marked by a horizontal line. Effects of acclimation, injection and time were assessed using 2-way ANOVAs followed by Tukey's HSD post-hoc comparisons, or Student's *t*-tests. Significant differences between groups are indicated with asterisks (*=P<0.05; **=P<0.01; ***=P<0.001). Sample sizes are n=7 for the initial and PBS-injected groups, and n=8 for the *A. sal.*-injected groups.

kDa) and a light (L; ~25 kDa) chain (Figure 4.4). This is consistent with previous findings in sablefish (Vasquez *et al.*, 2020) and other teleosts (Kaattari *et al.*, 2009, Mashoof *et al.*, 2016). The other bands detected likely represent various heterodimer complexes of the IgM chains (HL; ~100 kDa and HHHLLL; ~300 kDa) which have also been reported in lumpfish (*Cyclopterus lumpus*) (Gendron *et al.*, 2020). The specificity of the chicken anti-sablefish-IgM antibody for sablefish IgM was validated using Western blotting. The major bands detected with the anti-IgM antibody were the same in size (MW) as those observed with SDS-PAGE (Figure 4.4).

For the dELISA, a standard curve was developed with known IgM concentrations (0-2 μg mL^{-1}) and fitted with a 4-parameter sigmoidal regression [equation for a representative plate: $y=(0.15-3.16)/(1+(x/1.80)^{0.96})+3.16$ with a $R^2=0.9979$; Figure 4.5A]. At 6 wpi, there was no significant change in the total serum IgM level in any of the acclimation or injection groups ($P>0.05$; Figure 4.5B). However, at 10 wpi, the IgM level in bacterin-injected normoxia-acclimated sablefish was 48% higher as compared to their baseline level at 0 wpi (15.7±1.2 *vs.* 10.7±1.1 mg mL^{-1}, $P<0.01$), and 38% higher as compared to the level of PBS-injected normoxia-acclimated sablefish at 10 wpi (15.7±1.2 *vs.* 11.4±0.5 mg mL^{-1}, $P<0.01$). In contrast, no increase in IgM levels was observed in the hypoxia-acclimated sablefish 10 weeks after formalin-killed *A. salmonicida* injection ($P>0.05$).

4.4.3.2. Relative Abundance of Peripheral IgM^+ Cells

Whole blood flow cytometry did not reveal any significant effects ($P>0.05$) of formalin-killed *A. salmonicida* injection or hypoxia acclimation on the relative abundance

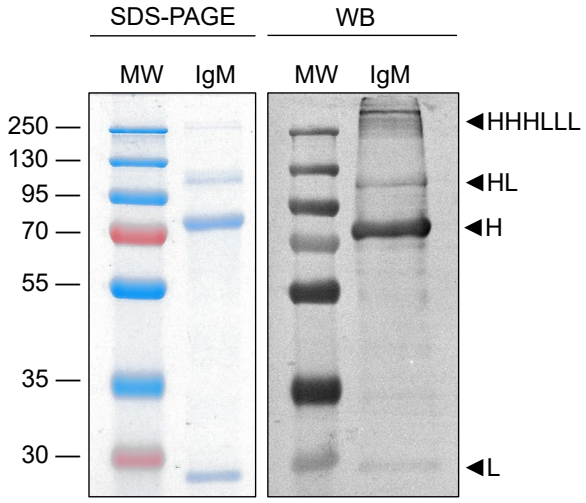

Figure 4.4. SDS-PAGE of purified sablefish IgM, and validation of anti-sablefish-IgM antibody specificity using Western blotting. (**Left**) Coomassie blue staining of purified sablefish IgM analysed with 10% SDS-PAGE under reducing conditions, alongside a pre-stained marker lane with the molecular weight (MW) of standard bands indicated in kDa. Bands observed in purified IgM (indicated with arrow heads) include heavy (H) and light (L) chain components, with an estimated molecular weight of ~75 and ~25 kDa, respectively. The remaining bands likely represent various heterodimer complexes of the IgM chains: HHHLLL (~300 kDa) and HL (~100 kDa), as previously reported for purified lumpfish IgM (Gendron *et al.*, 2020). (**Right**) Western blot of purified sablefish IgM using chicken anti-sablefish-IgM IgY primary antibody and rabbit anti-chicken-IgY-ALP conjugated secondary antibody. The major bands detected with the anti-IgM antibody were the same as those observed with Coomassie blue staining.

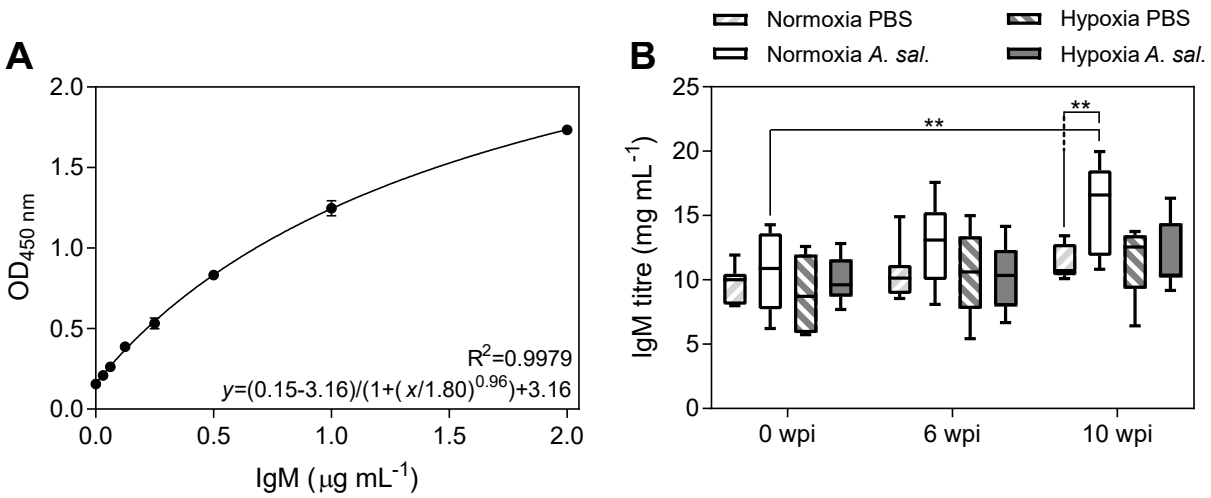

Figure 4.5. The effect of hypoxia acclimation on the sablefish's IgM titre response to formalin-killed *A. salmonicida* injection. (**A**) dELISA standard curve (concentrations of purified sablefish IgM: 2; 1; 0.5; 0.25; 0.125; 0.0625; 0.03125; 0 μg mL^{-1}) used to determine IgM levels in sablefish serum. The values were fitted with a 4-parameter sigmoidal regression, with the equation and R^2 value shown in the figure. Values are means±s.e.m. from one plate that is representative of other plates. (**B**) Serum IgM levels measured with dELISA in sablefish acclimated to normoxia or hypoxia (100% or 40% air saturation, respectively), at 0, 6 or 10 weeks post-injection (wpi) with PBS (control) or formalin-killed *A. salmonicida* (*A. sal.*). Effects of acclimation and time were assessed with 2-way ANOVAs followed by Tukey's HSD post-hoc comparisons, whereas the effect of injection (PBS *vs*. *A. sal.*) was assessed with Student's *t*-tests. Significant differences between groups are indicated with asterisks (**=P<0.01). Boxes and whiskers indicate the 25^{th}-75^{th} and 10^{th}-90^{th} percentiles, respectively, with the median value marked by a horizontal line. Samples sizes are n=7 for the PBS-injected groups, and n=8 and 9 for the *A. salmonicida* bacterin-injected normoxia- and hypoxia-acclimated groups, respectively.

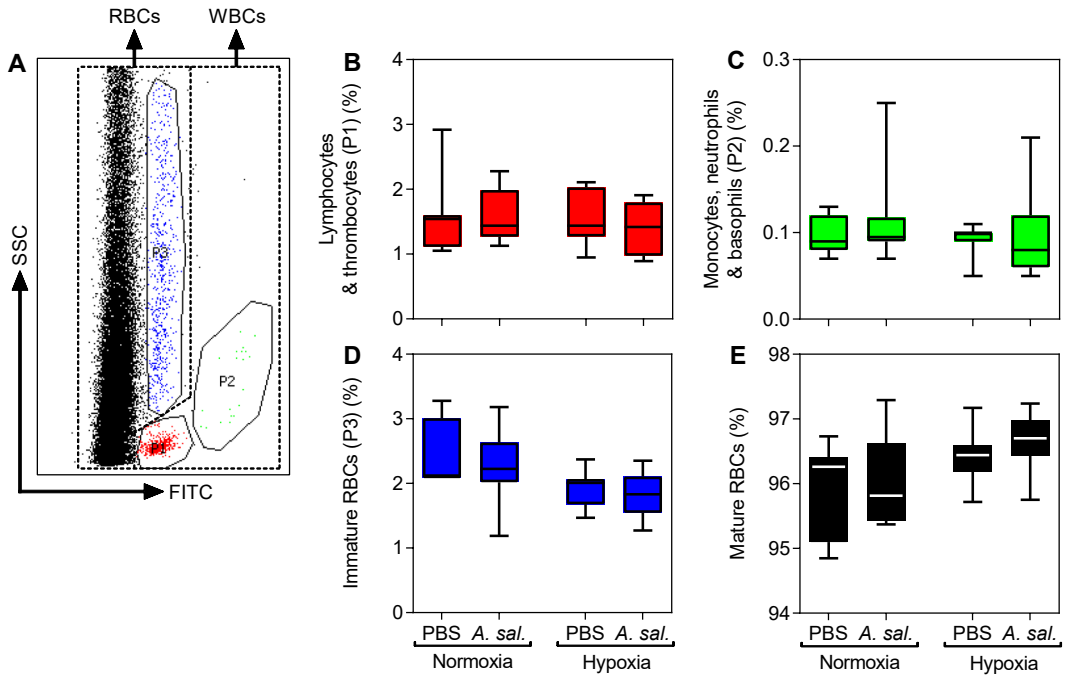

Figure 4.6. Flow cytometry analysis of $DiOC_6$ stained blood cells from sablefish, and the effect of hypoxia acclimation and injection with formalin-killed *A. salmonicida* on blood cell composition at 10 wpi. (**A**) Representative example of a green fluorescence (FITC) *vs.* side scatter (SSC) plot of $DiOC_6$ stained blood cells from a normoxia-acclimated PBS-injected sablefish. The dotted lines separate the red blood cells (RBCs) and white blood cells (WBCs), which are further divided into populations (P1-3). Shown are the relative abundance of (**B**) lymphocytes and thrombocytes (P1), (**C**) monocytes and granulocytes (P2), (**D**) immature RBCs (P3) and (**E**) mature RBCs (remaining cells). Effects of acclimation and injection were assessed with 2-way ANOVAs followed by Tukey's HSD post-hoc comparisons. There were no significant differences between the individual groups. Boxes and whiskers indicate the 25^{th}-75^{th} and 10^{th}-90^{th} percentiles, respectively, with the median value marked by a horizontal line. Sample sizes are n=7 for the PBS-injected groups, and n=8 and 9 for the *A. salmonicida* bacterin-injected (*A. sal.*) normoxia- and hypoxia-acclimated group, respectively.

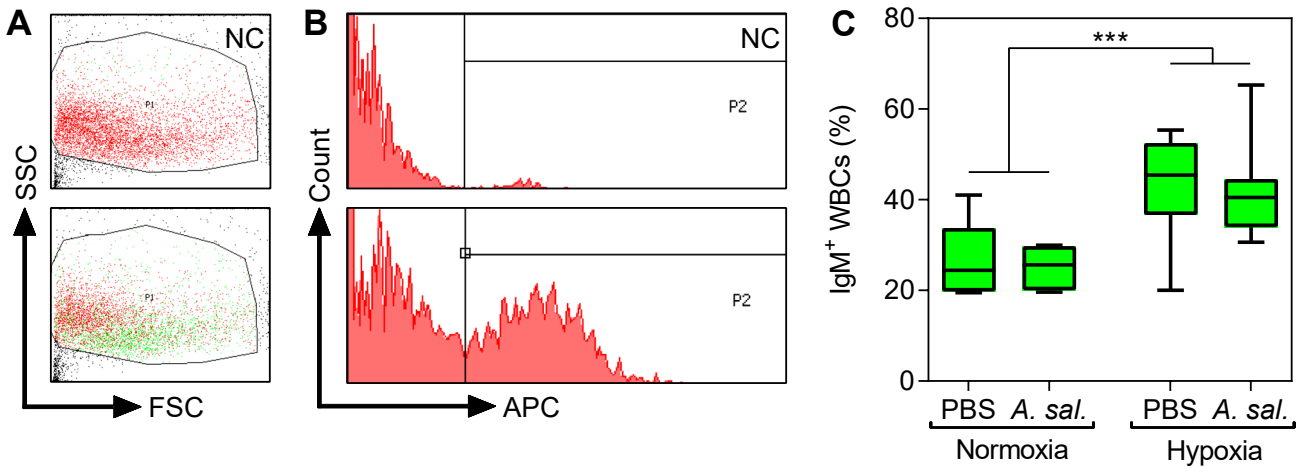

Figure 4.7. Detection of IgM$^+$ leukocytes in sablefish blood using flow cytometry, and the effect of chronic hypoxia and injection with formalin-killed *A. salmonicida* on the relative IgM$^+$ cell abundance at 10 wpi. Representative examples of (**A**) forward scatter (FSC) *vs.* side scatter (SSC) dot-plots and (**B**) red fluorescence (APC) *vs.* cell count plots for white blood cells (WBCs) from a hypoxia-acclimated *A. salmonicida* bacterin-injected (*A. sal.*) sablefish. Intact cells were gated out first (P1), then IgM$^+$ cells (P2) were distinguished based on their red fluorescence from Alexa Fluor 647 staining. Negative control (NC, no anti-sablefish-IgM), top panels; positive sample, bottom panels. (**C**) Relative abundance of IgM$^+$ WBCs. Effects of acclimation and injection were assessed using 2-way ANOVAs followed by Tukey's HSD post-hoc comparisons. The significant acclimation effect is indicated with asterisks (***=P<0.001), which was also significant within the injection groups (*=P<0.05 for PBS-injected and **=P<0.01 for *A. salmonicida* bacterin-injected fish). Boxes and whiskers indicate the 25th-75th and 10th-90th percentiles, respectively, with the median value marked by a horizontal line. Sample sizes are n=7 for the PBS-injected groups, and n=8 and 9 for the *A. salmonicida* bacterin-injected normoxia- and hypoxia-acclimated group, respectively.

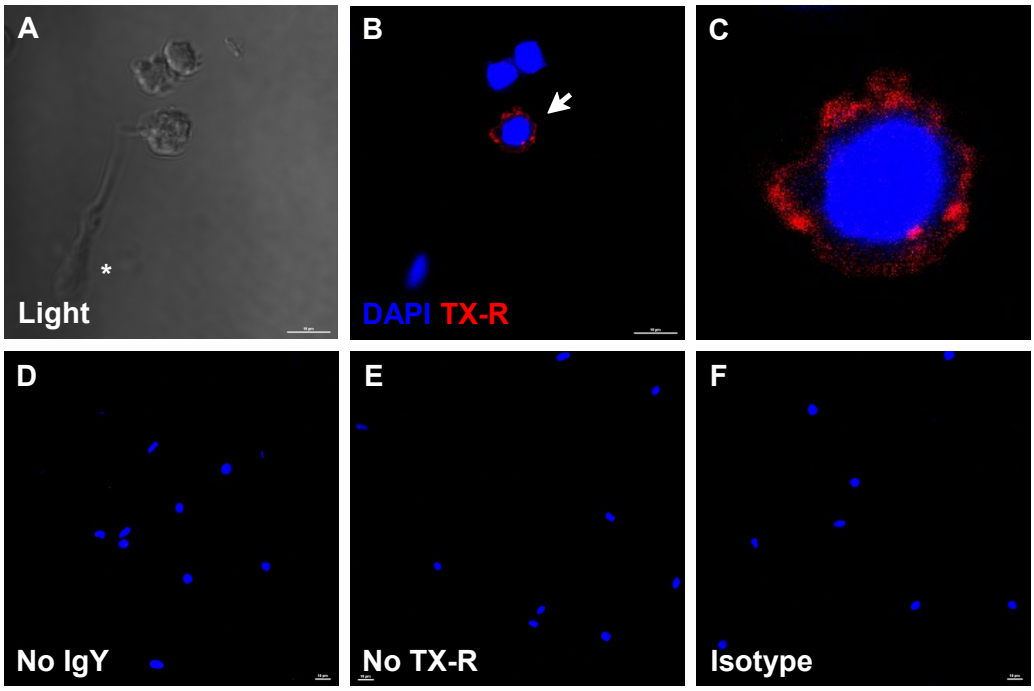

Figure 4.8. Fluorescent confocal microscopy images of IgM$^+$ leukocytes in sablefish blood. (**A**) Brightfield photo that corresponds with the fluorescent microscopy image shown in panel B. Cell indicated with asterisk (*) is a typical thrombocyte given the fusiform/spindle shape (Haugland *et al.*, 2012). (**B**) Immunostaining of WBCs using biotinylated chicken anti-sablefish-IgM IgY and avidin-Texas Red (TX-R), revealing an IgM$^+$ cell with red fluorescence (white arrow). Cell nuclei are stained blue with DAPI. (**C**) Close-up view of the IgM$^+$ cell from panel B showing the cell's general morphology and the immunolocalization of IgM (i.e., round cell shape, IgM appears to be mostly concentrated at the membrane). Panels D-F are images taken from negative controls: WBCs prepared without chicken anti-sablefish-IgM IgY (**D**); without TX-R (**E**); with chicken isotype IgY (**F**). No red fluorescence was detected in these samples. For more images of IgM$^+$ cells along with negative controls, see Figure S4.11. Scale bar=10 µm.

