Effects of Rearing Temperature on the Thermal Tolerance, Metabolic Capacity and Stress Response of Cultured Lumpfish (*Cyclopterus lumpus*)

by

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Abstract

Lumpfish (*Cyclopterus lumpus*) mortalities have been reported during the summer at some North Atlantic salmon cage-sites where they serve as 'cleaner fish'. To understand their physiology, and whether limitations in their metabolic capacity and thermal tolerance can explain this phenomenon, I compared the aerobic scope (AS) of 6°C-acclimated lumpfish using a critical swim speed (U_{crit}) test, a critical thermal maximum (CT_{Max}) test (rate of warming 2°C h^{-1}) and a chase to exhaustion. The U_{crit} and CT_{Max} of 45-75g lumpfish were 2.36 $\pm$ 0.08 body lengths s^{-1} and 20.6 $\pm$ 0.3°C, respectively. The AS of lumpfish was higher during the U_{crit} test (206.4 $\pm$ 8.5 mg O_2 kg^{-1} h^{-1}) vs. that measured in either the CT_{Max} test or after the chase (141.0 $\pm$ 15.0 and 124.7 $\pm$ 15.5 mg O_2 kg^{-1} h^{-1}). Next, I examined whether changing temperatures during incubation/rearing influenced the lumpfish's AS, CT_{Max} and IT_{Max} (incremental thermal maximum, measured by warming at 0.1-0.2°C day^{-1}), and stress physiology. Temperature combinations included 6°C/9°C (that used in standard production protocols), 8.5°C/9°C, 6-11°C/9°C, 8.5°C/9-11°C and 6-11°C/9-11°C, with ranges indicating stochastic changes. The lumpfish's upper thermal tolerance (CT_{Max}, 22.85 $\pm$ 0.12°C; IT_{Max}, 20.63°C) and AS were not influenced by incubation or rearing temperatures, and based on these values it does not appear that cage-site mortalities during the summer are related to high water temperatures.

General Summary

In the North Atlantic, aquaculture companies are using lumpfish (*Cyclopterus lumpus*) as 'cleaner fish' to control sea lice at marine cage-sites and improve salmon production and welfare. However, lumpfish mortalities have been reported at some cage-sites during the summer months. Thus, I compared the metabolic capacity of juvenile lumpfish using a number of standard methods [a critical swim speed (U_{crit}) test, a critical thermal maximum (CT_{Max}) test, and chasing to exhaustion] to better understand their metabolic capacity and physiology. Next, lumpfish eggs were incubated, and larvae and juveniles were reared, under different temperature regimes to determine if the upper thermal tolerance and welfare of lumpfish could be improved by altering rearing protocols. I found that while altering temperatures during egg incubation lowered lumpfish survival (hatching success), it did not affect their metabolic capacity, thermal tolerance or stress physiology as juveniles. Further, based on this research, it does not appear that summer water temperatures (< 20°C) are directly contributing to cage-site mortalities of lumpfish.

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Co-Authorship Statement

The research presented in this M.Sc. thesis was conducted by Rachel Eisenberg, under the supervision of Dr. A. Kurt Gamperl. Rachel Eisenberg shared in the selection and identification of the research topic, and experimental design, and was primarily responsible for: the rearing and care of experimental animals; conducting the experiments; the collection and analysis of data; and writing of the first draft of this thesis. This project was made possible via the collaboration of many individuals.

Rebeccah M. Sandrelli[1] assisted in the running of the experiments / sampling in both **Chapters 2 and 3.** Émile Vadboncoeur[1] assisted in the experiments / sampling in **Chapter 3**. Staff members of the Dr. Joe Brown Aquatic Research Building (JBARB; Denise Tucker[2], Jennifer Monk[2] and Jillian A. Porter[2]) provided training and assistance in lumpfish spawning, egg fertilization and rearing in **Chapter 3.** Danny Boyce[2] provided the experimental fish used in **Chapters 2 and 3** and research space in the JBARB facility for lumpfish rearing in **Chapter 3.** Dr. A. Kurt Gamperl[1] provided supervision, and input and expertise throughout the research program, including: the identification of research topics; experimental design; the execution of experiments; data collection and analysis; and providing edits and feedback on the thesis.

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List of Abbreviations and Symbols

%	Percent
°C	Degree centigrade
α	Significance
λ	Coefficient for shape of the fish
ϵ_s	Error due to solid blocking
τ	Dimensionless factor of 0.8
µm	Micrometer
A	Amplitude
A_0	Cross-sectional area of the fish
A_{exp}	Fractional area exponent
AS	Aerobic scope
ASR	Realistic aerobic scope
A_T	Cross-sectional area of the swimming chamber
BL	Body length
BMI	Body mass index
cirbp	Cold-inducible RNA binding protein
cm	Centimeter
CT_{Max}	Critical thermal maximum
dd	Degree days
ddpf	Degree days post-fertilization
ddph	Degree days post-hatch
dph	Days post-hatch
e.g.	For example
ELISA	Enzyme-linked immunosorbent assay
EPOC	Excess post-exercise oxygen consumption
G	Girth
g	Gram
h	Hour
HSI	Hepatosomatic index
hsp	Heat shock protein
Hz	Hertz
ID	Identification
i.e.	That is
IT_{Max}	Incremental thermal maximum
JBARB	Dr. Joe Brown Aquatic Research Building
kg	Kilogram
L	Litre
l	Length
LASCCR	Laboratory for Atlantic Salmon and Climate Change Research
LOE	Loss of equilibrium
lux	Unit of light intensity
m	Mass
mg	Milligram
min	Minute

mL	Millilitre
mm	Millimeter
MMR	Maximum metabolic rate
MO_2	Rate of oxygen consumption (mg O_2 kg^{-1} h^{-1})
n	Sample size
O_2	Oxygen
O_2 sat.	Oxygen saturation (%)
OCLTT	Oxygen- and capacity-limited thermal tolerance
p	Probability
PIT	Passive integrated transponder
PO_2	Partial pressure of oxygen
ppt	Parts per trillion
qPCR	Quantitative polymerase chain reaction
r	Radius
rpm	Rotations per minute
RMR	Routine metabolic rate
RVM	Relative ventricular mass
s	Second
SGR	Specific growth rate
SMR	Standard metabolic rate
T	Temperature
t	Time (days)
t_f	Elapsed time
TGC	Thermal growth coefficient
t_i	Time increment
TMS	Tricaine methanosulfonate
ucp	Uncoupling protein
U_{crit}	Critical swim speed
USA	United States of America
V	Velocity
v	Volts
V_F	Velocity at fish's maximum girth
V_i	Velocity increment
V_R	Velocity at the rear of the flume
vs.	Versus
W	Watts

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Chapter 1 - General Introduction

1.1: Climate Change

Current predictions are that global surface ocean temperatures will rise by approximately 1.5°C between 2030 and 2052 because of climate change, however, this will vary by region (IPCC, 2021). For example, it is estimated that the average summer and winter temperatures will increase by 3.0°C and 4.3°C, respectively, on Newfoundland's south coast by the middle of the century (https://www.gov.nl.ca/eccm/files/publications-the-way-forward-climate-change.pdf). Marine heatwaves have also become more severe and frequent over the past few decades (Frölicher et al., 2018; Oliver et al., 2018), with the cumulative number of days per year that marine heat waves occurred increasing globally by 30-50 days between the 20th and 21st centuries (Smale et al., 2019). These long- and short-term temperature increases could have far-reaching impacts on the physiology of organisms, the size, structure and distribution of marine species/populations, community species' composition, and the structure and functioning of ecosystems (Lotze et al., 2019; Hillebrand et al., 2018; Tittensor et al., 2010; Baum and Worm, 2009; Pörtner et al., 2008). The impact on particular species could include spawning and feeding distributions (Rose, 2005), shifts in their range towards higher latitudes and changes in fish abundance (Pörtner, 2021; Vergés et al., 2014; Doney et al., 2012; Beaugrand et al., 2008), and increased competition in niche areas like colder northern waters (Deutsch et al., 2015). For instance, if a heat wave occurs during the time of year coinciding with a population's pre-spawning period, that population's reproductive success may be diminished and this could cause cascading effects on its population strength and abundance (Pountney et al., 2020; Stillman, 2019).

1.2: Impacts of Differences in Environmental Temperature on Embryonic Development

Environmental variation prior to spawning / fertilization and during the embryonic period, including temperature changes, can affect embryonic development and significantly influence responses to environmental challenges later in life [see review by Beaman et al. (2016)]. These phenotypic changes, termed "developmental plasticity", can be positive or negative, and affect acclimation potential (also called 'reversible plasticity') (Lim et al., 2020; Rossi et al., 2020; Beaman et al., 2016). Thus, it is important to understand how various climate-driven environmental challenges encountered during the pre-zygotic (pre-fertilization), zygotic, and embryonic periods affect the biology and physiology of aquatic organisms (including fishes).

When sexually mature lumpfish were held at a temperature (14°C) above their thermal optimum (<10°C), a 50% reduction in sperm density was reported in males, and no eggs survived to the eyed stage (Pountney et al., 2020). These data show that embryonic development can be significantly impacted if the parents experience suboptimal high temperatures. Further, exposure to various environmental conditions during embryonic development can have major effects on this life stage. For example, lake whitefish (*Coregonus clupeaformis*) eggs exposed to warmer and more variable temperatures than normal during incubation experienced increased mortality, hatched earlier and had increased post-hatch metabolic costs (Eme et al., 2018). When lumpfish eggs were exposed to ambient (cold) temperatures (4-6°C) during incubation, the eggs experienced high mortality (38.5%) and low hatching success (46.1%). Whereas, while exposure to fluctuating temperatures (ambient to 10°C) resulted in high hatching success (74.9%), warm-exposure (to a constant temperature of 10°C) reduced the incubation period (by 9 days) and was associated with a high occurrence of deformities (34.7%) (Imsland et al., 2019). These latter data indicate that fish possess lower and upper thermal limits for embryonic development, and are consistent with a study

on golden pompano (*Trachinotus auratus*) where the exposure of eggs to temperatures (32°C) above optimum ($\leq$ 29°C) during incubation resulted in high rates of spinal deformity post-hatch (Han et al., 2020). These observations support the conclusion that deviations of temperatures from an individual's thermal optimum during embryonic development can have a number of, sometimes detrimental, effects on fish morphometrics, physiology and survival.

1.3: Impacts of Differences in Environmental Temperature on Later Life History Phenotypes

Altered environmental temperature during development can also impact the phenotype of fishes at later life history stages. For instance, lab-bred zebrafish (*Danio rerio*) exposed to a variable environment of $\pm$ 6°C while reared to adulthood had a higher thermal tolerance (critical thermal maximum; CT_{Max}) compared to zebrafish reared at constant temperatures (Schaefer & Ryan, 2006). Rearing embryos at 14 vs. 10°C increased the CT_{Max} of Chinook salmon (*Oncorhynchus tshawytscha*) by ~ 1.0°C (Del Rio et al., 2016), and when incubated and/or reared in warm temperatures (15°C) fry of this species had improved swimming endurance (Lim et al., 2020). Rossi et al. (2020) showed that exposure of tropical killifish (*Kryptolebias marmoratus*) to environmental fluctuations (cycles of immersion and emersion vs. constant emersion) during larval rearing can alter the adult phenotype and diminish phenotypic flexibility. Finally, Scott & Johnston, (2012) showed that incubating zebrafish embryos at relatively warm temperatures (27°C and 32°C) improved their critical swimming speed (U_{crit}) by ~15% when they swam at their respective incubation temperatures, suggesting that warming during brief embryonic windows may improve zebrafish's thermal tolerance at later life history stages. However, not all studies have shown positive effects of exposure to warmer temperatures during early life history stages.

For example Motson and Donelson (2017) showed that new recruits (i.e., early juveniles) of *Halichoeres malanurus, Halichoeres miniatus, and Thalassoma amblycephalum* reared at 29 vs. 31°C (the projected temperature in 2100) had reduced growth, as well as metabolic and swimming performance, and Illing et al. (2020) reported that rearing new recruits of three species of tropical fishes (*Acanthochromis polyacanthus, Amphiprion malanopus, Lates calcarifer*) at 30 vs. 28.5°C had no effects on their CT_{Max} or tissue citrate synthase activity (an index of aerobic capacity).

The majority of research regarding changes in rearing temperature, and its impacts on later life history phenotypes, has been conducted on warm water and/or eurythermal species (see Eme et al., 2018), and there is limited understanding of whether similar effects are seen in cold/cool water fishes. Further, it is not clear whether more ecologically relevant indices of thermal tolerance for these species are affected by changes in temperature during embryonic development. Much of the research in this area has exclusively used CT_{Max} as a metric to determine the upper thermal tolerance limits of fishes (Morgan et al., 2021; Illing et al., 2020; Ern et al., 2016; Del Rio et al., 2019; Leeuwis et al., 2019; Morgan et al., 2018). Although utilizing a CT_{Max} test does provide useful information regarding acute temperature tolerance, it is not representative of the long-term temperature changes that temperate marine fish species experience in the wild or in aquaculture (e.g. see; Burt et al., 2012; Gollock et al., 2006; Björnsson, 1997). Therefore, it is important that protocols such as the incremental temperature maximum (IT_{Max}) test be performed to determine how long-term temperature changes impact fishes (e.g., see Beemelmanns et al., 2021, Gamperl et al., 2020, Zanuzzo et al., 2019).

1.4: Lumpfish

The common lumpfish (*Cyclopterus lumpus*) is a semi-pelagic, and ecologically important, teleost found throughout the North Atlantic, including off the coasts of Norway, the United Kingdom, Greenland, Iceland and Canada (Jónsdóttir et al., 2018; Kennedy et al., 2015; Hedelholm et al., 2014; Pampoulie et al., 2014; Davenport, 1985). However, due to overfishing/harvesting, lumpfish have been designated as 'Threatened' by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC, 2017). The lumpfish is also a commercially important species due to its large geographical range, the demand for their roe as a substitute for sturgeon caviar, and most recently, their use as 'cleaner fish' in the Atlantic salmon (*Salmo salar*) aquaculture industry (Powell et al., 2018; Imsland et al., 2014). Lumpfish will feed on sea lice (*Lepeophtheirus salmonis*), and remove these damaging parasites from salmon in sea-cages (Imsland et al., 2019; Jónsdóttir et al., 2018). The utilization of lumpfish as a cold-water 'cleaner fish' is gaining traction quickly, as cleaner fish are an environmentally friendly ('green') and efficient option for removing sea lice from salmon in sea-cages as compared to medicated baths or other treatments (e.g., hydrogen peroxide, mechanical removal, and 'thermocycling') that can result in salmon mortality rates ranging from 15-30% (Imsland et al., 2019; Overton et al., 2019). However, lumpfish mortalities have been reported at some salmon sea-cages, especially in the summer months, and this raises concerns about lumpfish welfare (Rabadan et al., 2021; Geitung et al., 2020; Klakegg et al., 2020; Hvas et al., 2018). This is not surprising as lumpfish prefer temperatures of approximately 7°C (Geitung et al., 2020; Mortensen et. al, 2020), they experience significant mortalities (in addition to cataracts and erratic swimming behaviour) when acclimated to 18°C, and ocean temperatures where Atlantic salmon sea-cages are located can be as high as 18-20°C in the summer (Gamperl et al., 2021; Burt et al., 2012; Björnsson et al., 2007).

1.5: Effects of Rearing Temperature on Lumpfish Thermal Tolerance and Physiology

If incubation and/or rearing temperature can increase the upper thermal tolerance and metabolic capacity of, and reduce temperature-dependent stress in, lumpfish it is possible that this new phenotype will be better equipped to survive in warmer temperatures, and thus, alleviate (or at least diminish) the salmon industry's and the public's concerns about their welfare in salmon cages. An increase in this species' metabolic capacity could be a key response to alterations in incubation/rearing temperature as it is has been proposed that metabolic (aerobic) scope largely determines a fish's upper temperature tolerance [although this Oxygen and Capacity Limited Temperature Tolerance concept is presently controversial (Pörtner, 2021; Ern, 2019; Pörtner et al., 2017; Ern et al., 2016; Clark et al., 2013); and that it safeguards the energy put into growth and performance from the effects of environmental variation (Beaman et al., 2016)].