(%) of the main blood cell populations in sablefish at 10 wpi (Figure 4.6B-E). Regardless of injection and acclimation group, lymphocytes and thrombocytes (P1), monocytes and granulocytes (P2), immature RBCs (P3) and mature RBCs (rest) represented 1.53±0.08%, 0.10±0.01%, 2.10±0.09% and 96.27±0.12% of the blood cells, respectively. The relative abundance of all WBCs (P1+2) was 1.63±0.08%.

In contrast, flow cytometry analysis of isolated WBCs from sablefish at 10 wpi showed that there was a significant increase ($P<0.001$) in the relative abundance of IgM^+ cells in the hypoxia-acclimated groups as compared to the normoxia-acclimated groups (41.95±2.68% *vs.* 26.12±1.61%, respectively; Figure 4.7C). Interestingly, bacterin injection had no significant effect ($P>0.05$) on the percentage of IgM^+ cells in the blood at this time point. Based on their position on the FSC *vs.* SSC dot-plots, peripheral IgM^+ cells generally had a low internal complexity/granularity, but were more variable in size (Figure 4.7A). Immunostaining and confocal microscopy were used to visualize IgM^+ cells amongst the WBCs, and to allow for a general description of the cell morphology and immunolocalization of IgM (Figures 4.8 and S4.11). The IgM^+ cells typically had a round cell shape, with a high nucleus-to-cytoplasm ratio relative to other WBCs, and IgM fluorescence appeared to be mostly concentrated at the cell membrane (Figures 4.8A-C and S4.11A-C). This IgM^+ cell morphology is consistent with that of B lymphocytes and typical of mature, naïve B cells (Kaattari *et al.*, 2009).

4.5. Discussion

The worldwide spread of hypoxic zones in marine environments driven by climate warming and eutrophication (Gilbert *et al.*, 2005, Kemp *et al.*, 2005, Diaz *et al.*, 2008,

Rabalais *et al.*, 2010, Breitburg *et al.*, 2018, Claret *et al.*, 2018, Sampaio *et al.*, 2021) may have negative implications for the immune function of fish, and their capacity to defend against pathogen infection/disease. This is because exposure to acute aquatic hypoxia often has an immunosuppressive effect, and has been shown to increase disease susceptibility under experimental conditions (Walters *et al.*, 1980, Boleza *et al.*, 2001, Yada *et al.*, 2002, Evans *et al.*, 2003, Welker *et al.*, 2007, Bowden, 2008, Rodríguez *et al.*, 2016, Abdel-Tawwab *et al.*, 2019). However, research findings are conflicting about whether long-term exposure to low O_2 levels affects fish immunity (Cecchini *et al.*, 2002, Andersen *et al.*, 2010, Niklasson *et al.*, 2011, Kvamme *et al.*, 2013, Abdel-Tawwab *et al.*, 2014, 2015, Gallage *et al.*, 2016, Gallage *et al.*, 2017, Magnoni *et al.*, 2019, Martínez *et al.*, 2020, Krasnov *et al.*, 2021, Schäfer *et al.*, 2021). To address this issue, I subjected sablefish to chronic moderate hypoxia (5 months at ~40% air sat.), and then examined its effects on innate and adaptive immunity, including baseline function and the response to a formalin-killed *A. salmonicida* bacterin. I show that chronic hypoxia: (i) does not influence baseline immune function in sablefish; and likewise, (ii) does not substantially impact this species' innate immune response to inactivated *A. salmonicida*; whereas it (iii) modifies/impairs the adaptive immune response. Collectively, these results provide novel insights into the immunomodulatory nature of chronic hypoxia in sablefish/fishes. They show that the innate immune system of sablefish is robust despite prolonged exposure to unfavourable environmental conditions, but also that declining O_2 levels in coastal ecosystems may have deleterious consequences on the capacity of fishes to mount an appropriate adaptive immune response to bacterial pathogens.

4.5.1. Effect of Chronic Hypoxia on Innate Immunity

In this study, chronic moderate hypoxia did not influence baseline respiratory burst, haemolytic activity of the alternative complement pathway, and lysozyme concentration in the blood, or the expression of a panel of 16 known bacteria-responsive genes in the spleen of sablefish (Figures 4.2-4.3). Furthermore, these innate immune parameters were generally elevated after bacterin injection with only a few, minor (<2-fold change in the case of gene expression), differences in the responses between the normoxia- and hypoxia-acclimated sablefish.

In Nile tilapia (Gallage *et al.*, 2016), rainbow trout (Magnoni *et al.*, 2019) and Atlantic salmon (Zanuzzo *et al.*, 2020), chronic moderate hypoxia also did not affect alternative complement activity and/or lysozyme concentration in plasma, including baseline levels and after vaccine injection, and this is consistent with the findings in this study (Figure 4.2B-C). Further, in this study, long-term hypoxia did not affect the baseline respiratory burst activity of blood leukocytes or following the injection of formalin-killed *A. salmonicida* (Figure 4.2A). In contrast, Abdel-Tawwab *et al.* (2014, 2015) found that chronic hypoxia reduced both baseline blood respiratory burst activity and lysozyme concentration in Nile tilapia, and that the effect was dependent on the degree of hypoxia used. Abdel-Tawwab *et al.* used O_2 levels down to ~1 mg L^{-1} (~13% air sat.), and this may explain the disparate results. Interestingly, hypoxia-acclimated sablefish had a 2-fold higher leukocyte respiratory burst after PBS injection, and a similar result has been reported for Atlantic salmon (Zanuzzo *et al.*, 2020). Possibly, these fish are more sensitive/responsive to the acute stress associated with sham-injection when under hypoxic conditions, and may have experienced slightly higher post-handling stress

hormone levels, some of which (i.e., adrenaline and ACTH) are known to stimulate respiratory burst in other fish species (Tort, 2011). Finally, there was a small (25%), but significant, decline in the alternative complement activity in hypoxia-acclimated sablefish after *A. salmonicida* bacterin injection (compared to the sham-injection control), whereas complement activity did not respond to the bacterin in the normoxia-acclimated sablefish (Figure 4.2B). This effect has not been reported in the literature before, and its biological relevance is unclear.

The lack of an effect of long-term hypoxia on the constitutive expression of antibacterial response genes in sablefish (Figure 4.3) agrees with the findings (Zanuzzo *et al.*, 2020) for Atlantic salmon. However, chronic hypoxia significantly altered the baseline transcript expression of immune-relevant genes in coho salmon (Martínez *et al.*, 2020), including two genes (*il1b* and *tnfa*) which were also investigated in the current study. This disparity may be related to a difference in the species' hypoxia tolerance [the sablefish is very hypoxia-tolerant (Leeuwis *et al.*, 2019)], and the salmon in Zanuzzo *et al.* (2020) were only exposed to 70% air sat., whereas an increase in the constitutive expression of these genes in Martínez *et al.* (2020) was reported in the head kidney and spleen at lower O_2 levels (25-60% air sat.). After injection with the bacterin (i.e., at 24 hpi), transcript expression differed between the acclimation groups for 3 of the 16 studied genes in sablefish (i.e., mRNA levels for *ccl19*, *stat1a* and *tlr5b* were 1.7 and 1.4-fold higher, and 0.6-fold lower, respectively, in the hypoxia- *vs.* normoxia-acclimated fish) (Figure 4.3D, M, P). Because of the relatively small magnitude of these changes (<2-fold) compared to the overall transcriptional response to the bacterin, it is uncertain whether these would have any downstream physiological consequences. However, this would be

an interesting avenue for future research, in particular with regard to *ccl19* and *tlr5b* which showed the largest fold change (>1.5) among the three genes dysregulated by chronic hypoxia. The increased *stat1a* expression is also notable, because this signal transducer/transcription activator controls the expression of several genes in the IFNγ-induced JAK-STAT pathway (Murphy *et al.*, 2017b), and a comparable shift in the transcriptional response of *stat1b* was seen in hypoxia-acclimated Atlantic salmon injected with a polyvalent bacterial/viral vaccine (Zanuzzo *et al.*, 2020). Although *stat1* is characteristically involved in the antiviral response and has mostly been studied in this context [e.g., see Hori *et al.* (2012), Eslamloo *et al.* (2017), Zhang *et al.* (2017)], it has recently also been shown to be responsive to bacterial (*Edwardsiella ictaluri* and *Aeromonas hydrophila*) infection in channel catfish and Japanese eel (*Anguilla japonica*) (Jin *et al.*, 2018, Wang *et al.*, 2019). It appears that *stat1a* is similarly implicated in the antibacterial defence in sablefish, and further research could explore the modulation of its expression by hypoxia.

Nevertheless, this study shows that chronic moderate hypoxia does not substantially impair the sablefish's basal innate immune function and capacity to mount a robust response to pathogenic bacterial antigens. This result is in agreement with findings on a number of other teleost species (Gallage *et al.*, 2016, Magnoni *et al.*, 2019, Zanuzzo *et al.*, 2020, Krasnov *et al.*, 2021, Schäfer *et al.*, 2021), but in contrast to the frequently reported suppressive effect of acute hypoxia on fish innate immunity. Acute hypoxic exposure typically induces a stress response characterised by the release of cortisol, which mediates immune inhibition (Yada *et al.*, 2002, Welker *et al.*, 2007). In sablefish, cortisol levels after hypoxic acclimation (6 months at 40% air sat. followed by 3-4 weeks

at 20% air sat.) were not significantly elevated (compared to fish held at 100% air sat.) (Table S4.4), which may also apply to the sablefish in the present experiment. It is possible that, when a stressor (such as aquatic hypoxia) persists and becomes chronic, the stress response may be diminished (Bowden, 2008), and any endocrine suppression of innate immune function may dissipate. The finding that sablefish can mount a robust innate immune response at 40% air saturation, despite the constraints placed on this species' metabolic capacity at this O_2 level (Leeuwis *et al.*, 2021), may not be surprising as the majority of data published to date indicate that the innate immune response is not an energetically costly endeavour for teleosts. Studies in salmonids [e.g., Zanuzzo *et al.* (2015a), Zhang *et al.* (2019), Polinski *et al.* (2021)] have been unable to detect an increase in standard/routine metabolic rate over hours to months following bacterial antigen injection or viral infection. Even the 30% increase in routine metabolic rate in 27.5°C (but not 22°C) acclimated zebrafish (*Danio rerio*) reported by Bennoit *et al.* (2020) after heat-killed *Vibrio anguillarum* injection only represents ~17.5% of their aerobic scope (Lucas *et al.*, 1992). This high energetic efficiency of the innate immune defences would be advantageous, as teleosts may rely more heavily on innate, than adaptive, immunity to combat infectious agents (Tort *et al.*, 2003, Semple *et al.*, 2020). Nevertheless, to fully understand the impact of long-term aquatic hypoxia on the sablefish's immune function, the effects of O_2 limitation on adaptive immunity must also be considered (see below).

4.5.2. Effect of Chronic Hypoxia on Adaptive Immunity

Although chronic hypoxia did not affect initial total serum IgM levels in sablefish,

it completely inhibited the rise in IgM levels at 10 wpi with the *A. salmonicida* bacterin (Figure 4.5B). In addition, it increased the relative abundance of peripheral IgM^+ cells (from 26% to 42% of WBCs) at this time point (Figure 4.7C), while there was no change in the relative total WBC count (Figure 4.6B-C).

Under normoxic conditions, initial and peak IgM levels after bacterin injection in sablefish serum were estimated at 9.6-10.7 and 15.7 mg mL^{-1}, respectively (Figure 4.5B). These values are similar to those measured in other teleosts before and after vaccination [e.g., Japanese flounder (*Paralichthys olivaceus*): 3.3 and 7.8 mg mL^{-1} (Tang *et al.*, 2010); rainbow trout: 1.5 and 14 mg mL^{-1} (Chettri *et al.*, 2019); sea bass (*Dicentrarchus labrax*): 5-15 and 10-40 mg mL^{-1} (Coeurdacier *et al.*, 1997)]. Although, lower IgM levels have been reported in the fish literature [e.g., 0.8 and 1.6 mg mL^{-1} in healthy and infected Atlantic salmon (Magnadottir *et al.*, 1992)], including for the sablefish [i.e., 0.1 and 1.7 mg mL^{-1} before and after vaccination (Vasquez *et al.*, 2020)]. The discrepancy in serum IgM levels between the latter and present study may be attributable to differences in experimental design (e.g., inclusion of a boost) and fish size/age (125 and 600-750 g, respectively). Serum IgM concentrations are known to increase with repeated antigen exposure (Arkoosh *et al.*, 1991, Soto-Davila *et al.*, 2020) and size/age in a variety of teleosts [e.g., see Matsubara *et al.* (1985), Nakanishi (1986), Klesius (1990), Hirotoshi *et al.* (1991), Sánchez *et al.* (1993) and Hordvik (2015)].

Chronic hypoxia has been shown to reduce the antibody titre response to vaccination in sea bass (Cecchini *et al.*, 2002) and Nile tilapia (Gallage *et al.*, 2016, Gallage *et al.*, 2017), which is in line with the results presented here for sablefish (Figure 4.5B). Prior research by Vasquez *et al.* (2020) on sablefish injected with different *A.*

salmonicida vaccines indicated that peak IgM levels at 6-8 weeks post-vaccination correlate positively with immunoprotection (i.e., survival rate after *A. salmonicida* infection). Therefore, the impaired ability to raise antibody levels in sablefish experiencing continuous hypoxia may ultimately result in a higher disease susceptibility. IgM serves an essential function with regard to adaptive immunity in fishes by enhancing antigen-specific opsonisation and phagocytosis, and like in other vertebrates, this allows for a stronger and more efficient immune response to repeated pathogen exposures than is achievable through innate immunity (Ye *et al.*, 2013, Mashoof *et al.*, 2016). In an aquaculture setting, the efficacy of vaccines also relies on a well-functioning adaptive immune system (Semple *et al.*, 2020, Soto-Dávila *et al.*, 2020). Yet, immunological memory (incl. B cell proliferation, specific antibody production, etc.) may be an energy-intensive mechanism for fish, because of their poikilothermy which limits the energy available for immunocompetence (Tort *et al.*, 2003, Soto-Dávila *et al.*, 2020). The outcomes of this study appear to corroborate this high energetic cost of acquired immunity, because chronic hypoxia specifically constrained the adaptive immune response in sablefish (whereas innate immunity was largely unaffected). Schäfer *et al*. (2021) recently found a similar pattern/dichotomy in the effect of chronic hypoxia (28 days at 40% air sat.) on the immune status of pikeperch (*Sander lucioperca*), which maintained a robust innate immune response against inactivated *Aeromonas hydrophila*, while the adaptive immune response appeared to be more sensitive to the environmental stressor (i.e., the influx of lymphocytes to the peritoneal cavity was impaired). Bowden (2008) suggested that acute environmental stressors mostly affect innate immunity in fish, while chronic stressors affect adaptive immunity, and the present findings, along with the

current literature, indicate that this conceptual framework is potentially applicable to aquatic hypoxia.

The increased percentage of IgM^+ cells in the blood of hypoxia-acclimated sablefish is more difficult to interpret (Figure 4.7C). A key mediator of adaptive immunity is the B lymphocyte, and among teleosts, the most common B cell lineage expresses IgM at the cell membrane (mIgM) (Magadan *et al.*, 2015, Nakanishi *et al.*, 2018). Because B lymphocytes are the only cell type that produce and display Ig on their cell surface (Miller *et al.*, 1998, Mashoof *et al.*, 2016, Soto-Dávila *et al.*, 2020), I used mIgM as a B cell marker to test whether their abundance in the blood had declined as a result of chronic hypoxia; which could have provided a mechanistic explanation for the impaired IgM titre response. Interestingly, flow cytometry analysis indicated that there was a higher relative abundance of peripheral IgM^+ cells after hypoxia acclimation (Figure 4.7C), which is opposite to the hypothesized trend and appears contradictory to the reported serum IgM results. If this increase in relative abundance represents a rise in the absolute number of B lymphocytes (i.e., a type of lymphocytosis), then this suggests that B cells have a markedly lower antibody secretion activity under hypoxic conditions. This hypothesis could be investigated using qPCR, enzyme linked immunospot (ELISPOT) or ELISA assays, that quantify transcript levels or the secretion of antibodies by IgM^+ cells, respectively (Murphy *et al.*, 2017a). Lymphocytosis (an increase in the number/proportion of peripheral lymphocytes) has also been observed in rainbow trout exposed to acute hypoxia (Roh *et al.*, 2020), although there was no change in the blood lymphocyte percentage in channel catfish and pikeperch during acute/chronic hypoxia (Scott *et al.*, 1981, Schäfer *et al.*, 2021).