1.6: Research Goals

The main goal of my thesis was to determine how incubation and early rearing temperatures affect the phenotypic plasticity of lumpfish, with regard to: short- and long-term temperature tolerance (CT_{Max} and IT_{Max}, respectively); their metabolic capacity as measured by their maximum metabolic rate (MMR) and aerobic scope (AS); and their stress response as measured by plasma cortisol levels. Despite some information on variable hatching success, growth rates and changes in thermal niche with temperature (Imsland et al., 2019; Hvas et al., 2018; Nytrø et al., 2014), and data on lumpfish growth, metabolic capacity and the effects of hypoxia on its CT_{Max} (Jørgensen et. al, 2017; Ern et al., 2016; Killen et al., 2007), little is known about this species' thermal biology or whether temperature-induced changes during early life history (i.e., developmental plasticity) will influence their capacity to deal with environmental changes later in life (i.e., is there an influence on their 'reversible plasticity'/'acclimation capacity'). The proposed IT_{Max} experiments will be particularly informative with regards to the latter topic and for predicting how this species might be impacted by climate change, in addition to providing valuable information to the global Atlantic salmon aquaculture industry.

1.7: References

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Chapter 2 - Comparing Methods for Determining the Metabolic Capacity of Lumpfish (*Cyclopterus lumpus*)

2.1: Introduction

The metabolic limits/capacity of a species are often estimated by measuring its aerobic scope (AS). This parameter is calculated by subtracting an animal's minimum or 'standard' metabolic rate (SMR) from its maximum metabolic rate (MMR; Raby et al., 2020; Norin and Clark, 2016; Norin et al., 2016; Clark et al., 2013), and provides an estimate of the total amount of oxygen consumption / aerobic-based metabolism that an individual has available to meet increased energy demands above that required to support essential physiological functions. In contrast, the fish's routine metabolic rate (RMR; also known as resting metabolic rate), and by extension its ASR ('realistic' aerobic scope; determined by subtracting RMR from the MMR), considers context-specific oxygen consumption beyond basal levels that includes energy used for routine activity, growth, reproduction and/or other functions (Burton et al., 2011; Killen et al., 2007b).

Knowing a species' metabolic capacity (i.e., MMR, AS and ASR), and its limitations under various conditions/stressors, is essential to understanding a species' physiological constraints and their potential impacts on its biology. Many studies report significant differences in MMR and AS when comparing methods of 'exhaustion', and different types of stressors (e.g., exercise vs. heat stress: Raby et al., 2020; Zhang et al., 2020; Hvas and Oppedal, 2019; Paschke et al., 2018; Reidy et al., 1995). As various methods of 'exhausting' fish often provide different values for MMR, and by extension AS and ASR, it is important to determine which method provides the maximum/'true' value for metabolic capacity of a given species, and the relationship between these context-specific values. For example, one should determine: if the metabolic capacity of a fish species differs (and by how much) when exposed to these various challenges, each of which should maximize the fish's oxygen consumption under a particular set of conditions (i.e., when exposed to an exercise

vs. temperature stressor); and if it does, is the difference between them predictable such that it will allow one measure of metabolic capacity to be estimated/determined from another (i.e., can a 'correction factor' be applied)?

The common lumpfish (*Cyclopterus lumpus*) is a semi-pelagic, and ecologically important, teleost species found throughout the North Atlantic [including off the coasts of Norway, the United Kingdom, Spain, Greenland, Iceland and Canada (Jonsdottir et al., 2018; Kennedy et al., 2015; Hendelholm et al., 2014; Pampoulie et al., 2014; Bañón et al., 2008; Davenport, 1985)], that is being commercially produced at an increasing rate for use in the salmon aquaculture industry. Lumpfish are being used as 'cleaner fish' at Atlantic salmon (*Salmo salar*) cage-sites as a biological control for sea lice (*Lepeophtheirus salmonis*; Coates et al., 2021; Imsland et al., 2018; Powell et al., 2018; Imsland et al., 2014); one of the most serious production challenges to the production of this valuable species. They are effective in eating sea lice off the salmon, thus improving production at cage-sites and the salmon's health/welfare, and limiting mortalities (Solveig et al., 2023; Boissonnot et al., 2022; Imsland and Reynolds, 2022; Imsland et al., 2019; Eliasen et al., 2018; Imsland et al., 2018; Imsland et al., 2016; Imsland et al., 2014). However, it has recently been reported that a significant proportion of lumpfish stocked into sea-cages die annually (Foss and Imsland, 2022; Geitung et al., 2020). The proposed reasons for this mortality include high ocean temperatures, insufficient water exchange rates in the sea-cages, hypoxic stress, disease, handling stress, lack of attention to lumpfish welfare, and others (Reynolds et al., 2022; Garcia de Leanz, 2022; Rabadan et al., 2021; Geitung et al., 2020).

Few studies have examined the metabolic physiology of lumpfish (*Cyclopterus lumpus;* Hvas et al., 2018; Ern et al., 2016; Killen et al., 2007a, Killen et al., 2007b), and additional information is required to understand what capacity this species has to deal with the various

challenges they may face in sea-cages (e.g., high temperatures in the summer) or in the wild, and how their metabolic capacity compares to other North Atlantic species and relates to their lifestyle and ecology. Therefore, it is essential to understand how stressors that lumpfish face both at sea-cages and in nature limit their metabolic capacity. Lumpfish can be found at depths up to ~ 400 m (Kennedy et al., 2016), and thus, can avoid high temperatures in surface waters. However, when fish are held in sea-cages, they may not be able to avoid this thermal stressor as the depth in these cages is normally < 30 meters. In addition, as lumpfish are reported to be relatively poor swimmers, and prefer to use their sucker on their ventral side to stop and rest (Garcia de Leaniz et al., 2022; Imsland et al., 2015; Killen et al., 2007a), it is unclear what their true swimming capacity and metabolic capacity would be.

Thus, in this study, I compared three commonly used methods of 'exhausting' fish to determine the metabolic capacity of ~55 g cultured lumpfish under laboratory conditions: a chase to exhaustion, a critical swimming speed (U_{crit}) test and a critical thermal maximum (CT_{Max}) test. I predicted, based on the results of previous studies (Raby et al., 2020; Rummer et al., 2016) that: the U_{crit} test would provide the greatest values for MMR, AS and ASR; and that there would be significant relationships between these measures of metabolic capacity in the three tests when using the same individuals.

2.2: Materials and Methods

2.2.1: Experimental Animals

All procedures involving these fish were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland and Labrador (protocol #21-03-KG) and were performed in accordance with the Canadian Council on Animal Care Guidelines on the 'Care and Use of Fish in Research, Teaching and Testing' (Canadian Council on Animal Care, 2005).

Thirty lumpfish were maintained in a 445 L square tank, supplied with seawater at 6°C and a photoperiod of 14 h light: 10 h dark. Twelve of these lumpfish (range ~ 55-100 g) were individually anesthetized with 0.4 g L^{-1} tricaine methanesulfonate (TMS; Syndel Laboratories, Nanaimo, BC, Canada), placed on a wetted sponge, and had their gills irrigated with oxygenated seawater containing 0.2 g L^{-1} TMS. Then, each fish had two coloured beads sutured to the skin at the base of the dorsal fin for individual identification. A small 'tattoo' was also made in the middle of the ventral surface using a small syringe (1 mL, ½-inch 25-gauge needle) and red or black India Ink (Dr. Ph. Martin's Bombay India Ink Set, colours: sepia and terra cotta) in case a fish lost its beads during an experiment. Lastly, a small metal snap was sutured overtop of their ventral sucker (snaps were 0.18 g; Fig. 2.1A - C). These procedures took a maximum of 12 minutes per fish, and the fish were fitted with a snap to ensure that they could not adhere (stick) to solid surfaces, i.e., would swim during the U_{crit} and chase tests, and would lose equilibrium during the CT_{Max} tests. Once the beads and snaps were sutured to the fish, the fish were recovered in 6°C seawater, and returned to their tank to recover for one week. These 12 fish were then exposed to each of the three protocols below (in the order listed), with seven days between tests.