Alternatively, it is possible that chronic hypoxia indirectly impacted the B cells by its effects on T lymphocytes. As in mammals, antigen-specific antibody production by B cells in fishes appears to require the cooperation of T-helper ($CD4^+$) cells through major histocompatibility complex (MHC) II interaction (Magadan *et al.*, 2015, Nakanishi *et al.*, 2015, Ashfaq *et al.*, 2019). This means that, if the function and/or abundance of T-helper cells was diminished due to hypoxia, then this would also negatively affect B cell-mediated IgM production. Furthermore, a decline in the peripheral T lymphocyte count may be interpreted as a reduction in the IgM^- cell percentage [this is because T cells can be defined/their presence inferred by the lack of soluble/membrane IgM (Miller *et al.*, 1998, Nakanishi *et al.*, 2015)], and thus, perceived as an increased IgM^+ cell percentage without any actual change in the absolute number of B cells. Whether such a mechanism underlies the disparate effects of chronic hypoxia on IgM levels *vs*. relative IgM^+ cell abundance should be investigated, although the detection of an $IgM^-/CD4^+$ leukocyte subset would require the development and validation of an anti-CD4 antibody for sablefish. Additionally, it would be worthwhile to assess changes in IgM^+ cell abundance in other immune tissues (e.g., spleen, head kidney) following hypoxia acclimation and bacterin injection. In this study, the relative abundance of IgM^+ cells was measured in the blood, as typically, it is the dominant reservoir for this cell type in teleosts (because of the high ratio of $mIgM^+$ to total leukocytes, combined with the fish's large blood volume) (Kaattari *et al.*, 2009). However, both the spleen and head kidney have been proposed as sites of activated B cell proliferation and/or differentiation into plasma cells (Bromage *et al.*, 2004, Kaattari *et al.*, 2009, Castro *et al.*, 2013, Shibasaki *et al.*, 2019), and thus, an effect of *A. salmonicida* antigen exposure on IgM^+ cell abundance may be detected there

in particular. In sablefish, bacterin injection had no effect on the relative number of IgM^+ cells, and under normoxic conditions, they represented 26% of WBCs (Figure 4.7C). This is within the range reported for other fish species [e.g., 13% in Atlantic cod (*Gadus morhua*) (Rønneseth *et al.*, 2007), 42% in lumpfish (Rønneseth *et al.*, 2015), 62% in Atlantic salmon (Øverland *et al.*, 2010)] and exactly the same as reported for sea bass (Yang *et al.*, 2018).

4.5.3. Immune Gene Paralogues/Isoforms in Sablefish

This study is the first to assess the transcriptional response of immune-related genes in the sablefish to formalin-killed *A. salmonicida* injection using qPCR analyses with *de novo* designed primers (Table 4.2). These genes (incl. *cox1*, *cox2*, *ccl19*, *hamp*, *ifng*, *il1b*, *il8*, *stat1*, *tlr5* and *tnfa*) were selected based on previous research in other teleosts showing that they are upregulated in immune-relevant tissues or cells during *A. salmonicida* infection and/or after exposure to inactivated *A. salmonicida* preparations [e.g., see Ewart *et al.* (2007), Lin *et al.* (2007), Feng *et al.* (2009), Rebl *et al.* (2014), Zanuzzo *et al.* (2015b), Soto-Dávila *et al.* (2019)]. In sablefish, these antibacterial genes were found to be upregulated in the same manner, although it depends on the gene paralogue/isoform being considered (Figure 4.3). Based on the BLASTx searches and the alignments of sequences that have been identified for *cox1*, *hamp*, *il1b*, *il8*, *stat1* and *tlr5* in the current study (Table S4.1, Figures S4.2-S4.7), there appear to be two paralogues/isoforms for each of these immune genes that can have distinct expression profiles following injection with the bacterin. This was revealed using paralogue/isoform-specific qPCR primers (Table 4.2). For example: (i) *il8b* appeared to

be unresponsive to the bacterin, in contrast to *il8a* whose transcript levels increased by 3.3-fold (compared to the PBS-injected fish) (Figure 4.3I-J); and (ii) both *hampa* and *hampb* were upregulated, albeit to different extents (42 and 18-fold, respectively) (Figure 4.3E-F). Differences in the inducibility of paralogue expression by formalin-killed *A. salmonicida* have been observed for other immune-related genes [e.g., for cathelicidin and TNFα in steelhead trout (Zanuzzo *et al.*, 2015b)], and thus, the present results are not surprising. On the other hand, in the case of other gene paralogues in sablefish, such as *cox1a* and *cox1b*, the transcriptional response to the bacterin was very similar (both were unresponsive; Figure 4.3A-B).

The presence of multiple copies of immune genes is common in teleosts, and this is likely the result of a whole genome duplication that purportedly occurred in the early ray-finned fish lineage (Actinopterygii) around 320-350 million years ago (Amores *et al.*, 1998, Christoffels *et al.*, 2004, Hoegg *et al.*, 2004, Crow *et al.*, 2005, Volff, 2005, Koop *et al.*, 2008, Nakanishi *et al.*, 2018). Gene duplication is a process that can drive evolutionary changes in gene function among the duplicates, including sub- and neofunctionalization (Ohno, 1970, Force *et al.*, 1999, Steinke *et al.*, 2006, Conant *et al.*, 2008, Lien *et al.*, 2016). The present study did not involve the complete characterization of the aforementioned immune gene paralogues/isoforms in sablefish. However, one could speculate about their molecular evolution/function based on their differential expression in response to formalin-killed *A. salmonicida*. At 24 hours post-injection with the bacterin, and regardless of acclimation, the magnitude of the increase in transcript levels for *hampb* was much lower compared to the increase in *hampa* levels (18 *vs.* 42-fold, compared to the time-matched PBS control), and the same applies to the increase

in *tlr5a* and *tlr5b* levels (2.5 *vs.* 35-fold, compared to the time-matched PBS control) (Figure 4.3E-F, O-P). This suggests that *hampa* and *tlr5b* serve a more important/dominant role in the spleen antibacterial response. The complete CDS for *hampa* and *hampb*, and the partial CDS for *tlr5a* and *tlr5b*, are 70% and 66% identical, respectively, at the nucleotide level (Figures S4.3 and S4.7). Although it is possible that the peak in *hampb* and *tlr5a* expression was at a different time point or in a different immune tissue, this could be a case of subfunctionalization [i.e., partitioning of gene function caused by duplicate redundancy (Force *et al.*, 1999, Conant *et al.*, 2008)].

The two hepcidin-like gene copies in sablefish with differential expression are also noteworthy because in certain fish species [e.g., European sea bass, *Dicentrarchus labrax* (Neves *et al.*, 2015, Neves *et al.*, 2017); large yellow croaker, *Larimichthys crocea* (Mu *et al.*, 2018)] two functionally different Hamp types have been described (Hamp1: mostly involved in iron metabolism; Hamp2: predominantly has a antimicrobial role). Future studies could characterize the phylogeny, expression, regulation and biological function of sablefish *hampa* and *hampb*, to determine their relationship with Hamp1 and Hamp2 in other Acanthopterygians, and whether a similar kind of subfunctionalization has occurred. Further, the lack of an increase in transcription of *il8b* in the spleen in response to *A. salmonicida* bacterin antigens, suggests that this paralogue/isoform has potentially lost its original/ancestral function, while it may have been conserved in *il8a* (Figure 4.3I-J). The complete nucleotide CDS for sablefish *il8a* and *il8b* are 50% identical (Figure S4.5). Further investigation is required to test this idea of IL-8 paralogue divergence in sablefish (e.g., by measuring the inducibility of *il8a* and *il8b* expression with various pathogens or pathogen-like antigens).

Finally, the downregulation of *il1b-like* after bacterin injection (Figure 4.3H) is particularly interesting given that IL-1β, a pro-inflammatory cytokine, is normally strongly upregulated post-stimulation with bacteria or LPS (lipopolysaccharides) (Secombes *et al.*, 2011) and such a response is also observed for sablefish *il1b* (Figure 4.3G). A downward trend in *il1b* expression has previously been reported in the head kidney from rainbow trout infected with *A. salmonicida*, however, transcript levels did increase later during infection (Mulder *et al.*, 2007). There are other teleosts, such as the rainbow trout (Pleguezuelos *et al.*, 2000, Wang *et al.*, 2004) and common carp (*Cyprinus carpio*) (Engelsma *et al.*, 2003), in which more than one gene for *il1b* is also present; although it is important to note that these species are polyploid (pseudotetraploid and tetraploid, respectively) whereas the sablefish is diploid (Phillips *et al.*, 2013, Rondeau *et al.*, 2013). In sablefish, the complete CDS for *il1b* and *il1b-like* are 49% identical at the nucleotide level (Figure S4.4), and *il1b-like* transcriptome sequences translated to protein are 47-59% similar to *il1b* amino acid sequences from other Perciform fishes (Table S4.1). These percentages are the lowest amongst the paralogues/isoforms identified in this study. Given this, and that the *il1b-like* response is contrary to that typical for *il1b*, it is likely that *il1b-like* represents a gene that underwent neofunctionalization (Conant *et al.*, 2008). This topic could be further investigated by, for example, phylogenetic tree analysis [using sequences from a wide range of vertebrates, e.g., as in Havird *et al.* (2008) and Hall *et al.* (2015)] to gain insights into the timing of the gene duplication that gave rise to *il1b* and *il1b-like*.

4.5.4. Concluding Remarks

Given that there is limited, and often conflicting, published data on the effects of long-term O_2 limitation on fish immunity (Makrinos *et al.*, 2016), the findings of the present study enhance our knowledge and ability to predict the impact of worsening occurrences of aquatic hypoxia (Breitburg *et al.*, 2018, Sampaio *et al.*, 2021) on the health and disease susceptibility of fishes. The *a priori* hypothesis that chronic hypoxia has an overall immunosuppressive effect on sablefish immunity was only partly supported by the results, as it had a very limited impact on innate immunity, whereas it impaired the adaptive immune response to bacterial antigens (although with the current data, the underlying mechanism cannot be elucidated). While the various components of the immune system/response are similar among teleosts, they can also vary between lineages, and from species to species (Nakanishi *et al.*, 2018), and thus, their sensitivity to hypoxia may also be species-specific. This research on sablefish, an economically important (Goetz *et al.*, 2021) and hypoxia-tolerant (Leeuwis *et al.*, 2019) species, has: (i) expanded the relatively small number of fish species in which the effect of long-term hypoxia on immune function has been investigated; and (ii) provided thorough and comprehensive information on how both the innate and adaptive branches of immunity are impacted by this environmental challenge.

Nonetheless, this research has some limitations and raises several key questions that could be addressed in future studies. For instance, the sablefish's innate immune response to the bacterin was assessed at a single time point (24 hours) post-injection, which was carefully chosen to represent the maximum response for the majority of the measured parameters based on prior studies in other fish species exposed to

live/inactivated *A. salmonicida* [e.g., in zebrafish, Lin *et al.* (2007); in Atlantic cod, Feng *et al.* (2009); in steelhead trout, Zanuzzo *et al.* (2015b); and in Atlantic salmon, Zanuzzo *et al.* (2020)]. However, more sampling time points would allow for any potential effect of chronic hypoxia on the timing of the innate immune response to be elucidated. Furthermore, while the sablefish's capacity for, and limitations in, mounting an immune response to inactivated *A. salmonicida* under hypoxic conditions have now been characterised, these results cannot be immediately extrapolated to predict the susceptibility to furunculosis (or other diseases). No live pathogen studies were conducted as a part of this thesis, and live *A. salmonicida* can use virulence factors to undermine the host's immune defences during infection (Ellis, 1999, Frey *et al.*, 2016, Soto-Dávila *et al.*, 2019). Some pathogens (e.g., *Shewanella putrefaciens*, a facultative anaerobic bacterium) can also become more abundant in the water under hypoxic conditions, which may promote disease outbreaks (e.g., in European eel *Anguilla anguilla*) (Esteve *et al.*, 2017). These studies, along with the present findings, highlight that the health status of fishes is a dynamic trait determined by complex interactions between the host, pathogens/parasites, and the environment. Our results demonstrate that hypoxic conditions will likely play an important role in shaping the disease susceptibility and health of fishes experiencing global climate change-related warming and coastal eutrophication.

4.6. References

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Table S4.1. Summary of BLASTx search results for the identification of sablefish transcripts included in the qPCR studies. For *cox2*, a contig was constructed using GAJJ01046020 and GAJJ01047049, and thus, BLASTx results for both sequences are shown.

GenBank accession no. or Trinity ID	**Gene name(s) (*gene symbol*) (species name and accession no. of best BLASTx hit)**	**% ID (align)**	**E-value**
Genes of interest			
GAJJ01016042	prostaglandin G/H synthase 1-like isoform X1 (alias cyclooxygenase 1 a) (*cox1a*) (*Sander lucioperca*, XP_031161696)	88 (514/583)	0
GAJJ01016043	prostaglandin G/H synthase 1-like (alias cyclooxygenase 1 b) (*cox1b*) (*Anarrhichthys ocellatus*, XP_031716110)	91 (547/600)	0
GAJJ01046020	prostaglandin G/H synthase 2 (alias cyclooxygenase 2) (*cox2*) (*Cottoperca gobio*, XP_029285061)	95 (170/179)	5.00E-124
GAJJ01047049	cyclooxygenase-2, partial (*cox2*) (*Acanthopagrus schlegelii*, QED90009)	95 (162/170)	2.00E-117
BT082212	C-C motif chemokine 19-like (*ccl19*) (*Monopterus albus*, XP_020450731)	68 (73/108)	7.00E-31
GAJJ01029649	hepcidin-like (*hampa*) (*Cottoperca gobio*, XP_029306823)	88 (76/86)	3E-49
GAJJ01004264	hepcidin-1 (*hampb*) (*Epinephelus lanceolatus*, XP_033495956)	93 (65/70)	6.00E-38

Table S4.1. Summary of BLASTx search results for the identification of sablefish transcripts included in the qPCR studies (continued). For *il1b* and *il1b-like*, contigs were constructed using TR28839_c0_g1_i1 with TR28839_c0_g2_i5, and TR21996_c0_g1_i1 with TR21996_c0_g2_i3, respectively, and thus, BLASTx results for each sequence are shown. Transcriptome sequences with corresponding Trinity IDs are provided in Figure S4.1.

GenBank accession no. or Trinity ID	**Gene name(s) (*gene symbol*) (species name and accession no. of best BLASTx hit)**	**% ID (align)**	**E-value**
Genes of interest			
TR28839_c0_g1_i1	interleukin-1 beta-like (*il1b*) (*Cyclopterus lumpus*, XP_034398416)	61 (78/127)	7.00E-47
TR28839_c0_g2_i5	interleukin-1 beta (*il1b*) (*Anarrhichthys ocellatus*, XP_031726439)	84 (165/196)	1.00E-114
TR21996_c0_g1_i1	interleukin-1 beta (*il1b-like*) (*Trematomus bernacchii*, XP_033978886)	47 (34/73)	3.00E-07
TR21996_c0_g2_i3	interleukin-1 beta-like (*il1b-like*) (*Anarrhichthys ocellatus*, XP_031730975)	59 (136/230)	5.00E-82
BT082167	interleukin-8 (*il8a*) (*Dicentrarchus labrax*, CAM32186)	84 (83/99)	1.00E-53
JO666101	interleukin 8 isoform 1 (*il8b*) (*Oplegnathus fasciatus*, AGR27883)	89 (93/104)	7.00E-59
TR26914_c0_g1_i1	interferon gamma-like isoform X1 (*ifng*) (*Cyclopterus lumpus*, XP_034382228)	70 (130/185)	2.00E-81
TR33543_c1_g1_i14	signal transducer and activator of transcription 1-alpha/beta isoform X2 (*stat1a*) (*Anarrhichthys ocellatus*, XP_031707651)	96 (713/740)	0

Table S4.1. Summary of BLASTx search results for the identification of sablefish transcripts included in the qPCR studies (continued). For *pabpc1a*, a contig was constructed using GAJJ01013600 and JO671032, and thus, BLASTx results for both sequences are shown. Transcriptome sequences with corresponding Trinity IDs are provided in Figure S4.1.

GenBank accession no. or Trinity ID	**Gene name(s) (*gene symbol*) (species name and accession no. of best BLASTx hit)**	**% ID (align)**	**E-value**
Genes of interest			
TR36335_c1_g1_i1	signal transducer and activator of transcription 1-alpha/beta-like (*stat1b*) (*Anarrhichthys ocellatus*, XP_031695063)	82 (498/607)	0
BT083097	lipopolysaccharide-induced tumor necrosis factor-alpha factor homolog isoform X2 (*tnfaa*) (*Gasterosteus aculeatus aculeatus*, XP_040047401)	80 (82/102)	4.00E-37
TR29278_c0_g1_i1	toll-like receptor 5 (*tlr5a*) (*Cyclopterus lumpus*, XP_034383273)	82 (644/782)	0
TR29636_c0_g1_i2	toll-like receptor 5 (*tlr5b*) (*Anarrhichthys ocellatus*, XP_031717546)	88 (563/640)	0
ECs			
GAJJ01033263	elongation factor 1-alpha isoform X1 (*ef1a2*) (*Toxotes jaculatrix*, XP_040921216)	95 (434/457)	0
TR33310_c0_g1_i8	elongation factor 1-alpha (*ef1a4*) (*Etheostoma cragini*, XP_034748252)	97 (433/448)	0
JO688912	eukaryotic translation initiation factor 3 subunit D isoform X2 (*etif3d*) (*Sander lucioperca*, XP_031174486)	99 (524/530)	0
GAJJ01013600	polyadenylate-binding protein 1b isoform X1 (*pabpc1a*) (*Sander lucioperca*, XP_031152059)	97 (505/522)	0

Table S4.1. Summary of BLASTx search results for the identification of sablefish transcripts included in the qPCR studies (continued).