2.2.2: Chase Test

The day before a chase test, two lumpfish at a time were placed in a floating 'cage' (Fig. 2.2) to prevent feeding. The next morning the fish were placed in a shallow circular tank (75 cm in diameter x 15 cm deep) filled to a depth of ~5 cm with 6°C seawater and subjected to an eight-minute chase (Fig. 2.3A; Hvas et al., 2018), after which they were immediately placed into seawater filled 1150 mL (15 cm diameter x 6.5 cm high) custom-made glass respirometry chambers with a 'false' bottom (perforated plastic disk; Fig. 2.3B). These chambers were then submersed in a temperature-controlled shallow tank that was positioned over top of a multi-position stir plate (IKA RT 15 Magnetic Stirrer, Wilmington, North Carolina, USA; Fig. 2.3C), and located in a blacked-out enclosure with indirect lighting (Fig. 2.3D). Mixing of water inside the respirometry chambers was accomplished using a stir bar (rotated at 240 rpm) placed underneath the 'false bottom' (Rodgers et al., 2016).

Oxygen consumption (MO_2) was measured for two hours using automated intermittent closed respirometry (Killen et al., 2021; Rodgers et al., 2016; Svendsen et al., 2016), with 'flush' and 'closed' periods of eight and seven minutes, respectively. This allowed for measurement of the fish's RMR, MMR, AS and Excess Post-Exercise Oxygen Consumption (EPOC; i.e. the total amount of oxygen consumed above that of a resting fish during the recovery period) (see below). The chambers were intermittently flushed by automatically turning on/off an Eheim 5 L min^{-1} submersible pump (Model 1048; Eheim GmbH Co., Deizisau, Germany) using a computer program written by Tommy Norin (Norin and Gamperl, 2017) with the flush rate matched to the size of the respirometry chambers so that water in the chambers returned to > 95% air saturation between oxygen measurements. Water oxygen partial pressure (PO_2) inside the respirometry chambers was recorded at 0.1 Hz using fibre-optic dipping probes connected to a FireSting optical

oxygen meter (Pyro Science GmbBH, Resenberg, Germany) and a computer running Pyro Oxygen Logger, version 3.314 (2019 by Firmware 2.30; Aachen, Germany).

To account for background respiration when calculating fish MO_2, blank MO_2 measurements were taken in each respirometry chamber after the fish were removed. Background respiration was subtracted from all recorded values of MO_2.

2.2.3: Swim Speed (U_{crit}) Test

Each fish was removed from the floating 'cage,' placed in a 6.8 L Blazka swim tunnel (14.0 cm internal diameter, 61.4 cm long swimming section; Fig. 2.4A) at a velocity of 2 cm s^{-1} (~ 0.2 body lengths per second, BL s^{-1}) and at a temperature of 6°C and allowed to recover overnight. The following morning, the fish underwent a 20-minute 'training session,' where speed was gradually increased to 8 cm s^{-1} (~ 1.2 - 1.4 BL s^{-1}) over 10 minutes and maintained at this velocity for another 10 minutes (Fig. 2.5). When the fish rested against the back grid of the swim tunnel, the back of the swim tunnel was tapped, followed by providing a mild electrical current to the grid (~ 0.1 A, 5 v; Power / Mate Corp. Power supply, BPA-40D; Hackensack, New Jersey, USA) when necessary, to ensure that the fish did not rest at the back of the swim tunnel. After this brief 'training session,' the fish was allowed to recover for four hours at a velocity of 2 cm s^{-1} (0.2 BL s^{-1}). This was adequate for RMR to return to baseline levels based on the results of the chase tests (see Fig. 2.8A, C).

Once the fish was fully recovered from the training session, an initial 20-minute measurement of MO_2 was made. MO_2 was measured manually by stopping the flow of water into the swim tunnel, then calculating MO_2 from the slope of the decrease in PO_2 over the final eight minutes of the stop-flow period at rest, and over the final five minutes of the stop-flow period at

each swimming speed; the oxygen level in the swim tunnel measured continuously using a fiber-optic dipping probe connected to a PreSens O_2 meter (Fibox3 LCDV3; version 2.0.1.0 PreSens Precision Sensing GmBH) and a laptop computer running PreSens software. Speed was then gradually increased to 8 cm s^{-1} (~1 BL s^{-1}) over a 5-minute period (ramp). After 10 minutes, MO_2 was recorded for another 10 minutes, followed by a 5-minute open period. Thereafter, water velocity was increased by 2 cm s^{-1} (~ 0.2 BL s^{-1}) every 20 minutes until the fish exhausted (i.e., when the fish repeatedly fell back on the rear grid of the swim tunnel and remained there for several seconds; Hvas et al., 2018). The tunnel was kept 'open' for the first 5 minutes, so the fish could adjust to the new speed, 'closed' for 10 minutes so that MO_2 could be recorded, and then 'opened' again for another 5 minutes before swimming velocity was increased (Fig. 2.5). When the fish became exhausted the fish's velocity and MO_2 were recorded, and the velocity was decreased to 2 cm s^{-1} for an additional 2 hours to determine the fish's EPOC. Finally, a MO_2 measurement was made after the fish was removed from the swim tunnel to account for background respiration, which was subtracted from all measurements of MO_2.

Critical swimming speed (U_{crit}) was calculated as:

$$U_{crit} = V + [(t_f \times V_i)/t_i] \quad (1)$$

Where V = the last velocity at which the fish swam for the entire increment; t_f = time elapsed from the last change in current velocity to exhaustion; V_i = velocity increment (0.2 BL s^{-1}); t_f = time elapsed from the last change in current velocity to exhaustion; and t_i = time increment, the time between step increases in velocity (20 minutes). Then, it was corrected for the solid blocking effect of the fish (Kline et. al, 2015; Bell & Terhune, 1970) using the formula:

$$V_F = V_R (1 + \epsilon_s) \quad (2)$$

Where V_F = water velocity at the position of the fish's maximum girth, V_R= water velocity at the rear of the flume, and ϵ_s = the error due to solid blocking. With ϵ_s calculated as:

$$\epsilon_s = \tau\lambda (A_0 / A_T)^{Aexp} \quad (3)$$

Where τ = a dimensionless factor for tunnel cross-section (0.8), and λ is a factor (coefficient) related to the shape of the fish calculated as total body length divided by the total body thickness (Fulton, 2007); A_0 = the cross-sectional area of the fish and was calculated as $0.25G^2\pi^{-1}$ (where G = girth of the fish, and $G\pi^{-1}$ = thickness); A_T is the cross-sectional area of the swimming chamber calculated as πr^2, where r = radius and is 100 mm, and A_{exp} = the fractional area exponent (1.5; Kline et al., 2015).

2.2.4: Critical Thermal Maximum (CT_{Max}) Test

Fish were removed from their floating cages in the morning, and placed in the same respirometry chambers that were used following the chase protocol (Fig. 2.3B-D) so that standard metabolic rate (SMR; see below) could be measured overnight (i.e., every 15 minutes from 6:00 pm to 8:00 am) at an initial temperature of 6°C. The next morning, an immersion heater (Intelligent Heater LLC, QDNTY-1.8-1, 1000 W) was used to increase the water temperature by 2°C h^{-1}, with the MO_2 recorded at every 1°C increase (Fig. 2.6; using the same 'open' and 'closed' intervals as used following the chase protocol) until each fish lost equilibrium (i.e., reached its CT_{Max}) as determined by watching live video footage from a Go-Pro® (model HERO 6.0 Black) camera placed in front of the chambers (Fig. 2.7). Using this protocol, we measured maximum metabolic

rate (MMR), routine metabolic rate (RMR), standard metabolic rate (SMR), aerobic scope (AS) and realistic aerobic scope (ASR; Bowden et al., 2018; Poletto et al., 2017; Kelly et al., 2014). After each fish reached its CT_{Max}, it was immediately removed from the respirometer and euthanized with an overdose of TMS (0.8 g L^{-1}).

2.2.5: Calculation of Metabolic Parameters

Measurements of oxygen consumption were made considering the recommendations for aquatic respirometry as detailed in Rodgers et al. (2016), Svendsen et al. (2016) and Killen et al. (2021). After each test was completed, the time and PO_2 data were imported into LoggerPro® 3.1 (Vernier Software & Technology, Beaverton, Oregon, USA). This program created a graph of % air saturation as a function of time, and at each measurement point, the slope of the decline in oxygen (in units of % air sat. s^{-1}) was determined, and this was used to calculate MO_2:

$$MO_2 \text{ (mg } O_2 \text{ kg}^{-1} \text{ h}^{-1}) = \frac{\text{slope (\% air sat. s}^{-1}) \text{ x } 3600 \text{ (s h}^{-1}) \text{ x } 10.1 \text{ (mg } O_2 \text{ L}^{-1}) \text{ x [volume of chamber (L) – mass of fish (L)]}}{\text{mass of the fish (kg)}} \quad (4)$$

Note: 1) 10.1 mg L^{-1} is the oxygen content of 100% air saturated seawater of a salinity of 32 ppt at 6°C.
2) We assumed that 1 g of fish = 1 mL of seawater.

During the CT_{Max} experiments, the oxygen content of 100% air saturated seawater changed with temperature. Therefore, MO_2 during the CT_{Max} experiments was calculated considering the change in oxygen content with temperature:

$$MO_2 \text{ (mg } O_2 \text{ kg}^{-1} \text{ h}^{-1}) = \frac{\text{slope (\% air sat. s}^{-1}) \text{ x 3600 (s h}^{-1}) \text{ x } O_2 \text{ Saturation}^6 \text{ (mg } O_2 \text{ L}^{-1}) \text{ x [volume of chamber (L) – mass of fish (L)]}}{\text{mass of the fish (kg)}} \quad (5)$$

$$\% \text{ air saturation} = [0.0034 \text{ x temperature } (°C)^2] - [0.2723 \text{ x temperature } (°C) + 11.575 \quad (6)$$

Standard metabolic rate (SMR) was calculated by taking an average of the lowest 10% of the MO_2 recordings from the overnight measurements prior to the CT_{Max} test. It was not possible to take overnight measurements in the swim tunnel, as measurements of MO_2 were taken manually by opening and closing the swim tunnel.

The routine metabolic rate (RMR) of each fish during the U_{crit} and the CT_{Max} tests was recorded as the first (basal) measurement before the start of each protocol. For the U_{crit} protocol, this measurement was the first one taken at a velocity of 2 cm s^{-1} (~0.2 BL s^{-1}). For the CT_{Max} test, this measurement was the first taken at 6°C, before any increase in temperature. The RMR of each fish after the chase test was recorded as the lowest metabolic rate measured during recovery.

The maximum metabolic rate (MMR) of each fish during the U_{crit} and CT_{Max} tests was recorded as the highest metabolic rate achieved for each individual. For the chase test, MMR was calculated by back extrapolating the relationship between MO_2 and time for each fish back to the exact time at which the chase ended (i.e., Time 0; it took between two to three minutes to get the fish in the respirometry chambers, to seal them, and to achieve steady (reliable) decreases in water oxygen level. To account for background respiration when calculating fish MO_2, blank measurements of MO_2 were taken in each respirometry chamber, after the fish was removed. However, background respiration was negligible in all cases.

The fish's aerobic scope (AS) was calculated by subtracting the SMR of each fish prior to the CT_{Max} test from its respective MMR during each particular test. This value represents the

capacity of fish to increase their metabolic rate beyond the oxygen consumption required for maintaining essential cellular and physiological functions. The realistic aerobic scope (ASR) was calculated in all the tests by subtracting the RMR of each fish from its respective MMR (Norin and Clark 2016; Norin et al., 2015; Clark et al., 2013).

2.2.6: Statistical Analyses

Linear mixed-effects models, followed by least squares post-hoc tests were used to identify differences in metabolic parameters measured using the 3 experimental protocols. Fish ID was used as a fixed variable in this analysis to account for the fact that the same fish (n = 12) were used in all three protocols (i.e., the measurements were not independent of each other). Statistical analyses were conducted in RStudio 4.1.2 (RStudio Team, 2021), using lme4 (Bates et al., 2015) and Prism v.9 (GraphPad Software, USA, graphpad.com). Significance was set at $p < 0.05$, and data in figures, tables and throughout the text are means $\pm$ 1 standard error of the mean.

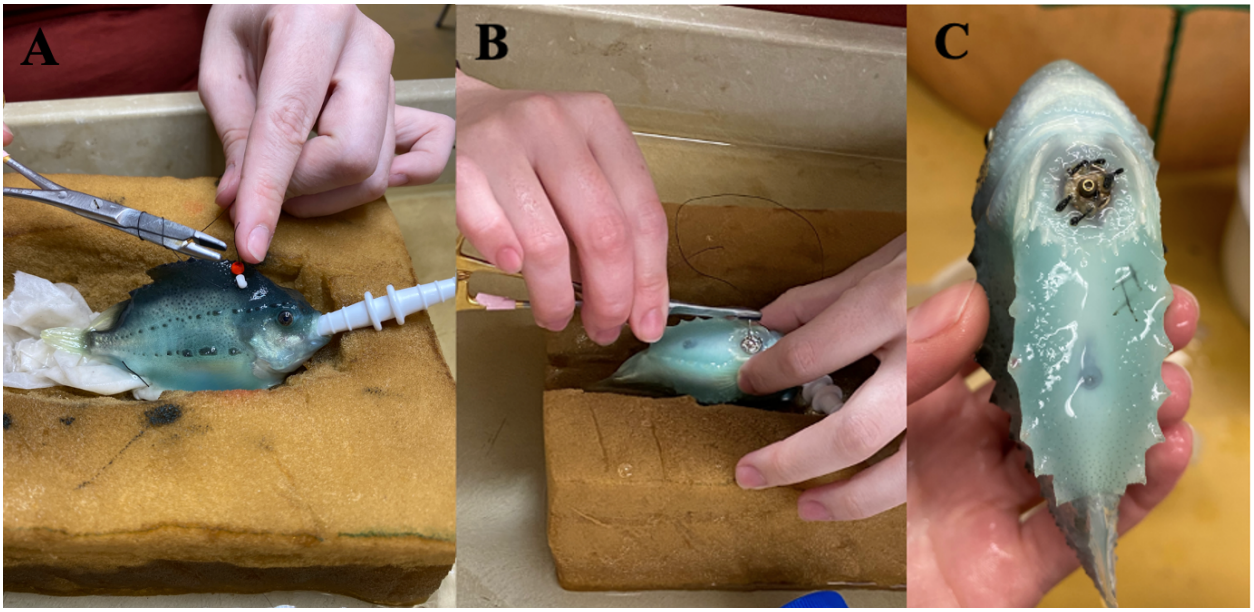

Figure 2.1: Photographs showing the suturing of two coloured beads to the anterior region of the lumpfish's dorsal hump (A), the suturing of a small metal 'snap' over the sucker of the lumpfish (B), and the ventral side of the fish with attached 'snap' and ink 'tattoo' used as a back-up method of identification (C).