GenBank accession no. or Trinity ID	Gene name(s) (*gene symbol*) (species name and accession no. of best BLASTx hit)	% ID (align)	E-value
ECs			
JO671032	poly A binding protein, cytoplasmic 1 b, partial (*pabpc1a*) (*Epinephelus coioides*, AEG78330)	97 (129/133)	7E-71
GAJJ01026238	polyadenylate-binding protein 1A (*pabpc1b*) (*Cyclopterus lumpus*, XP_034384844)	99 (632/635)	0
BT082131	60S ribosomal protein L32 (*rpl32*) (*Etheostoma cragini*, XP_034732374)	99 (134/135)	8.00E-84

Table S4.2. Endogenous control (EC) testing and selection. For each of the six candidate EC genes, the C_T values (means±s.e.m.) were measured in all six treatment groups (each represented by a subset of four samples), and geNorm M values were determined. Based on their lowest M values, *ef1a4* and *pabpc1b* were the most stably expressed transcripts, and were selected as EC genes for the experimental qPCR studies. See Section 4.3.6.5 and Table 4.2 for further details.

C_T value	***ef1a2***	***ef1a4***	***etif3d***	***pabpc1a***	***pabpc1b***	***rpl32***
0 hpi						
Normoxia-acclimated	30.97±0.19	18.89±0.19	22.42±0.16	25.66±0.23	20.92±0.23	18.77±0.23
Hypoxia-acclimated	31.24±0.19	18.90±0.15	22.42±0.13	25.79±0.10	20.98±0.19	18.82±0.16
24 hpi						
Normoxia-acclimated						
PBS-injected	31.18±0.09	18.68±0.14	22.18±0.16	25.54±0.20	20.76±0.13	18.76±0.19
A. salmonicida bacterin-injected	30.88±0.10	18.65±0.09	22.06±0.14	25.62±0.19	20.72±0.11	18.84±0.14
Hypoxia-acclimated						
PBS-injected	31.12±0.16	18.73±0.14	22.22±0.13	25.72±0.07	20.85±0.15	18.96±0.14
A. salmonicida bacterin-injected	30.94±0.08	18.69±0.18	21.94±0.14	25.24±0.15	20.62±0.16	18.84±0.14
GeNorm M value	**0.262**	**0.108**	**0.115**	**0.199**	**0.105**	**0.152**

Table S4.3. Cycle threshold (C_T) values for the TOIs and ECs measured in the experimental qPCR assays. For each qPCR study, three TOIs and two ECs (*pabpc1b* and *ef1a4*) were run on each linked plate (there were six studies in total). The six treatment groups include normoxia- and hypoxia-acclimated fish sampled before (initial) and 24 hpi with PBS or formalin-killed *A. salmonicida* (*n*=7-8/group). Values are means±s.e.m.

TOI / EC C_T	**Initial**		**24 hpi**			
	Normoxia	Hypoxia	Normoxia PBS	Normoxia *A. sal.*	Hypoxia PBS	Hypoxia *A. sal.*
Study 1						
cox2	33.85±0.75	32.93±0.29	32.62±0.22	30.47±0.47	32.26±0.33	30.71±0.35
tlr5a	31.05±0.19	31.25±0.25	30.28±0.15	29.03±0.18	30.89±0.26	29.30±0.20
tlr5b	31.97±0.41	31.94±0.40	30.79±0.22	25.99±0.22	30.41±0.76	26.59±0.24
pabpc1b	20.16±0.05	20.12±0.08	20.06±0.05	20.19±0.06	20.15±0.06	20.11±0.06
ef1a4	18.19±0.03	18.17±0.06	18.02±0.03	18.11±0.06	18.21±0.06	18.18±0.06
Study 2						
hampa	31.80±0.69	31.44±0.59	29.32±0.49	23.39±0.44	30.79±0.80	22.50±0.25
ifng	29.88±0.20	29.75±0.17	29.51±0.15	27.52±0.22	29.61±0.16	27.08±0.35
cox1b	29.10±0.18	29.14±0.30	28.51±0.22	28.45±0.32	29.08±0.26	29.05±0.32
pabpc1b	20.57±0.07	20.54±0.12	20.28±0.05	20.36±0.05	20.34±0.09	20.37±0.06
ef1a4	18.64±0.05	18.53±0.10	18.30±0.03	18.41±0.04	18.44±0.08	18.49±0.06
Study 3						
hampb	28.27±0.25	27.96±0.34	26.04±0.35	21.54±0.11	26.88±0.43	21.60±0.21
il1b-like	27.50±0.10	27.48±0.12	27.50±0.10	28.57±0.11	27.83±0.10	28.61±0.08
il8a	25.98±0.12	25.95±0.11	26.32±0.07	24.65±0.16	26.23±0.23	24.41±0.12
pabpc1b	20.22±0.05	20.31±0.09	20.21±0.06	20.31±0.05	20.30±0.08	20.33±0.09
ef1a4	18.40±0.05	18.39±0.08	18.26±0.04	18.37±0.04	18.45±0.07	18.50±0.07
Study 4						
il8b	26.95±0.16	26.75±0.21	25.59±0.27	26.28±0.31	26.96±0.41	26.72±0.50
stat1b	28.61±0.13	28.62±0.15	27.89±0.06	26.24±0.14	28.04±0.25	25.97±0.32
cox1a	25.40±0.21	25.39±0.17	25.21±0.11	24.93±0.10	24.76±0.15	25.02±0.17
pabpc1b	20.89±0.10	20.86±0.14	20.59±0.03	20.69±0.08	20.64±0.08	20.75±0.09
ef1a4	18.52±0.08	18.48±0.11	18.21±0.05	18.34±0.06	18.28±0.07	18.46±0.07

Table S4.3. Cycle threshold (C_T) values for the TOIs and ECs measured in the experimental qPCR assays (continued).

TOI / EC C_T	**Initial**		**24 hpi**			
	Normoxia	Hypoxia	Normoxia PBS	Normoxia *A. sal.*	Hypoxia PBS	Hypoxia *A. sal.*
Study 5						
tnfaa	23.08±0.09	23.24±0.14	22.91±0.10	22.28±0.08	23.14±0.12	22.18±0.11
ccl19	22.07±0.18	22.07±0.19	21.76±0.15	18.94±0.20	21.84±0.25	18.21±0.23
stat1a	24.75±0.16	24.94±0.15	24.65±0.14	23.42±0.17	24.76±0.11	22.89±0.18
pabpc1b	20.65±0.06	20.67±0.11	20.61±0.06	20.78±0.05	20.84±0.07	20.67±0.08
ef1a4	18.25±0.06	18.23±0.07	18.16±0.05	18.24±0.05	18.36±0.06	18.33±0.07
Study 6						
il1b	31.43±0.36	31.10±0.29	30.67±0.21	25.77±0.25	30.25±0.41	25.75±0.35
ef1a4	19.94±0.06	20.00±0.08	19.91±0.04	19.94±0.03	20.02±0.09	20.00±0.07
pabpc1b	18.67±0.06	18.56±0.07	18.50±0.05	18.63±0.07	18.68±0.07	18.62±0.09

Table S4.4. Plasma cortisol levels in sablefish acclimated to normoxia (~100% air sat.) or chronic hypoxia (6 months at ~40% air sat. followed by 3-4 weeks at ~20% air sat.). These are unpublished data that were collected from sablefish in a different study [see Gerber *et al.* (2019)]; although the sablefish in the present chronic hypoxia/immunology experiment underwent a similar acclimation protocol (see Section 4.3.1). Plasma samples were taken from sablefish that were euthanized (by a blow to the head) right after netting from the tank [see Gerber *et al.* (2019)], and were immediately frozen in liquid N_2, stored at -80°C and analysed as described by Leeuwis *et al.* (2021). Specifically, cortisol levels were measured in duplicate using ELISA kits (Neogen Life Sciences, cat. $^{\#}$402710, Lexington, KY, USA). There was no significant difference (P>0.05) between the acclimation groups (assessed by a Mann-Whitney U test). Values are means±s.e.m., and sample sizes are n=9 and 10 for the normoxia- and hypoxia-acclimated group, respectively.

	Acclimation treatment	
	Normoxia-acclimated	Hypoxia-acclimated
Plasma cortisol (ng mL^{-1})	49.2±13.2	56.9±22.3

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TCGGTGTTTCTGGGTTTTCACGGCCGACACTGCTGCCCCTAGCCAGGTAACAGAAAGAG

Figure S4.1. Sablefish transcriptome sequences (in FASTA format) utilized for qPCR primer design. Transcriptome sequences for the following genes are shown: *il1b* (TR28839_c0_g1_i1/c0_g2_i5), *il1b-like* (TR21996_c0_g1_i1/c0_g2_i3), *ifng* (TR26914_c0_g1_i1), *stat1a* (TR33543_c1_g1_i14), *stat1b* (TR36335_c1_g1_i1), *tlr5a* (TR29278_c0_g1_i1), *tlr5b* (TR29636_c0_g1_i2) and *ef1a4* (TR33310_c0_g1_i8). After *de novo* transcriptome assembly, each transcript received an ID (e.g., TR29278_c0_g1_i1) specifying the Trinity read cluster (TR29278_c0), gene (g1) and isoform (i1). BLASTx analysis was performed to identify these transcripts (Table S4.1). See Section 4.3.6 for details.

```
cox1a       CTCAAGTGTTGTGAATCCCTGTTGTTATTACCCGTGTCAGAACTCAGGAGTGTGTGTGAG      60
cox1b       CTCCAGTACAGTGAACCCATGCTGTTATTTCCCCTGTCAGCACTGGGGTGTGTGTGTCAG      60
cox2        CTGCGAAGGGGGTAACCTATGTTGCTCAGATCCATGTCAGAACAGGGGCGTTTGTACAGC      60
            **        *  ** *  ** ** *     ** ****** **   ** ** ***


cox1a       ATTTGGTACAGATGGTTACCAATGTGACTGCACTCGCACCGGCTTCTATGGAGAGAACTG      120
cox1b       GTATGGTGAAGACAAGTATGAGTGTGACTGCACTCGTACCGGCTTCAGCGGAGAAAACTG      120
cox2        GCTTGGATCTGATAATTATGAGTGTGATTGCACACGAACAGGATTTCATGGACAAAACTG      120
               ***    **    **  * ***** ***** ** ** ** **    *** * *****


cox1a       CACTGTTCCGGAGCTCTGGACCAGAGTTCGTCGCACGCTGAAGCCGACCCCTTCAGTAGT      180
cox1b       CACCGACCCGGAGCTTTTGACCAGACTGCGTCACTTTTTCAAGCCCAGCCCTGATGTGTT      180
cox2        CACAACTCCTGAATTCCTCACCTGGGTCAAAATATCCCTGAAGCCATCGCCCAACACTGT      180
            ***    ** **  *    *** *  *           * *****    **        *

                     Forward
cox1a       TCACTTCATCCTCACCCACTTCCAGTGGTTCTGGGACCTCGTCAACAA---CTCTTTCCT      237
cox1b       GCATTACATACTCACTCACTTTCAATTGCTCTGGGACATCATCAACTA---CACCTTCCT      237
cox2        CCACTACCTTCTCACCCACTTCAAGGGCTTCTGGAACATCATCAACAACATCTCCTTTCT      240
             ** * * * ***** *****  *     ***** ** ** ***** *   * * ** **
                                  Forward
                                                          Reverse
cox1a       ACGAGACACAGTCATGAGATTGGTGCTGACGGTCCGAAGCGACCTTATTCCAAGCCCTCC      297
cox1b       ACGGGACGTCCTCATGCGGCTGGTTCTAACAGTGAGGGCAAACTTAATACCCAGCCCGCC      297
cox2        CAGGGATGCCATCATGAGATATGTGCTGACATCTCGATCCCACTTGATTGATAGTCCGCC      300
              * **     ***** *    ** ** **     *     ** * **    ** ** **
                                                          Reverse

cox1a       GACCTACAATACCAAGTATGGATACCTCAACTGGGAGGCGTACTACAACATCTCCTACTA      357
cox1b       AACCTACAACTCCAAATATGGCTACCTCAGCTGGGAATCCTACTATAACCTGAGCTACTA      357
cox2        GACCTTCAATGCAGATTATGGTTACAAAAGCTGGGAAGCATATTCCAACCTCTCCTACTA      360
             **** ***  *  * ***** ***   * ******  * ** *  *** *   ******


cox1a       CACCCGTCTCCTCCCTCCGGTACCTGAAGACTGCCCTCTGCCGATGGGAACTAAAGGTAA      417
cox1b       CACCCGGCTTCTGCCCCCGGTGCCGAAGGACTGCCCTACACCTATGGGGGTCAAAGGCAA      417
cox2        TACACGCACCCTCCCCCCTGTGTCAGAGGATTGCCCAACCCCTATGGGAGTAGAAGGTAA      420
             ** **    ** ** ** **  *  * ** *****    ** *****     **** **


cox1a       ATCTGTTCTTCCTGATCCCAAAGTGTTGACTGAGAGGTTTTTCAAGAGGAAGACATTTAG      477
cox1b       GGCTGGGATGCCTGACCCTGAGCTGCTGGTTGAGCGTCTGCTGAAGAGAAGGACGTTCAG      477
cox2        AAAGGAGCTGCCAGACGCTAAGCTATTGGCAGAGAAGCTTCTTATGAGAAGACAGTTCAT      480
                *   * ** **  *  *  *  **   ***    *  * * *** *     ** *


cox1a       GCCAGACCCTCAGGGAACCAATCTGATGTTTGCCTTCATGGCCCAACACTTCACCCACCA      537
cox1b       ACCCGACCCCCAGGGCTCCAACCTCATGTTCGCCTTCTTCGCACAGCACTTCACCCACCA      537
cox2        CCCGGACCCACAGGGCACCAGCCTGATGTTTGCATTCTTCGCACAGCATTTCACCCACCA      540
             ** ***** *****  ***  ** ***** ** *** * ** ** ** ***********


cox1a       GTTTTTCAAGACAGACCATAAAGTTCAAGGTGGCTTCACCAAGGCTTTAGGACATGGGGT      597
cox1b       GTTCTTTAAGACCTACAATCGCATGGGTGTGGGCTTCACCAAGGCTCTAGCGCACGGGGT      597
cox2        GTTCTTCAAATCTGATATGAAGAAAGGACCTGCTTTTACTGCAGCTAAAGGCCACGGGGT      600
            *** ** **  *  *               *  ** **    ***  **  ** *****
```

```
                                                                  Forward
cox1a       GGATGCGGGCAATATCTACGGAGACAACCTCATTAGACAGCACCACCTCCGGCTTCATAC      657
cox1b       GGATGCAGGACACGTTTACGGAGACAACCTGGAACGTCAGCTCCGGCTCAGGCTTCACAA      657
cox2        GGACCTCAGCCATATTTATGGAGACACCCTGGAGAGGCAACACAAGCTCAGACTCTTTAA      660
            ***     *  *  * ** ******* ***     * ** * *   *** * **    *


cox1a       AGATGGAAAGCTGAAATATCAGTTGGTAAATGGTGACATATACCCTCCTACTGCATCTGA      717
cox1b       AGACGGAAAACTCAAGTATCAGTTAGTAGACGGCGATATGTACCCTCCCTCTGTAGCTGA      717
cox2        GGATGGCAAGCTTAAATATCAGATCATGGATGGAGAGATGTATCCCCCAACAGTAAAGGA      720
             ** ** ** ** ** ****** *  *  * ** ** ** ** ** ** **  * * *   **

                Reverse
cox1a       GGTCCCTGTGCACATGGTGTATCCTGAAAACTTCCCTCCAGAGAAGCGTCTGGTCATCGG      777
cox1b       TGCACCCGTTAAGATGAGCTACCCCCCCGGGATCCCTCCTGAGGCTCAGATGGCGATTGG      777
cox2        AGTGGGCGCTGACATGCACTACCCTCCTCACGTTCCCGAGTCTCACCGCTTCGCTGTGGG      780
             *     *   * ***   ** **        * **          *   * *   * **


cox1a       TCAGGAGGTGTTTGGCCTCCTGCCGGGCCTCACCATGTACGCCACCATATGGCTGAGGGA      837
cox1b       TCAGGAAGTGTACGGACTCCTCCCGGGTTTGGGAATGTTTGCAACGCTGTGGCTCAGGGA      837
cox2        CCACGAGGCGTTCGGCCTGGTCCCCGGTCTGATGATGTACGCCACCATCTGGCTACGAGA      840
             ** ** * **  ** **  * ** **  *    ****  ** **  * *****  * **


cox1a       GCATAACAGAGTTTGTGACATCCTGAAGTCAGAACATCCCACCTGGGACGACGAGCAGCT      897
cox1b       GCATAACCGAGTATGTGACATCTTGAAGGTGGAGCATCCCACCTGGGACGATGAGCAGCT      897
cox2        ACACAACCGAGTGTGTGATGTGTTGAAGGAGGTCCACCCGGACTGGGATGACGAAAGGCT      900
             ** *** **** *****  *  *****   *  ** **   ****** ** **   ***


cox1a       CTTCCAGACCACCAGACTCATCATCATTGGAGAGATCATCAACATTATAATAGAAGAGTA      957
cox1b       TTTCCAGACCACACGCTTAATCATCATCGGTGAGACAATAAAGATAGTGATAGAGGAGTA      957
cox2        CTTCCAGACCACCCGGCTCATTCTGATTGGAGAGACCATCAAGATTGTGATCGAGGACTA      960
             **********  *  * **  * ** ** ****  ** ** **  * ** ** ** **


cox1a       CGTGCAGCATCTCAGTGGTTACCTGCTGAATCTGAAGTTCGATCCCACCTTGCTCTTCAG     1017
cox1b       CGTACAGCACCTCAGCGGATACCTGCTGCAGCTGAAGTTTGATCCCGCCCTTCTGTTTAA     1017
cox2        TGTGCAGCACCTGAGCGGCTATAACTTCAAGCTCAAGTTTGACCCCGAGCTACTGTTCAA     1020
             ** ***** ** ** ** **     *  * ** ***** ** ***    * ** ** * *


cox1a       C     1018
cox1b       C     1018
cox2        C     1021
            *
```

Figure S4.2. Alignment of the CDS for cyclooxygenase-like cDNAs from sablefish included in the qPCR studies. Nucleotide sequences were aligned using the multiple sequence alignment tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Identities are 71.6% (*cox1a vs. cox1b*), 64.4% (*cox1a vs. cox2*), and 63.9% (*cox1b vs. cox2*). When all three sequences are identical, this is indicated with an asterisk (*), and gaps are indicated with a dash (−). The sequences of the primers used in the qPCR assays are highlighted in grey (*cox1a*), blue (*cox1b*), or pink (*cox2*). The GenBank accession numbers for *cox1a* and *cox1b* are GAJJ01016042 and GAJJ01016043, respectively. For *cox2*, a contig was constructed using two sequences with GenBank accession numbers GAJJ01046020 and GAJJ01047049. In the case of GAJJ01046020, the reverse complement is shown. For all three sequences, the CDS is partial.