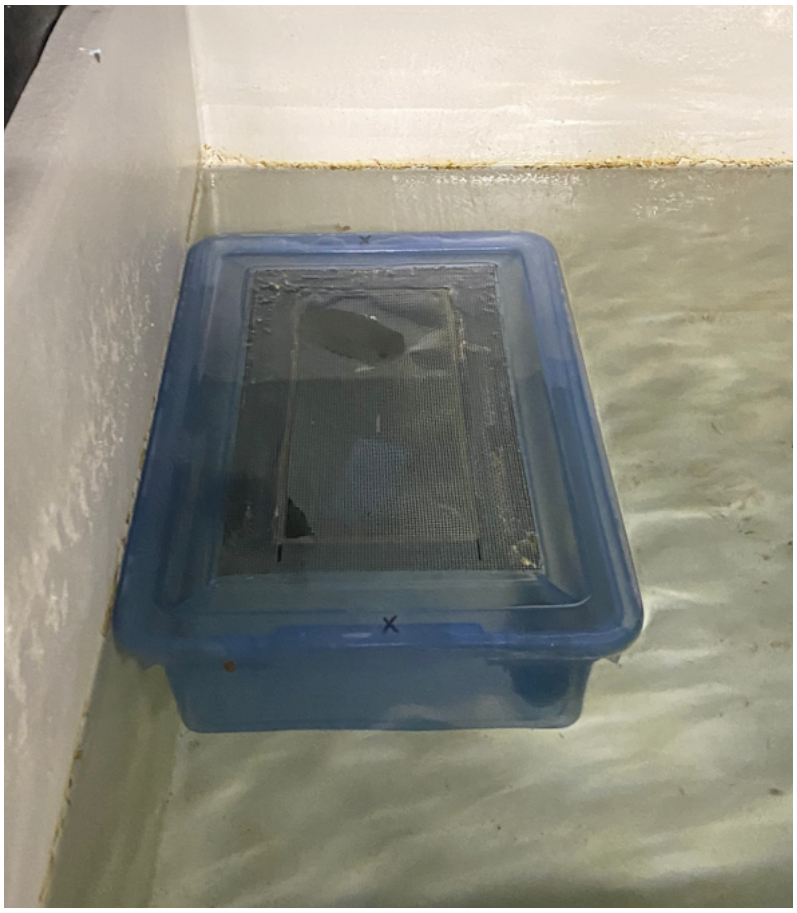

Figure 2.2: Two lumpfish in a floating ‘cage,’ one day before they were used in an experiment.

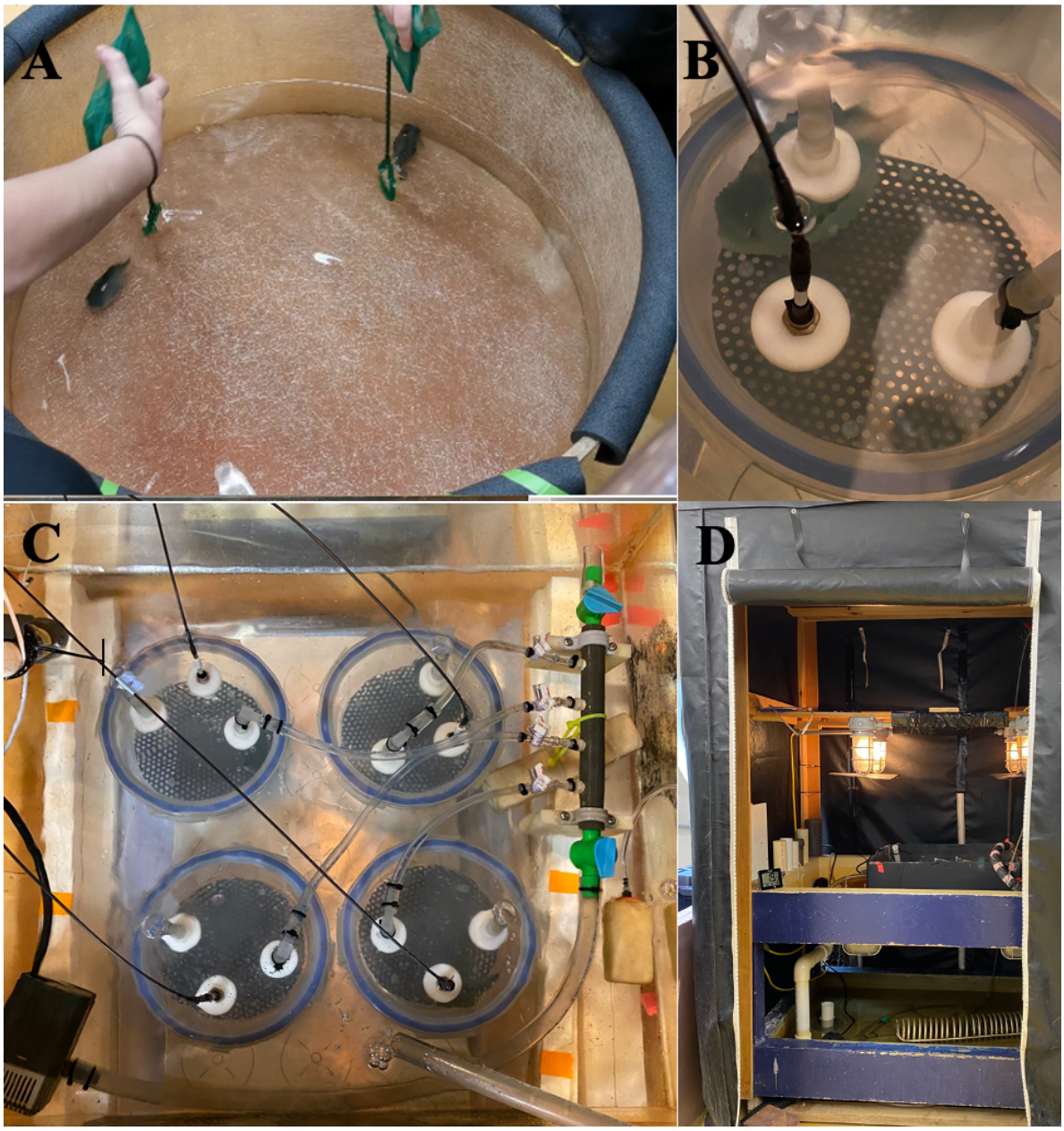

Figure 2.3: Pictures of the equipment / set-up used to chase the fish and make measurements of lumpfish MO_2 (oxygen consumption) following a chase, and during the critical thermal maximum (CT_{Max}) test. Two lumpfish being chased (A), lumpfish in the respirometers (B and C), these respirometers fitted with an oxygen probe and inlet and outlet water lines and containing a false mesh bottom and a stir bar. Note: the stir-plate below the respirometers in (C). The experimental set-up with the opening to the 'blacked-out' enclosure rolled up (D).

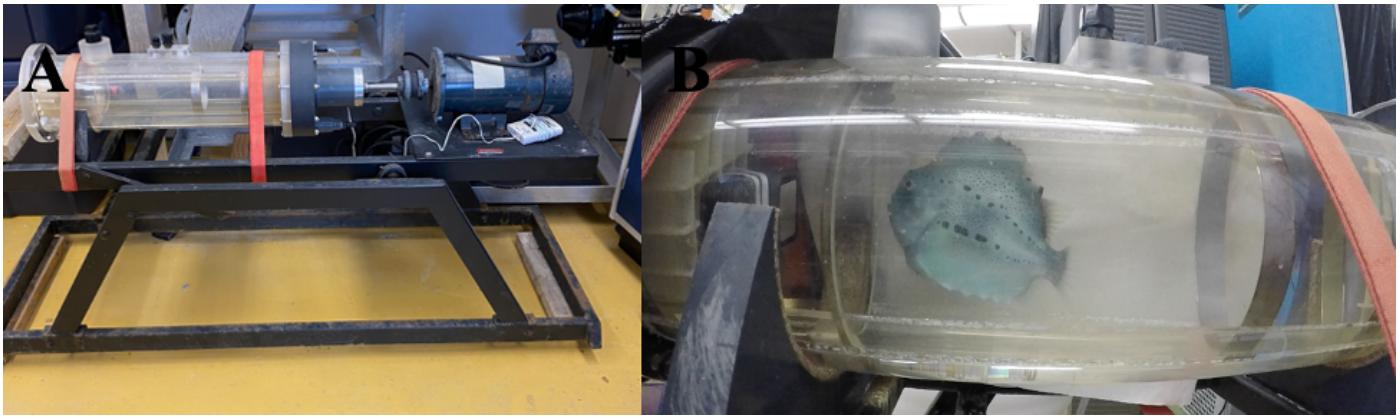

Figure 2.4: The 6.8 L Blazka swim tunnel used in these experiments (A), and a lumpfish swimming in the swim tunnel (B).