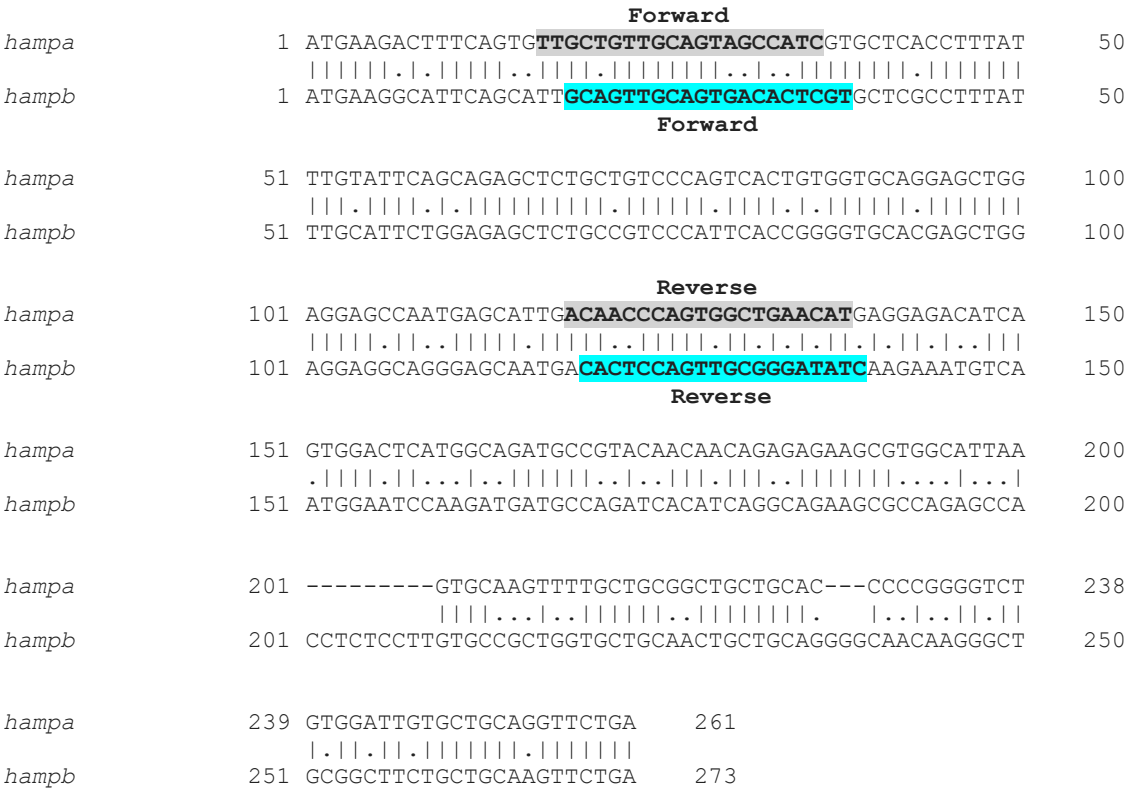
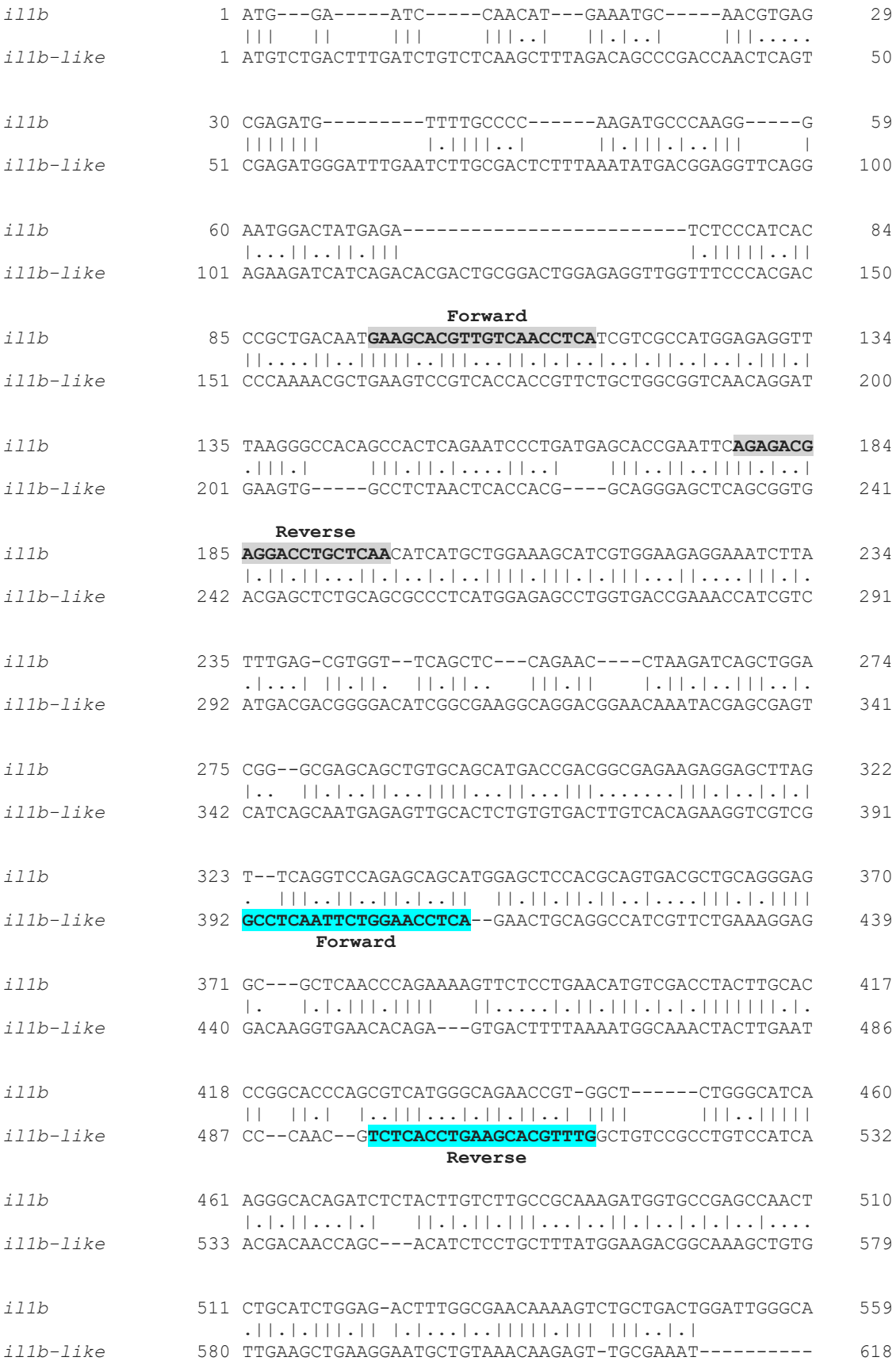


Figure S4.3. Alignment of the CDS for hepcidin-like cDNAs from sablefish included in the qPCR studies. Nucleotide sequences were aligned using the pairwise sequence alignment tool EMBOSS Stretcher (https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/). Identity is 69.6%. Identical sequences are indicated with a line (|), different sequences are indicated with a period (.) and gaps are indicated with a dash (−). The sequences of the primers used in the qPCR assays are highlighted in grey (*hampa*) or blue (*hampb*). The GenBank accession numbers are GAJJ01029649 (*hampa*) and GAJJ01004264 (*hampb*). In the case of GAJJ01004264, the reverse complement is shown.


il1b 1 ATG---GA-----ATC-----CAACAT---GAAATGC-----AACGTGAG 29
il1b-like 1 ATGTCTGACTTTGATCTGTCTCAAGCTTTAGACAGCCCGACCAACTCAGT 50
il1b 30 CGAGATG---------TTTTGCCCC------AAGATGCCCAAGG-----G 59
il1b-like 51 CGAGATGGGATTTGAATCTTGCGACTCTTTAAATATGACGGAGGTTCAGG 100
il1b 60 AATGGACTATGAGA-------------------------TCTCCCATCAC 84
il1b-like 101 AGAAGATCATCAGACACGACTGCGGACTGGAGAGGTTGGTTTCCCACGAC 150
Forward
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il1b-like 151 CCCAAAACGCTGAAGTCCGTCACCACCGTTCTGCTGGCGGTCAACAGGAT 200
il1b 135 TAAGGGCCACAGCCACTCAGAATCCCTGATGAGCACCGAATTCAGAGACG 184
il1b-like 201 GAAGTG-----GCCTCTAACTCACCACG----GCAGGGAGCTCAGCGGTG 241
Reverse
il1b 185 AGGACCTGCTCAACATCATGCTGGAAAGCATCGTGGAAGAGGAAATCTTA 234
il1b-like 242 ACGAGCTCTGCAGCGCCCTCATGGAGAGCCTGGTGACCGAAACCATCGTC 291
il1b 235 TTTGAG-CGTGGT--TCAGCTC---CAGAAC----CTAAGATCAGCTGGA 274
il1b-like 292 ATGACGACGGGGACATCGGCGAAGGCAGGACGGAACAAATACGAGCGAGT 341
il1b 275 CGG--GCGAGCAGCTGTGCAGCATGACCGACGGCGAGAAGAGGAGCTTAG 322
il1b-like 342 CATCAGCAATGAGAGTTGCACTCTGTGTGACTTGTCACAGAAGGTCGTCG 391
il1b 323 T--TCAGGTCCAGAGCAGCATGGAGCTCCACGCAGTGACGCTGCAGGGAG 370
il1b-like 392 GCCTCAATTCTGGAACCTCA--GAACTGCAGGCCATCGTTCTGAAAGGAG 439
Forward
il1b 371 GC---GCTCAACCCAGAAAAGTTCTCCTGAACATGTCGACCTACTTGCAC 417
il1b-like 440 GACAAGGTGAACACAGA---GTGACTTTTAAAATGGCAAACTACTTGAAT 486
il1b 418 CCGGCACCCAGCGTCATGGGCAGAACCGT-GGCT------CTGGGCATCA 460
il1b-like 487 CC--CAAC--GTCTCACCTGAAGCACGTTTGGCTGTCCGCCTGTCCATCA 532
Reverse
il1b 461 AGGGCACAGATCTCTACTTGTCTTGCCGCAAAGATGGTGCCGAGCCAACT 510
il1b-like 533 ACGACAACCAGC---ACATCTCCTGCTTTATGGAAGACGGCAAAGCTGTG 579
il1b 511 CTGCATCTGGAG-ACTTTGGCGAACAAAAGTCTGCTGACTGGATTGGGCA 559
il1b-like 580 TTGAAGCTGAAGGAATGCTGTAAACAAGAGT-TGCGAAAT---------- 618

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il1b            560 CGAGAATTGGCTTGGACAGCGACTTGGTGCGGTTTCTCTTCTACAGACAG    609
                    ||..||...||..|.|||.||||.|..||.||||||||||||   |
il1b-like       619 -----ATCAGCGCTGATGGAGACATGGTCCATTTCCTCTTCTACAG---G    660


il1b            610 GACACCGGGGTGAACA---TCACCACCCTCATGTCCGTCGCCCAACCCAA    656
                    .|.||...||..|.||   |.|.|||..|...||||||.......|||..
il1b-like       661 AAAACTATGGGAACCAGTGTGAACACTTTTGAGTCCGTTAAATGTCCCGG    710


il1b            657 CTGGTACATCAGCACGGCGCAGCAGGACAACAGGCCGTTGGCGATGTGCC    706
                    |||||.|||||||||..|....|||||..|||.|.|..|||.||||||..
il1b-like       711 CTGGTTCATCAGCACCTCCTGTCAGGATGACAAGTCAGTGGAGATGTGTA    760


il1b            707 ---TGGAGTCCACCCAGCACTCCCGCATCTTCAGCATCCGAGAGGAGGTC    753
                       ||||.||||.||||..|.||.||...|.||.| |||...|..|.
il1b-like       761 AAGTGGACTCCAGCCAGGCCCCCAGCCGTTGCACC-TCCTTCAAAAT---    806


il1b            754 GAACGTCAGGGATAA    768
                    ||||        |||
il1b-like       807 GAAC--------TAA    813
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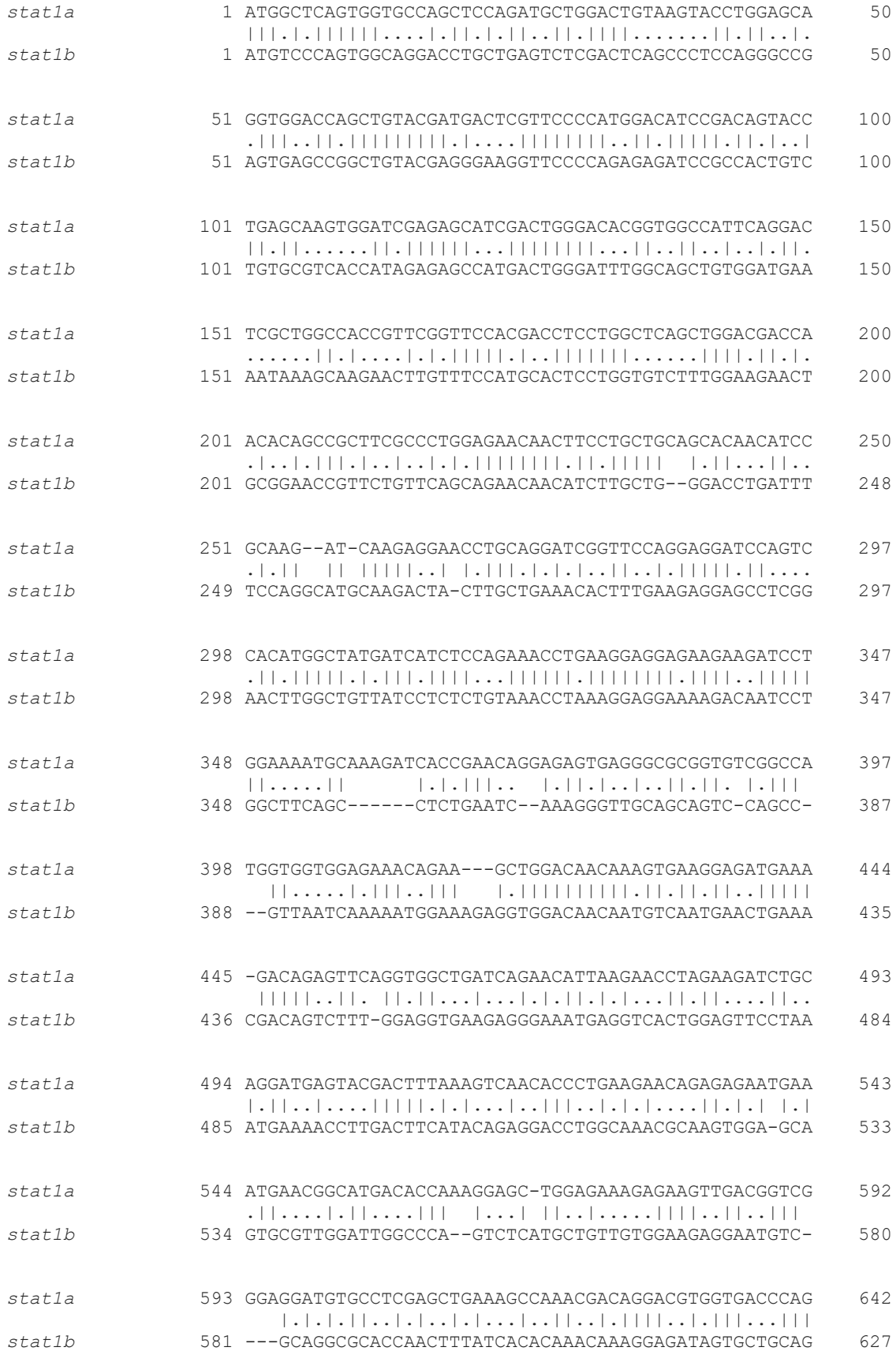
Figure S4.4. Alignment of the CDS for interleukin 1 beta-like cDNAs from sablefish included in the qPCR studies. Nucleotide sequences were aligned using the pairwise sequence alignment tool EMBOSS Stretcher (https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/). Identity is 49.4%. Identical sequences are indicated with a line (|), different sequences are indicated with a period (.) and gaps are indicated with a dash (−). The sequences of the primers used in the qPCR assays are highlighted in grey (*il1b*) or blue (*il1b-like*). For *il1b*, a contig was constructed using two sequences with Trinity IDs TR28839_c0_g1_i1 and TR28839_c0_g2_i5. For *il1b-like*, a contig was constructed using two sequences with Trinity IDs TR21996_c0_g1_i1 and TR21996_c0_g2_i3. In the case of TR21996_c0_g2_i3, the reverse complement is shown.

Figure S4.5. Alignment of the CDS for interleukin 8-like cDNAs from sablefish included in the qPCR studies. Nucleotide sequences were aligned using the pairwise sequence alignment tool EMBOSS Stretcher (https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/). Identity is 50.4%. Identical sequences are indicated with a line (|), different sequences are indicated with a period (.) and gaps are indicated with a dash (−). The sequences of the primers used in the qPCR assays are highlighted in grey (*il8a*) or blue (*il8b*). The GenBank accession numbers are BT082167 (*il8a*) and JO666101 (*il8b*).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stat1a 398 TGGTGGTGGAGAAACAGAA---GCTGGACAACAAAGTGAAGGAGATGAAA 444
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stat1a 494 AGGATGAGTACGACTTTAAAGTCAACACCCTGAAGAACAGAGAGAATGAA 543
stat1b 485 ATGAAAACCTTGACTTCATACAGAGGACCTGGCAAACGCAAGTGGA-GCA 533
stat1a 544 ATGAACGGCATGACACCAAAGGAGC-TGGAGAAAGAGAAGTTGACGGTCG 592
stat1b 534 GTGCGTTGGATTGGCCCA--GTCTCATGCTGTTGTGGAAGAGGAATGTC- 580
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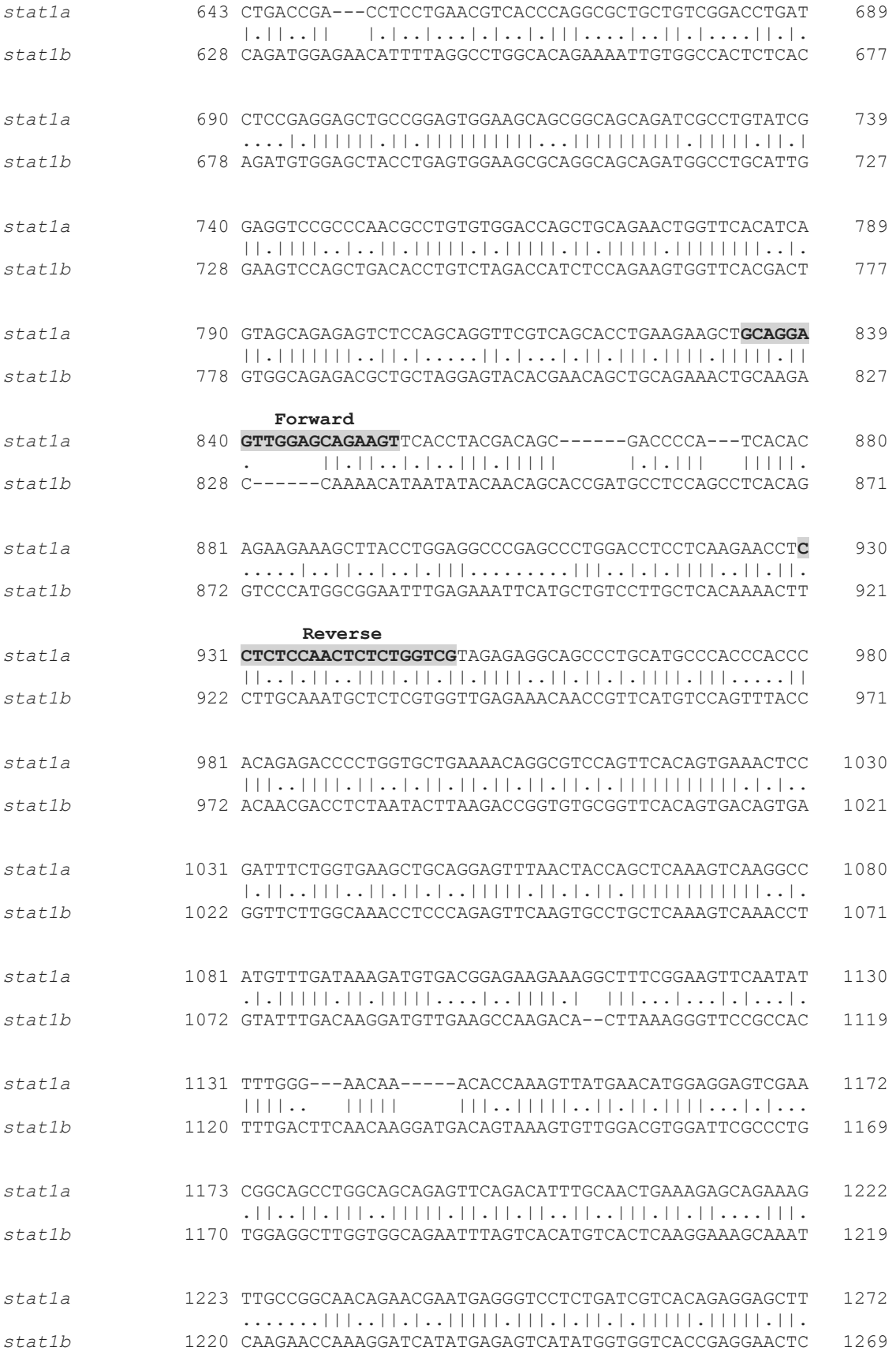
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Forward
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Reverse
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stat1b 1120 TTTGACTTCAACAAGGATGACAGTAAAGTGTTGGACGTGGATTCGCCCTG 1169
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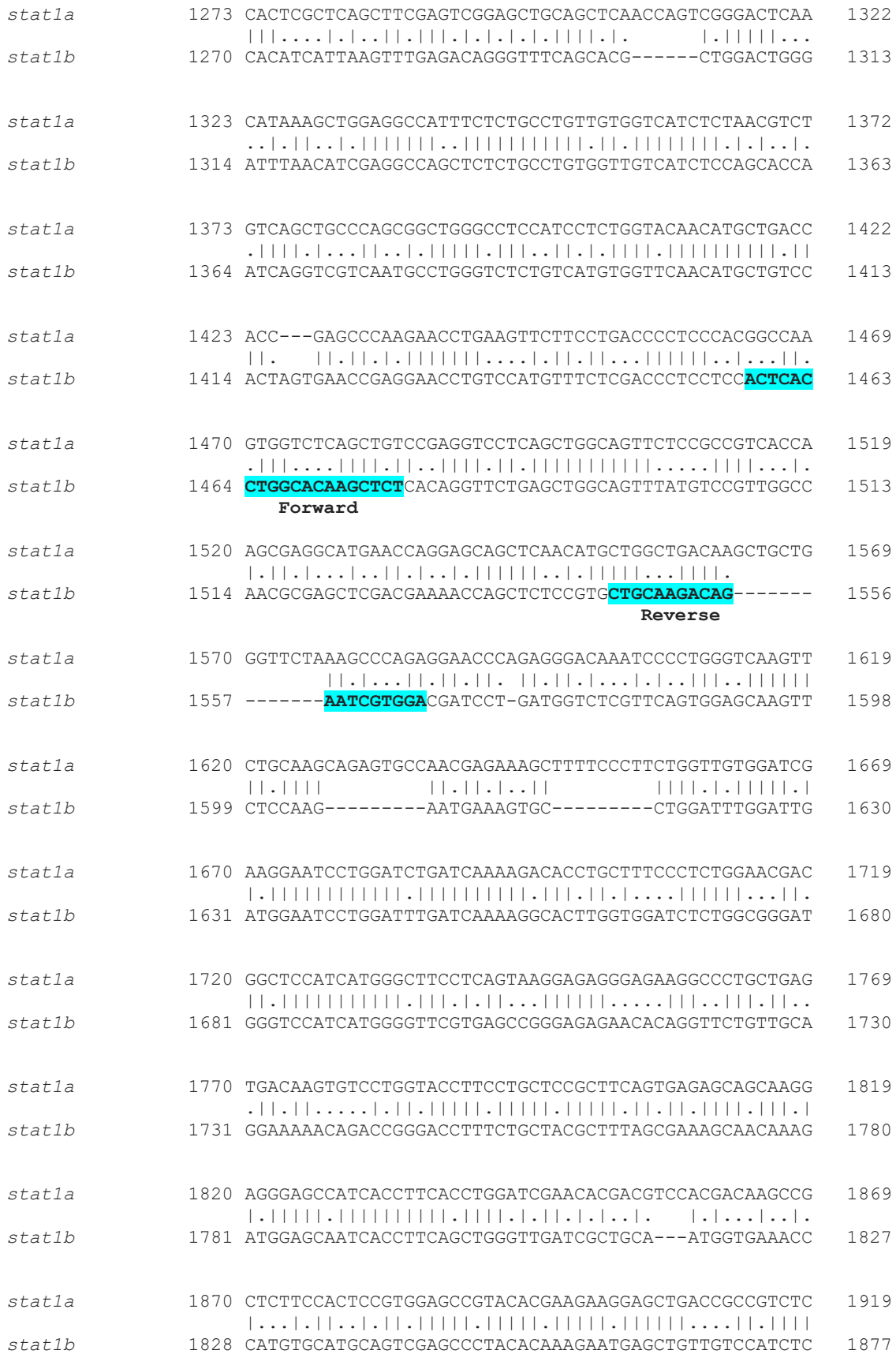
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stat1a 1373 GTCAGCTGCCCAGCGGCTGGGCCTCCATCCTCTGGTACAACATGCTGACC 1422
stat1b 1364 ATCAGGTCGTCAATGCCTGGGTCTCTGTCATGTGGTTCAACATGCTGTCC 1413
stat1a 1423 ACC---GAGCCCAAGAACCTGAAGTTCTTCCTGACCCCTCCCACGGCCAA 1469
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stat1a 1470 GTGGTCTCAGCTGTCCGAGGTCCTCAGCTGGCAGTTCTCCGCCGTCACCA 1519
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Forward
stat1a 1520 AGCGAGGCATGAACCAGGAGCAGCTCAACATGCTGGCTGACAAGCTGCTG 1569
stat1b 1514 AACGCGAGCTCGACGAAAACCAGCTCTCCGTGCTGCAAGACAG------- 1556
Reverse
stat1a 1570 GGTTCTAAAGCCCAGAGGAACCCAGAGGGACAAATCCCCTGGGTCAAGTT 1619
stat1b 1557 -------AATCGTGGACGATCCT-GATGGTCTCGTTCAGTGGAGCAAGTT 1598
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stat1b 1781 ATGGAGCAATCACCTTCAGCTGGGTTGATCGCTGCA---ATGGTGAAACC 1827
stat1a 1870 CTCTTCCACTCCGTGGAGCCGTACACGAAGAAGGAGCTGACCGCCGTCTC 1919
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stat1b          1878 TGTGCCGGACATCATTTACCACTACAGTCTGAGAGCCCAGGGGAGAA-AG   1926

stat1a          1968 CCCGGAGAACCCGCTCCGCTTCCTCTACCCCAACATCCCCAAAGACAAGG   2017
                     |.|.| |||.||.||...||.|||||||||..||||...|||||||...|
stat1b          1927 CACAG-GAATCCACTGATCTACCTCTACCCAGACATAGACAAAGACGCTG   1975

stat1a          2018 CCTTCGGGAAGTACTACCCCAAACCC---------TCAGAGACTCCAGAG   2058
                     ||||   .||...||||..||||||.         ||.|..|||..|.||
stat1b          1976 CCTT---TAAACGCTACTACAAACCTCGTGAAATGTCGGCAACTAAAAAG   2022

stat1a          2059 CC---AATGGACGTGGAGAACGGTCCGGAGAAGAGCGGCTACATGAAGAC   2105
                     ..   ||..|||...|.||||.|..|...|.....||.|| ||...|..|
stat1b          2023 GTCGTAAGTGACTACGTGAACAGAACTATGGTTCCCGTCT-CAGACAATC   2071

stat1a          2106 GGAGCTCATATCCGTCTC---------AGAAGTACATCC-GTCCA--GAC   2143
                     ..|...||...|||||||         |||.|..|||.. |..||  |||
stat1b          2072 CTACGCCACCGCCGTCTCCACCGACCGAGATGCCCATTATGGACATGGAC   2121

stat1a          2144 -TGCAC---GAC--------AACA-----TGATGCCCATGTCTCC--TGA   2174
                      ||.||   |||        ||||     |.||||..|..|||.|  |||
stat1b          2122 ATGGACATGGACCCTGGAATAACACTGGATAATGCTGAACTCTTCGCTGA   2171

stat1a          2175 TGACTACAAGGTTCTG-TCGCAGTACGTCAGTCCC---------------   2208
                     ...||.|...|.|.|. |||...|||.|.|...||
stat1b          2172 ACTCTTCGCTGATTTACTCGACATACCTGAACACCCCGACTTAATGACAA   2221

stat1a          2209 -------AGAGACATCG-------ATGCTGTGG------TGAGTTCCTCT   2238
                             |||.|||.|.       ||.|..|.|      |.||||.|||.
stat1b          2222 TTTATCAAGAAACAGCATCAACTCATACCTTCGGCGATCTTAGTTGCTCC   2271

stat1a          2239 TTGTGA   2244
                     ||.|||
stat1b          2272 TTCTGA   2277
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Figure S4.6. Alignment of the CDS for signal transducer and activator of transcription 1-like cDNAs from sablefish included in the qPCR studies. Nucleotide sequences were aligned using the pairwise sequence alignment tool EMBOSS Stretcher (https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/). Identity is 56.6%. Identical sequences are indicated with a line (|), different sequences are indicated with a period (.) and gaps are indicated with a dash (−). The sequences of the primers used in the qPCR assays are highlighted in grey (*stat1a*) or blue (*stat1b*). The Trinity IDs are TR33543_c1_g1_i14 (*stat1a*) and TR36335_c1_g1_i1 (*stat1b*). In the case of TR36335_c1_g1_i1, the reverse complement is shown.

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tlr5b      1 GGCCTTCGACTGGAGCCGCAGGCTTTTGTCGGACTGTCCGGTTTGCAGAA     50

                                    Forward
tlr5a     51 CCTCTTTCTGGATTATTGCGATTTGTCAGACTCCATATTAGCAGACAGCT    100
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tlr5b     51 TCTCTACCTGGATTACTGTTCGCTTCAAGACTCCATACTTATGGAGAACT    100

tlr5a    101 ATATGGAGCCGCTTTTGTCCTTGGAAACGCTCGACCTCTTCGGCAATAAA    150
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tlr5b    101 ATCTGGAGCCACTGTCCTCCTTAGAGACACTTGACCTCTTTGGTAACAAG    150

                   Reverse
tlr5a    151 ATAGTGAGTGTTCGACCGGGACCATTCTTTTCGAGACTTACAAAGTTCAC    200
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tlr5b    151 ATAAAAAGACTCCAGCCCTCAATGTTCTTTAGAAACATTACTAATTTGAA    200

tlr5a    201 ACAGCTAAACCTCAAATTGAATCAGATTGAAAGATTGTGCGAAGATGATC    250
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tlr5b    201 AGATTTGAATCTCAAGCTGAATCCAATTGACAAAATATGTGAGTCTGATC    250

tlr5a    251 TTGTTGGTTTCCGGGGCAAATACTTCAAACTCCTGAACTTGCATTCCAAT    300
             |.||||||||.|..||.||..|||||||..|||||||.||..|.||...|
tlr5b    251 TGGTTGGTTTTCAAGGGAAGGACTTCAAGGTCCTGAATTTAGACTCTGTT    300

tlr5a    301 AAATTCTATATCGGCTATGGTGAAGGCTTTGACTGGGAAAGATGCGGGAA    350
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tlr5b    301 CACCTTAAGGCCATGTTTAATGAAAAGTTTGACAGGCAGGAATGTGGGAA    350

tlr5a    351 CCCTTTCAGAGGGATGGCTTTTAAAACACTCGACTTATCCACCAATGGGT    400
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tlr5b    351 TCCTTTCAGGGGAATGTCCTTTCAGACACTCGACATGTCCCACAACGGGT    400