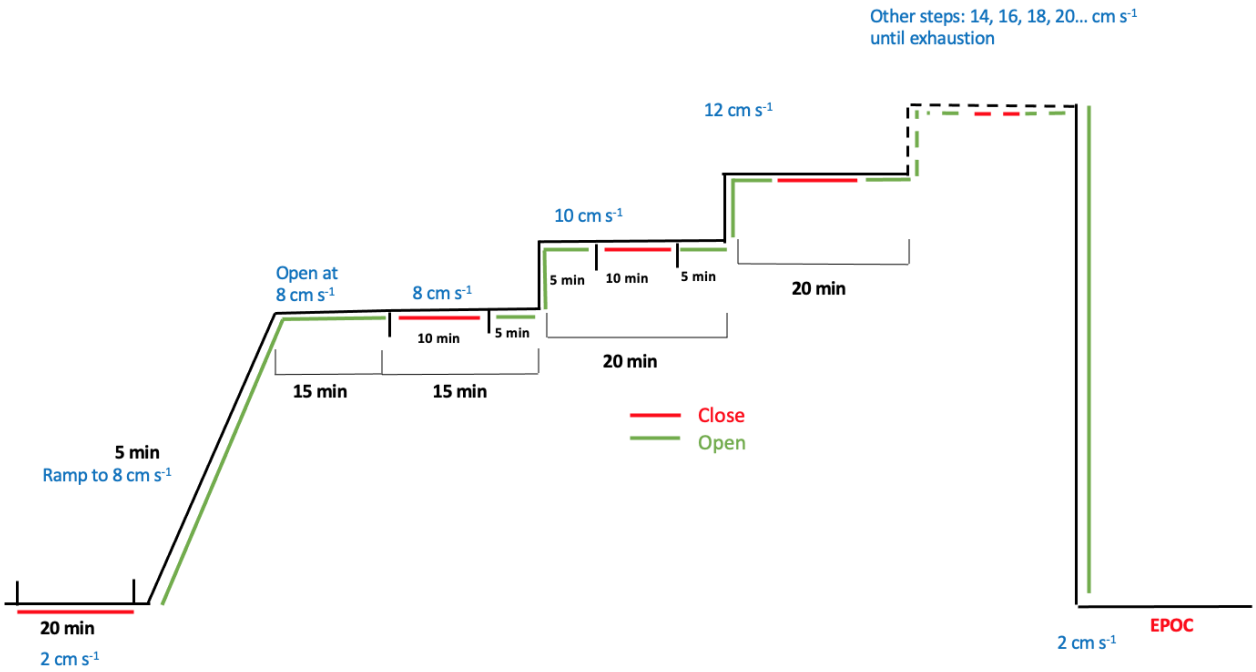

Figure 2.5: A schematic diagram of the critical swim speed (U_{crit}) test. The dotted lines indicate that the number of steps required to exhaust the fish varied between individuals. After the fish was determined to be exhausted (to have reached its U_{crit}), excess post-exercise oxygen consumption (EPOC) measurements were taken for 2 hrs at 2 cm s^{-1} (~ 0.2 BL s^{-1}).

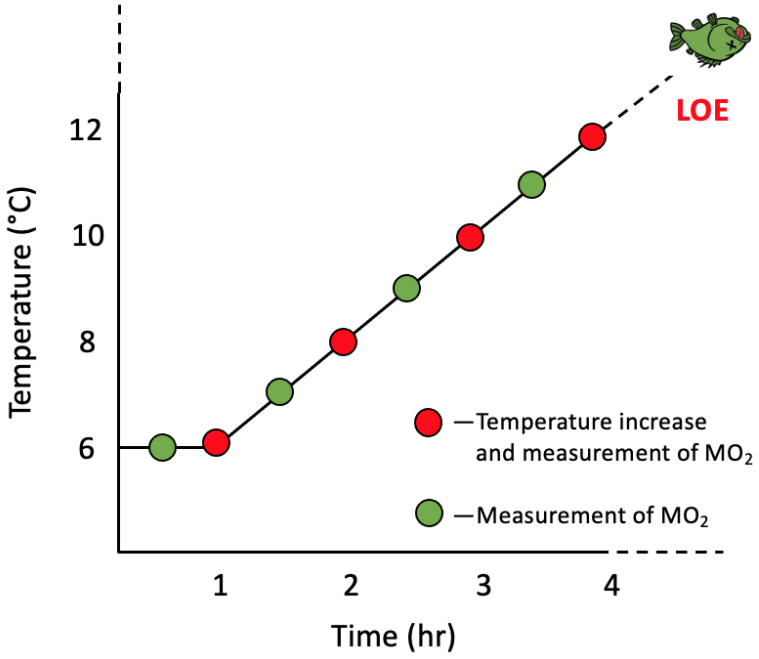

Figure 2.6: A schematic diagram of the critical thermal maximum (CT_{Max}) test, showing an increase in temperature of 2°C h^{-1} until the fish lost equilibrium (LOE). The dotted lines indicate an indeterminate number of steps.

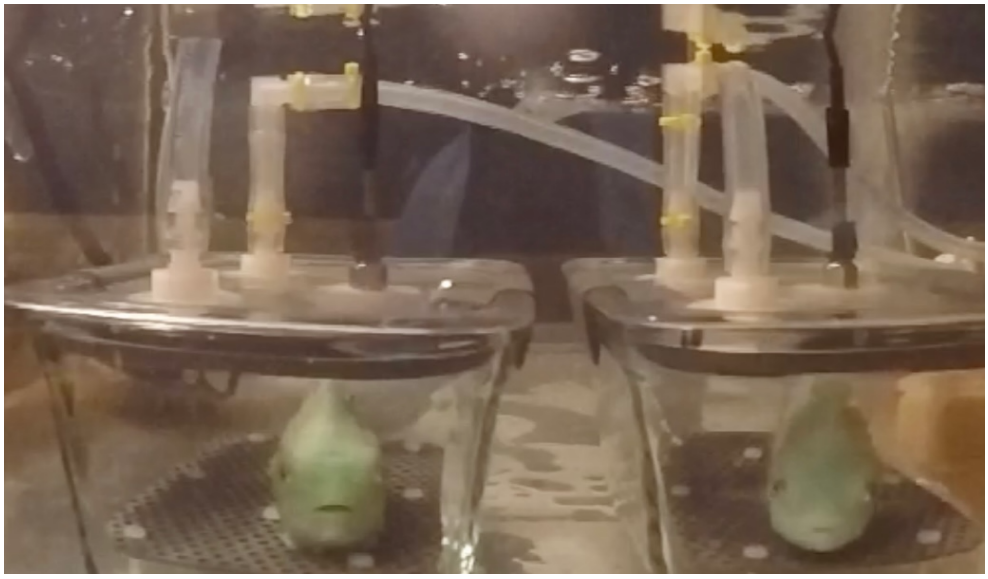

Figure 2.7: A screenshot of lumpfish during the critical thermal maximum (CT_{Max}) test acquired using a GoPro® video camera. The GoPro® was placed in front of the static respirometry chambers and remained on for the duration of the CT_{Max} trial. This ensured that the fish could still be observed with the enclosure closed; i.e., to limit outside disturbances (visual and sound).

2.3: Results

The objective of this study was to determine the MMR, RMR, AS and ASR of lumpfish using three different methods of 'exhaustion': a chase test, a U_{crit} test and a CT_{Max} test (n = 12). SMR was only measured in lumpfish prior to the CT_{Max} test, as overnight measurements could not be taken in the swim tunnel used in these studies. The SMR of lumpfish in the static respirometers averaged 36.6 $\pm$ 3.1 mg O_2 kg^{-1} h^{-1}. This value was used to calculate the AS obtained from each method of exhaustion (Table 2.1). RMR prior to the CT_{Max} test (48.9 $\pm$ 4.0 O_2 kg^{-1} h^{-1}) was 25% higher than the fish's SMR prior to the CT_{Max} test (36.6 $\pm$ 3.1 mg O_2 kg^{-1} h^{-1}; $p < 0.05$). The RMR of lumpfish at rest in the swim tunnel (109.1 $\pm$ 6.4 mg O_2 kg^{-1} h^{-1}) was 45% higher than the RMR of the same lumpfish 2 h post-chase and prior to the start of the CT_{Max} test (65.8 $\pm$ 4.0 and 48.9 $\pm$ 4.0 mg O_2 kg^{-1} h^{-1} respectively). RMR prior to the chase and CT_{Max} tests was not significantly ($p = 0.095$) but were both significantly different than the RMR of lumpfish in the swim tunnel ($p < 0.05$).