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tlr5a    451 ATTGCTCATCTTATATTAACTGGACCCATGGGCAAAGGCTTCTCATATGA    500
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tlr5a          748 CACACATTTGCCAATCTGACGGAGCTTTGGGTGTTGGATTTATCTTACAA    797
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tlr5a          798 TCACATAGCTGTATTGGGACACAAAGCATTCAGCGGTCTTCCAAATTTAC    847
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tlr5b          798 TCACATTGGTGCTCTGGGCCATGGCTCATTTAGTGGACTACCACAGTTAA    847

tlr5a          848 AACATCTATATCTGACGGGAAATTCACTGCGAGCCCTGGGTTATCCGGCG    897
                   ||....||..|||.||.||||||.||..||||||.|||.||.|..||.||.
tlr5b          848 AATCATTAATTCTCACAGGAAACTCTTTGCGAGACCTCGGCTTCCCTGCA    897

tlr5a          898 CCATTGCCAAGCTTAGATTTTCTTCTTTTGAACGACAATAAATTAA---A    944
                   .|..|.||.||||||||||.|||.||.||||||.|||||||.|.|.|   |
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tlr5a          945 ATCTCTATGGAACATTGAAT------TTGGCAAGAACAGTATTCACCTGG    988
                   .|....|..||.||.|...|      |||.|...||.|.||||||.||||
tlr5b          948 GTTGTCAGTGAGCAGTCTCTCACGCTTTGCCGGTAATATTATTCATCTGG    997

tlr5a          989 ATGTTGCAGACAACAGATTAACGAACTTGGAGGACGTTTATGTTATTTTA   1038
                   |..||...|||||||||||||.||||.|.|.|||||||||..|...||||.
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                                                Forward

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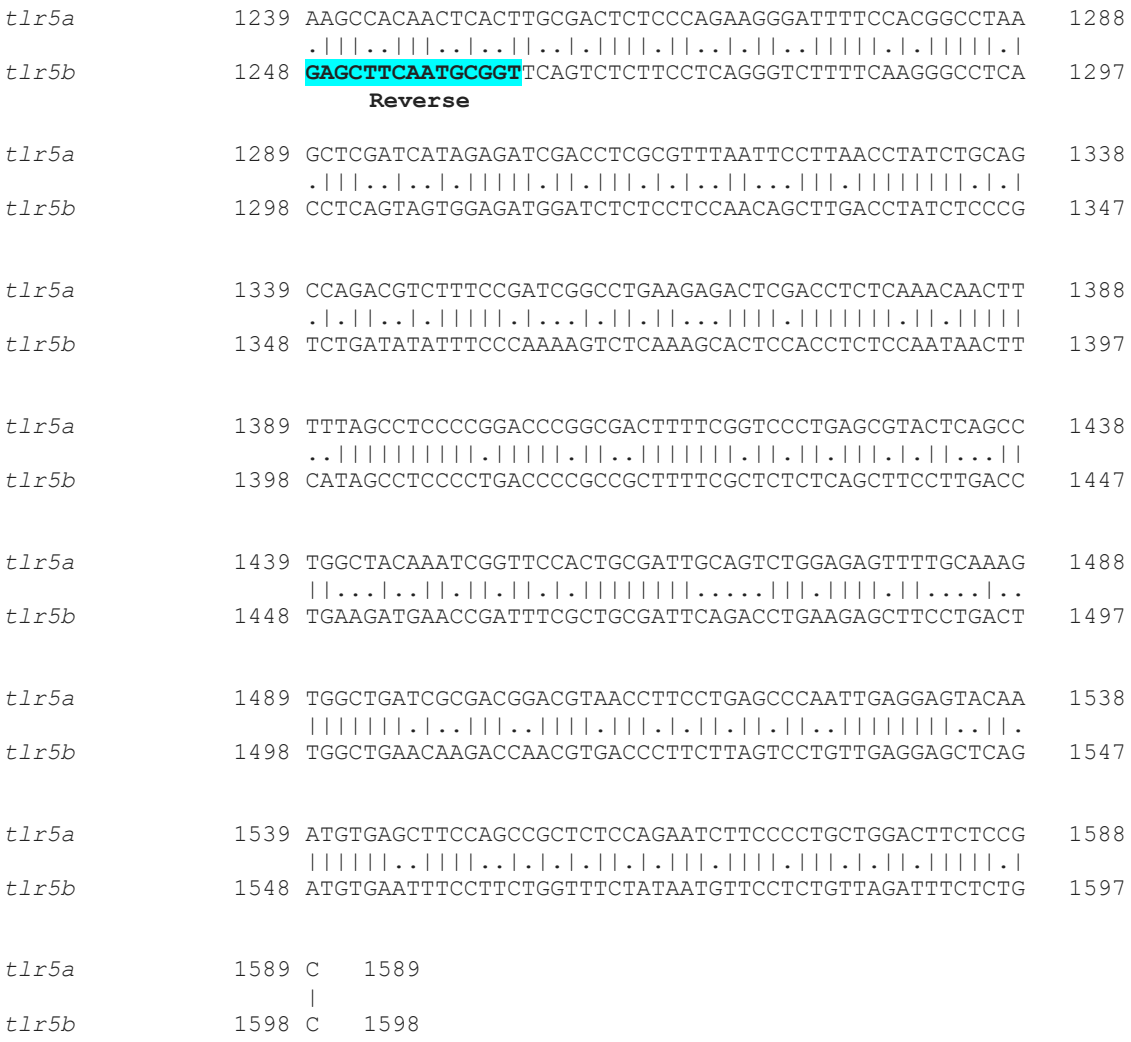

Figure S4.7. Alignment of the CDS for toll-like receptor 5-like cDNAs from sablefish included in the qPCR studies. Nucleotide sequences were aligned using the pairwise sequence alignment tool EMBOSS Stretcher (https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/). Identity is 66.0%. Identical sequences are indicated with a line (|), different sequences are indicated with a period (.) and gaps are indicated with a dash (−). The sequences of the primers used in the qPCR assays are highlighted in grey (*tlr5a*) or blue (*tlr5b*). The Trinity IDs are TR29278_c0_g1_i1 (*tlr5a*) and TR29636_c0_g1_i2 (*tlr5b*). In the case of both sequences, the CDS is partial, and the reverse complement is shown.

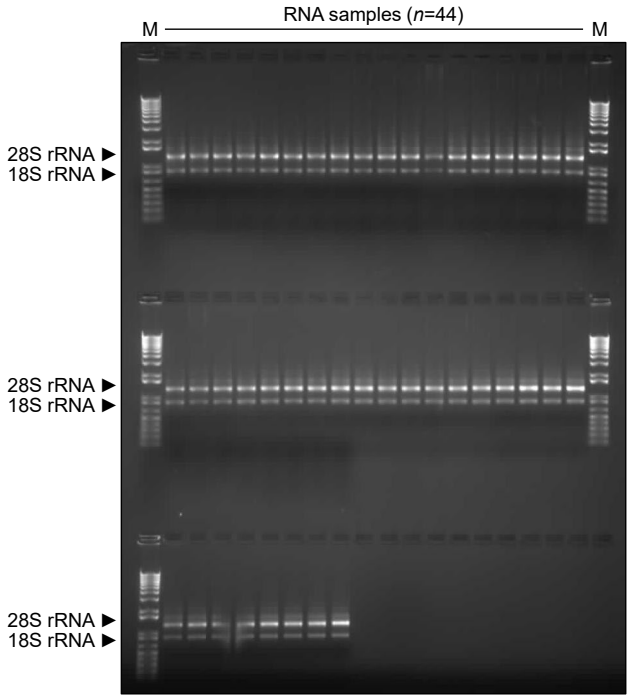

Figure S4.8. Verification of RNA integrity of column-purified RNA samples using 1% agarose gel electrophoresis. All RNA samples in this study (*n*=44) were loaded on a single gel alongside a marker lane (M) with standards. Arrow heads indicate 28S and 18S ribosomal RNA (rRNA) bands. All samples were of high RNA integrity (i.e., tight 28S and 18S rRNA bands, with 28S being approximately twice as intense as 18S).

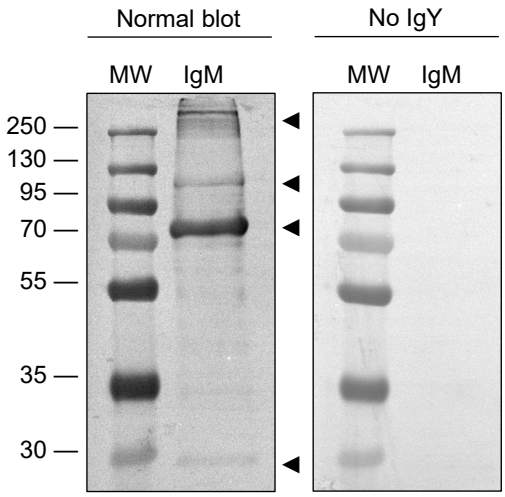

Figure S4.9. Validation of the Western blot assay for the detection of sablefish IgM by the anti-sablefish-IgM antibody. (**Left**) Normal blot with pre-stained marker lane with the molecular weight (MW) of standard bands indicated in kDa, and where arrow heads indicate observed bands (see Figure 4.4 for details). (**Right**) Negative control blot where no primary antibody (chicken anti-sablefish-IgM IgY) was added. The absence of bands confirms that there is no non-specific binding of sablefish IgM by the secondary antibody (rabbit anti-chicken-IgY IgG-ALP).

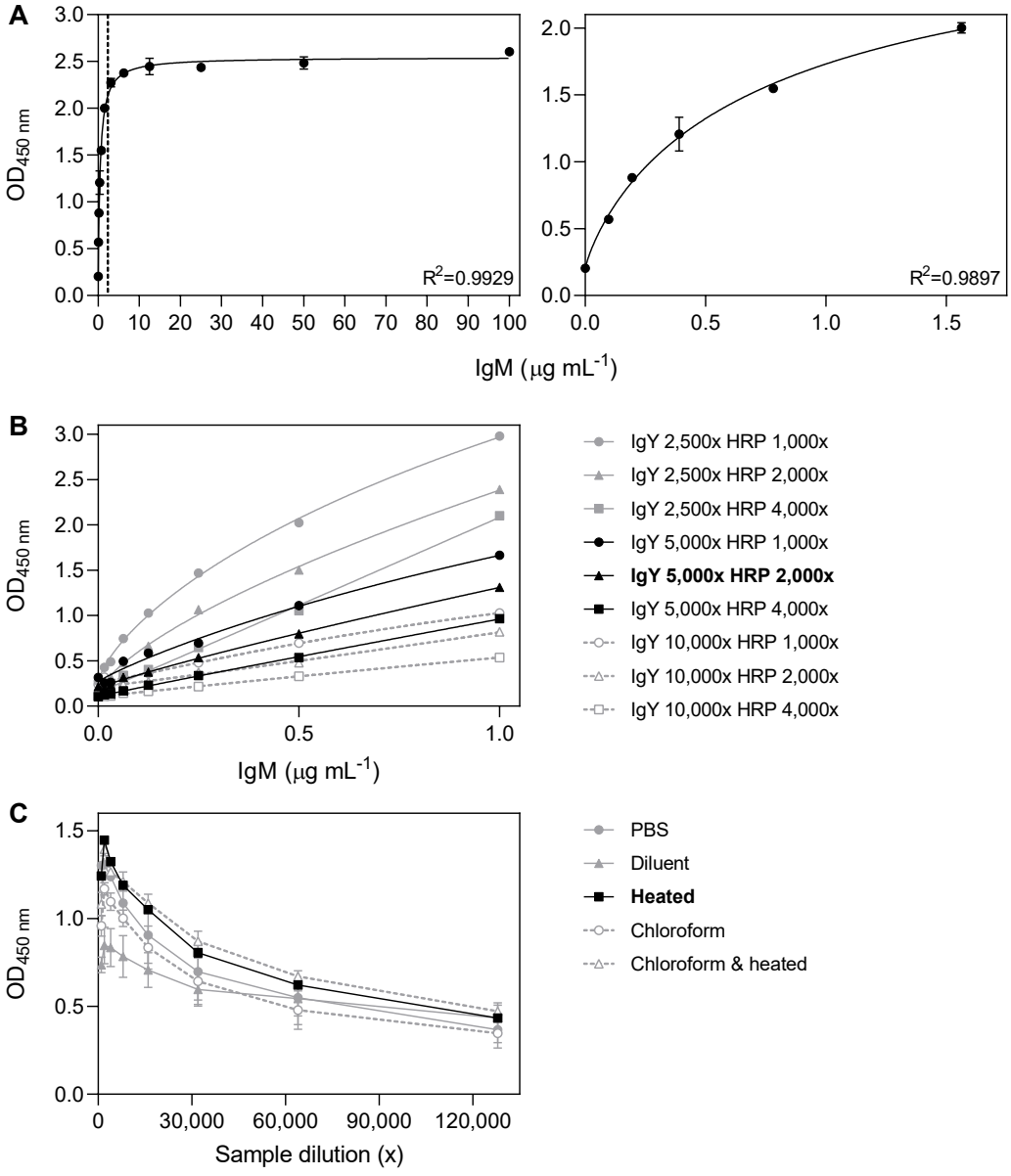
A
B
C
OD450 nm
IgM (μg mL-1)
R2=0.9929
R2=0.9897
IgY 2,500x HRP 1,000x
IgY 2,500x HRP 2,000x
IgY 2,500x HRP 4,000x
IgY 5,000x HRP 1,000x
IgY 5,000x HRP 2,000x
IgY 5,000x HRP 4,000x
IgY 10,000x HRP 1,000x
IgY 10,000x HRP 2,000x
IgY 10,000x HRP 4,000x
PBS
Diluent
Heated
Chloroform
Chloroform & heated
Sample dilution (x)

Figure S4.10. Examples of optimization tests during dELISA method development. (**A**) Optimization of the standard curve range. (**Left**) The relationship between a wide range of known IgM concentrations (0-100 µg mL^{-1}) *vs.* absorbance ($OD_{450\ nm}$). Left of the dotted line is where the relationship was the strongest (i.e., widest OD range and high assay sensitivity). (**Right**) Close-up view of the standard curve range (~0-2 µg mL^{-1}) used for the dELISA method (see Figure 4.5A). The values were fitted using a 4-parameter sigmoidal regression, with the R^2 shown in each panel. (**B**) Optimization of the primary antibody (IgY) and enzyme conjugate (HRP) dilutions. For each reagent, three dilutions were tested using standard IgM concentrations. Dilutions of 5,000x and 2,000x for IgY and HRP, respectively (shown in bold in the legend) were selected because these provided large signal:background values, while also using reagents economically and avoiding extremely high ODs (>2.0). (**C**) Optimization of sample dilution and pre-treatment for inhibitory matrix removal, using a sample pool. Matrix interference of the OD signal was sufficiently removed once samples were diluted at least ~2,000x. Several sample pre-treatments were tested: use of a commercial diluent, heating and/or chloroform extraction. Compared to no pre-treatment (PBS) and the diluent, heating was determined as optimal (bolded in the legend) as it allowed for a high signal, while chloroform extraction appeared to have no effect. All tests were performed in duplicate, and values are means±s.e.m., except for panel B (no duplicates included).

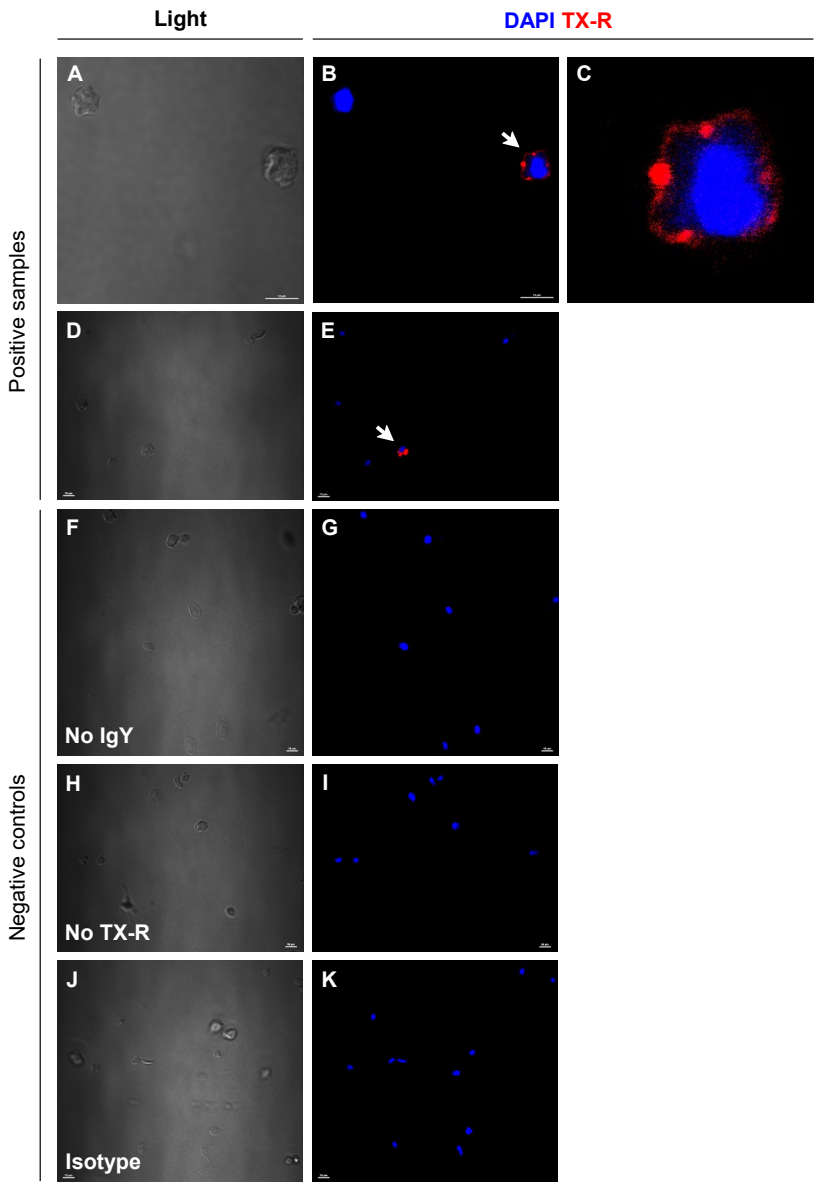

Figure S4.11. Additional confocal microscopy images of IgM$^+$ leukocytes in sablefish blood. (**Left**) Bright field and (**Right**) corresponding fluorescent microscopy photos. (**A**-**E**) Immunostaining of WBCs using anti-sablefish-IgM IgY and TX-R, revealing IgM$^+$ cells with red fluorescence (white arrows). Cell nuclei are stained blue with DAPI. (**C**) Magnified image of the IgM$^+$ cell from panel B showing details of the general morphology and immunolocalization of IgM. (**F**-**K**) Photos taken from negative controls: WBCs prepared (**F**-**G**) without anti-sablefish-IgM IgY; (**H**-**I**) without TX-R; (**J**-**K**) with isotype IgY. No red fluorescence was detected in these samples. Scale bar=10 µm.

Chapter 5: **Summary & Discussion**

The overarching aim of this dissertation was to improve our fundamental understanding of the effects of high temperatures and hypoxia, as two key climate-related stressors, on the cardiorespiratory physiology and immunology of fishes. Using sablefish as a study species (which has significant ecological and economic importance), I obtained a broad physiological dataset that addressed various pressing questions in the field of fish ecophysiology, and this dataset provides evidence for a negative impact of hypoxia on both fish cardiorespiratory and immunological responses. This is an important step towards acknowledging, and better understanding, the different ways that global warming and the spread of hypoxic zones can affect the physiology of both wild and farmed fishes.

In the following sections of my final thesis chapter, I will highlight the main findings of each individual data chapter, their contributions/implications for the field of fish ecophysiology, and their relevance for the areas of fish ecology, fisheries and aquaculture (Section 5.1). After this, I suggest future research directions/approaches that could further advance our knowledge about the physiology of sablefish, and about the effects/consequences of climate-related stressors on the cardiorespiratory and immune function of fishes in general (Section 5.2).

5.1. Thesis Highlights

In Chapter 2, I characterised the environmental tolerances and metabolic physiology of the sablefish, using Atlantic salmon (*Salmo salar*) as a comparative species, and this research was an important foundation for the rest of my thesis. Specifically, I collected data on the tolerance of both species to acute exposure to high temperatures and hypoxia, and on their metabolic response to these two environmental conditions. I found

that, as adults, the sablefish's upper thermal tolerance is very similar to that of Atlantic salmon (CT_{max} ~25°C *vs.* ~26°C, respectively), whereas the sablefish was much more hypoxia tolerant (O_2 level at LOE ~5% *vs.* ~24% air sat., respectively). In fact, the sablefish's P_{crit} (~16% air sat.) is comparable to that of other hypoxia-tolerant species like the goldfish (*Carassius auratus*) (Fu *et al.*, 2011) and tidepool sculpin (*Oligocottus maculosus*) (Mandic *et al.*, 2009). This is the first time that these tolerance metrics have been determined for the sablefish, and they establish this species as being very tolerant to both of these particular climate stressors.

In Chapter 2, I also explored whether metabolic parameters, such as AS_T and RMR, could explain differences in thermal and hypoxia tolerance, respectively, between life-stages (juvenile *vs.* adult) and species (sablefish *vs.* Atlantic salmon). This idea was based on the OCLTT concept (Pörtner, 2010, Pörtner *et al.*, 2017) which postulates that AS is a primary determinant of fish thermal tolerance, while hypoxia tolerance is generally considered to be dependent on RMR (Farrell *et al.*, 2009, Mandic *et al.*, 2009, Claireaux *et al.*, 2016, Killen *et al.*, 2016). Interestingly, AS_T was the same across all tested groups (~180 mg O_2 $kg^{-0.80}$ h^{-1}) despite significant differences in CT_{max} among life-stages and species, and the RMR of adult sablefish was also only slightly (~20%) lower than in adult salmon. The former findings add to the ongoing, and rapidly evolving, debate about the usefulness of the OCLTT concept (e.g., see Jutfelt *et al.*, 2018, Pörtner *et al.*, 2018), and are in line with other studies that have challenged the OCLTT concept (e.g., Ekström *et al.*, 2016, Ern *et al.*, 2016, Lefevre, 2016, Lefevre *et al.*, 2021).

High temperatures and hypoxia often co-occur in marine and freshwater environments (e.g., Kemp *et al.*, 2005, Breitburg *et al.*, 2018, Del Rio *et al.*, 2019).

However, prior to this thesis research, only one study (Ern *et al.*, 2016) had examined the effect of hypoxia on fish thermal tolerance, and the consequences of hypoxia for cardiorespiratory responses to acute warming had not been investigated; despite the fact that cardiac function appears to be a primary factor in determining the upper thermal limits of fishes (e.g., see Wang *et al.*, 2007, Eliason *et al.*, 2017, Farrell *et al.*, 2017). Therefore, in Chapter 3, I addressed this major knowledge gap using the sablefish as a model, and discovered that acute hypoxia (40% air sat.) prevented the temperature-dependent increases in f_H and $\dot{Q}$ that normally occur in fishes (i.e., under normoxic conditions), and that this was associated with reductions in MMR and CT_{max} of 48% and 3°C, respectively. These findings further corroborate that cardiac function is a primary determinant of AS_T and thermal tolerance, and suggest that there is a 'physiological chain of demand' whereby the depression of f_H by hypoxia (bradycardia; Farrell, 2007, Gamperl *et al.*, 2014) appears to overrule the requirement for increasing f_H (tachycardia) with warming. These results are highly novel in the field of fish cardiorespiratory physiology, and identify hypoxia as a climate stressor that can severely constrain a fish's ability to respond to, and tolerate, high temperatures.

In Chapter 3, I also tested whether acclimation to hypoxia (4-6 months at 40% air sat.) alters the sablefish's temperature-dependent physiological responses and improves this species' CT_{max} under hypoxic conditions. I found: 1) that while hypoxia-acclimated sablefish no longer experience bradycardia when exposed to acute hypoxia, their f_H and $\dot{Q}$ still failed to increase with temperature; and 2) that despite having an AS_T under hypoxia that was slightly higher as compared to normoxia-acclimated fish (~87 *vs.* ~59 mg O_2 kg^{-1} h^{-1}), their scope for enhancing O_2 extraction ($\dot{M}O_2/\dot{Q}$) and their CT_{max} were not

different. Thus, hypoxic acclimation did not fully offset the negative effects of acute hypoxia on cardiorespiratory function and thermal tolerance (i.e., there is limited plasticity), and I found no evidence of cross-tolerance (i.e., hypoxic acclimation did not improve thermal tolerance). These findings provide valuable insights into the level of phenotypic plasticity in fishes (e.g., Muñoz *et al.*, 2015, Sandblom *et al.*, 2016, Morgan *et al.*, 2020, White *et al.*, 2020), and whether there is cross-tolerance between these two O_2-limiting conditions (e.g., Levesque *et al.*, 2019, Collins *et al.*, 2021, Del Rio *et al.*, 2021, McDonnell *et al.*, 2021). Limitations in the sablefish's physiological plasticity when chronically exposed to hypoxia may be, in part, due to the fact that this species often inhabits OMZs as adults, and thus, it might already be well-adapted to low O_2 levels and unable to further enhance/optimize certain responses/traits. These results underline the interaction/interplay between plasticity, evolution, and environmental stressors (e.g., see Diamond *et al.*, 2016, Donelson *et al.*, 2019).

Before the completion of this thesis research, the literature was already in agreement that acute hypoxia impairs immune function in fishes (Yada *et al.*, 2002, Bowden, 2008, Abdel-Tawwab *et al.*, 2019). However, research findings about the immunomodulatory nature of chronic hypoxia were equivocal (e.g., Kvamme *et al.*, 2013, Gallage *et al.*, 2016, Magnoni *et al.*, 2019, Martínez *et al.*, 2020, Zanuzzo *et al.*, 2020). Thus, the aim of Chapter 4 was to gain a better understanding of how long-term hypoxia affects fish immunity. Similar to Chapter 3, I acclimated sablefish to 40% air sat. (for up to 5 months). Subsequently, I measured several innate and adaptive immunity parameters before and after injection with antigens from a relevant bacterial pathogen (i.e., a formalin-killed *Aeromonas salmonicida* bacterin). I found that chronic hypoxia did not

influence baseline innate immunity, and that there was no substantial change in the innate immune response to the bacterin. On the other hand, I found that chronic hypoxia significantly modified the sablefish's adaptive immune response. This included completely preventing the rise in total serum IgM levels at 10 weeks following bacterial antigen exposure, and increasing the relative abundance of peripheral IgM^+ cells (from ~26% to ~42% of WBCs) at this time point regardless of bacterin injection. These results highlight that distinct branches of the fish immune system can respond differently to environmental O_2 limitation; specifically, that there is a dichotomy in the sensitivity to long-term hypoxia exposure whereby innate immunity is largely unaffected, but adaptive immunity is strongly constrained. Interestingly, my findings in sablefish are consistent with a recent paper by Schäfer *et al.* (2021) which reported similar effects of chronic hypoxia on the immune status of pikeperch (*Sander lucioperca*).

To be able to assess immune function in sablefish, I developed various species-specific assays in Chapter 4, including qPCR primers for 16 bacteria-responsive innate immune-relevant genes, and a direct ELISA for the measurement of serum IgM levels. This is another important contribution of this thesis chapter, because novel tools are now available to enhance research on sablefish immune function. Prior to this dissertation these had been limited to assessing disease susceptibility and vaccine efficacy (Arkoosh *et al.*, 2015, Arkoosh *et al.*, 2018), and to measuring lysozyme concentration (Kim *et al.*, 2017a-b). Finally, the qPCR study in Chapter 4 revealed that for a number of innate immunity genes (*cox1*, *hamp*, *il1b*, *il8*, *stat1* and *tlr5*) there appear to be two paralogues/isoforms in sablefish, which can have divergent transcriptional responses. This finding suggests a number of additional research projects aimed at better understanding

the evolutionary trajectory of immune gene paralogues that may have undergone subfunctionalization or neofunctionalization.

5.2. Future Research Directions

This dissertation has provided several findings/observations which show that sablefish have significant innate ability and plasticity to cope with both acute and chronic hypoxia. For instance, in Chapter 2, I showed that the O_2 level at LOE for adult sablefish was as low as ~5% air sat., and in Chapters 3 and 4, I was able to acclimate this species to moderate hypoxia (40% air sat.) for up to 5-6 months while individuals continued to feed and grow (depending on the experiment, weight gain was ~55-88%). Sablefish can also survive weeks of exposure to 20% air sat. (Gerber *et al.*, 2019). Although these findings are not surprising given that sablefish are known to inhabit OMZs in the wild (Mason *et al.*, 1983, Moser *et al.*, 1994, Doya *et al.*, 2017), it is not entirely clear what mechanisms might confer their high hypoxia tolerance. The information collected in this thesis related to cardiorespiratory physiology does not provide a sufficient explanation. For example, the sablefish's heart (RVM ~0.08%; Chapters 2 and 3) is not unusually large for fishes (Farrell *et al.*, 2017), their Hct (~22%; Chapter 3) is intermediate compared to other fish species [e.g., Atlantic salmon: ~30% (Deitch *et al.*, 2006, Harter *et al.*, 2019), flatfish: ~15% (Wood *et al.*, 1979, Turner *et al.*, 1983)], their RMR is only ~20% lower than that of Atlantic salmon (Chapter 2), and finally, the sablefish did not utilize anaerobiosis to endure acute or chronic hypoxia (plasma lactate levels were barely detectable, <0.1 mM; Chapters 2 and 3). On the other hand, I found that sablefish can greatly enhance O_2 extraction (>2-fold increase in $\dot{M}O_2/\dot{Q}$; Chapter 3), and Gerber *et al.* (2019) showed that

this species maintains cardiac mitochondrial function despite chronic exposure to hypoxia. Likely, it is a combination of evolved adaptations like these that give the sablefish its innate hypoxia tolerance. Elucidating these adaptations is an exciting opportunity for future research, because the sablefish appears to be a suitable hypoxia-tolerant model to gain insights into mechanisms of hypoxia tolerance in fishes in general.

A key finding in Chapter 3 was that sablefish that initiated bradycardia when acutely exposed to hypoxia were unable to/did not increase f_H or $\dot{Q}$ in response to warming. Recently this result has been confirmed in Atlantic salmon (Gamperl *et al.*, in prep.). The consistency of this phenomenon in these two phylogenetically distant species, suggests that it may have broad application to teleosts, and this warrants further investigation. A crucial question that future studies will need to answer, is what mechanism(s) is/are responsible for the suppression of f_H during warming. Because hypoxic bradycardia in fishes is induced by an increase in cholinergic tone (Farrell, 2007, Farrell *et al.*, 2017) on the heart, a plausible explanation is that these fish are unable to remove/reduce this nervous tone, and that this prevents increases in f_H. A second candidate mechanism is that adenosine, produced by the myocardium when O_2 supply is limited, depresses f_H (Sundin *et al.*, 1999, Stecyk *et al.*, 2007).

Another important finding in Chapter 3 was that, because there was no increase in $\dot{Q}$ during hypoxic warming, sablefish relied solely on enhanced O_2 extraction to meet their elevated metabolic O_2 demands. In sablefish warmed under normoxia, $\dot{Q}$ doubled, but they still depended on O_2 extraction to the same extent to increase $\dot{M}O_2$, which is also true for European eel (Claësson *et al.*, 2016). This demonstrates the vital importance of

O_2 extraction as a means to deliver more O_2 to the tissues during acute warming in a number of fishes. However, the role of O_2 extraction in determining thermal tolerance (at least in some fish species and under certain circumstances) has been underappreciated to date. Furthermore, it is still unclear what processes are facilitating arteriovenous extraction in this species, especially when hypoxia coincides with warming. The sablefish lacks β-NHE on their red cell membranes (Rummer *et al.*, 2010), and this would make them even more sensitive to the role that PaCA could play in tissue O_2 offloading/extraction (e.g., see Rummer *et al.*, 2013, Randall *et al.*, 2014, Alderman *et al.*, 2016, Harter *et al.*, 2019). However, the increased $\dot{M}O_2/\dot{Q}$ was partially mediated by a ~25% increase in blood Hb levels (which could have resulted from splenic red cell release, as seen in exhaustively exercised fish; e.g., see Gallaugher *et al.*, 1992) and this likely elevated blood O_2 content/carrying capacity. Further, we do not know if/how hypoxia affects Hb-O_2 affinity or the magnitude of the Root effect in this species.

The findings in Chapter 4 also lead to new questions that could be addressed in the future. For instance, chronic hypoxia affected the transcript levels of only 3 of the 16 studied innate immunity-relevant genes (*ccl19*, *stat1a* and *tlr5b*) in the sablefish spleen after *A. salmonicida* bacterin injection, and while these were also relatively small changes (<2-fold) compared to the overall transcriptional response to the bacterin, it is still worthwhile to investigate whether these have any downstream physiological consequences. Among the three dysregulated genes, *ccl19* and *tlr5b* showed the largest fold change (>1.5), while the effect on *stat1a* is notable because: (i) this signal transducer/transcription activator controls the expression of several genes in the IFNγ-induced JAK-STAT pathway (Murphy *et al.*, 2017); and (ii) a comparable shift in

stat1b expression after vaccination was reported in Atlantic salmon exposed to chronic hypoxia (Zanuzzo *et al.*, 2020). RNA sequencing (RNA-seq) could be a suitable tool to explore whether the modulation of *stat1a* transcript levels by hypoxia results in altered expression of JAK-STAT pathway genes. In addition, I found that long-term hypoxia prevented the increase in sablefish IgM levels in response to *A. salmonicida* bacterin injection, which may ultimately result in a higher susceptibility to furunculosis. The latter is likely, given that peak IgM levels correlated positively with immunoprotection (survival) in sablefish vaccinated against *A. salmonicida* and subsequently infected with this pathogen (Vasquez *et al.*, 2020). Furthermore, Nile tilapia (*Oreochromis niloticus*) held under hypoxic conditions had lower IgM titres after vaccination against the bacterial pathogen *Streptococcus agalactiae*, and suffered from higher mortality rates when then challenged with live *S. agalactiae* (Gallage *et al.*, 2017). Nevertheless, the hypothesis that impaired post-vaccination antibody production due to O_2 limitation has direct consequences for the health and disease resistance of sablefish needs to be tested using live pathogens.

The above list of future research questions is not exclusive, and in the individual data chapters, further research possibilities are discussed in more detail. However, there is one final future research perspective that arises when considering the overall results of this dissertation. Both acute and chronic hypoxia had negative effects on the sablefish's cardiorespiratory and immune function, and acute thermal tolerance, despite this species being hypoxia-tolerant. This illustrates, and emphasizes, the importance of hypoxia as a climate stressor that is capable of strongly shaping the physiology, distribution and survival of fishes. Interestingly, this agrees with the meta-analysis recently performed by

Sampaio *et al.* (2021). These authors compared the effects of hypoxia with ocean warming and acidification effects on marine ectotherms, and indicated that hypoxia has been understudied as an environmental condition and elicits the most consistent negative effects on biological performance across taxonomic groups, including fish. Altogether, these findings call for fish ecophysiologists to include hypoxia as a single, or combined, stressor in experimental studies about climate change. This is essential for improving our scientific knowledge with regard to the consequences of this global change on fishes. They are the largest group of vertebrates (Helfman *et al.*, 2009), and often have a central position in conservation efforts and are of major socio-economic importance worldwide.

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