During the U_{crit} test, MO_2 increased steadily until the fish reached exhaustion (Fig 2.8C); the mean U_{crit} of these lumpfish was 20.9 $\pm$ 0.80 cm s^{-1} (2.39 $\pm$ 0.081 BL s^{-1}). In contrast, during the CT_{Max} test, MO_2 increased in a linear fashion until approximately 16°C and was then maintained at this level until the lumpfish lost equilibrium at 20.6 $\pm$ 0.25°C (Fig. 2.8B). Mean MMR values in the chase, U_{crit} and CT_{Max} tests were 176.0 $\pm$ 8.4, 243.0 $\pm$ 7.7 and 183.9 $\pm$ 14.4 mg O_2 kg^{-1} h^{-1}, respectively, with the MMR values for the chase and CT_{Max} tests not statistically different from each other, but statistically lower ($p < 0.05$) than the MMR value obtained in the U_{crit} test. Similar to the results for MMR: while the AS for the chase and CT_{Max} tests were not different (124.7 $\pm$ 15.5 and 141.0 $\pm$ 15.0 mg O_2 kg^{-1} h^{-1}, respectively), both these values were less than measured after the U_{crit} protocol (206.4$\pm$ 8.5 mg O_2 kg^{-1} h^{-1}; Table 2.1). However, none of the

ASR values for the chase, U_{crit}, or CT_{Max} tests were significantly different from each other (Table 2.1). In the CT_{Max} test, all three measures of metabolic capacity (MMR, AS and ASR) were positively ($0.10 > p < 0.05$) related to the lumpfish's thermal tolerance. (Fig. 2.12). In contrast, none of these parameters of metabolic capacity were related to the fish's U_{crit} (Fig. 1.13; $0.40 > p < 0.10$). Interestingly, there were no significant correlations ($0.88 > p > 0.14$) between MMR, ASR or AS as measured using the 3 different methods of 'exhaustion' (Figs. 2.9, 2.10 and 2.11 A-C).

After MMR was reached in the chase and U_{crit} tests, MO_2 decreased rapidly and returned to pre-exercise RMR values within approximately 45-60 minutes (Figs. 2.8A and C). EPOC values following the chase and U_{crit} tests were 42.0 and 38.7 mg O_2 kg^{-1}, respectively (Table 2.1).

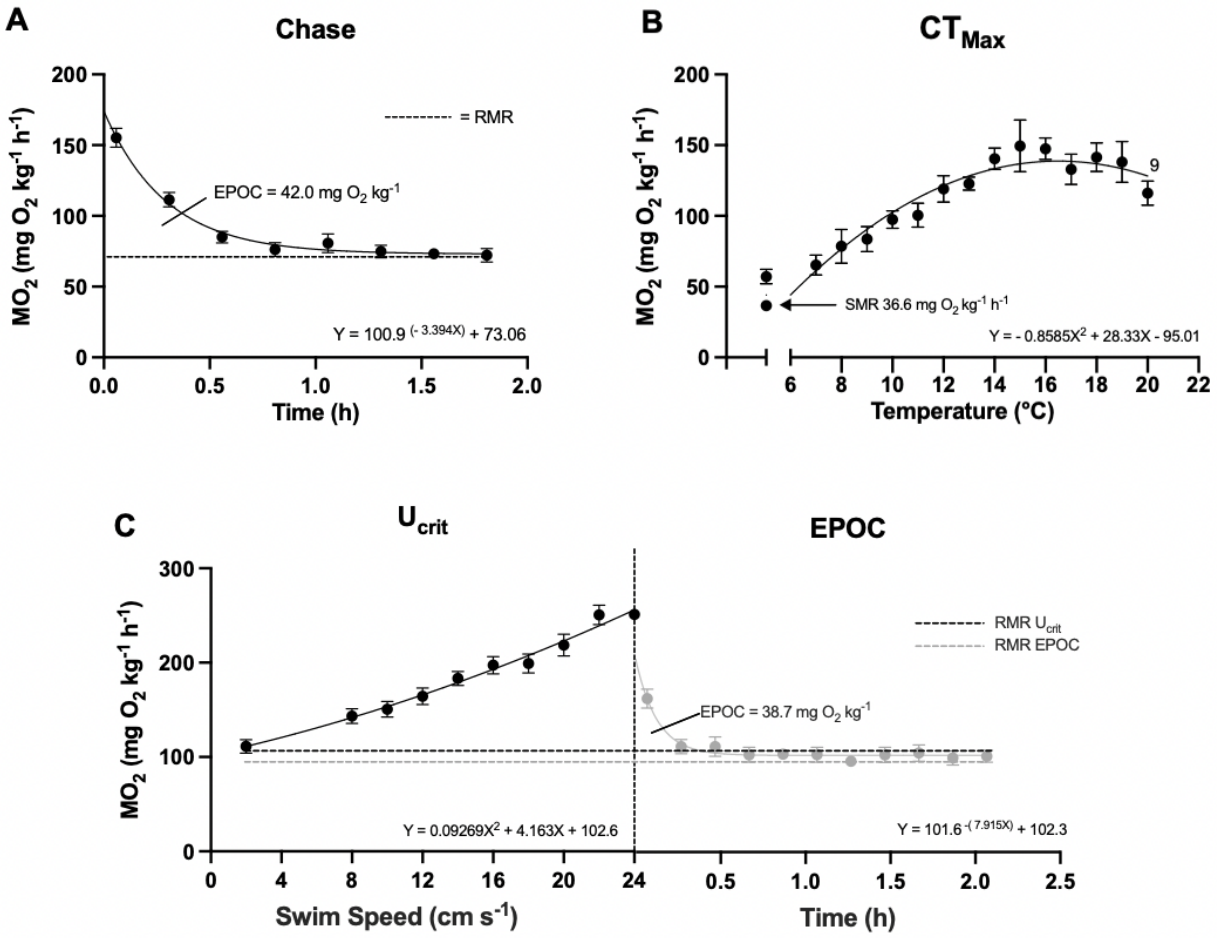

Figure 2.8: MO_2 measurements during each method of 'exhaustion'. EPOC was measured after the chase and U_{crit} tests only (A, C) and is indicated on the figures. SMR was only calculated during the CT_{Max} experiment (B) and was measured as the mean of the lowest 10% of measurements taken overnight before a trial. The mean CT_{Max} of lumpfish reared at 6°C was 20.6 $\pm$ 0.25°C. Symbols in all the plots represent means $\pm$ 1 SE. The number above the symbols in panel B indicate when the number of fish was < 12.

Table 2.1: Mean metabolic parameters for lumpfish subjected to 3 different methods of 'exhaustion'; a chase, a U_{crit} test and a CT_{Max} test. Absolute aerobic scope (AS) was calculated by subtracting SMR from the MMR obtained in each protocol. Realistic aerobic scope (ASR) was calculated by subtracting RMR from the MMR. Values without a letter in common in each row are statistically different ($p < 0.05$). Statistical differences could not be determined for the EPOC measurements as these values were calculated from the mean data plotted in Figs. 2.8 A and C. SMR = standard metabolic rate; RMR = routine metabolic rate; MMR = maximum metabolic rate; AS = aerobic scope, ASR = realistic aerobic scope; EPOC = excess post-exercise oxygen consumption.

Metabolic Parameter	**Chase**	**U_{crit}**	**CT_{Max}**
SMR (mg O_2 kg^{-1} h^{-1})	-	-	36.6 ± 3.1
RMR (mg O_2 kg^{-1} h^{-1})	65.8 ± 4.0[a]	109.1 ± 6.4[b]	48.9 ± 4.0[a]
MMR (mg O_2 kg^{-1} h^{-1})	176.0 ± 8.4[a]	243.0 ± 7.7[b]	183.9 ± 14.4[a]
AS (mg O_2 kg^{-1} h^{-1})	124.7 ± 15.5[a]	206.4 ± 8.5[b]	141.0 ± 15.0[a]
AS_R (mg O_2 kg^{-1} h^{-1})	110.2 ± 9.4[a]	134.0 ± 5.9[a]	134.6 ± 15.0[a]
EPOC (mg O_2 kg^{-1})	42.0	38.7	-

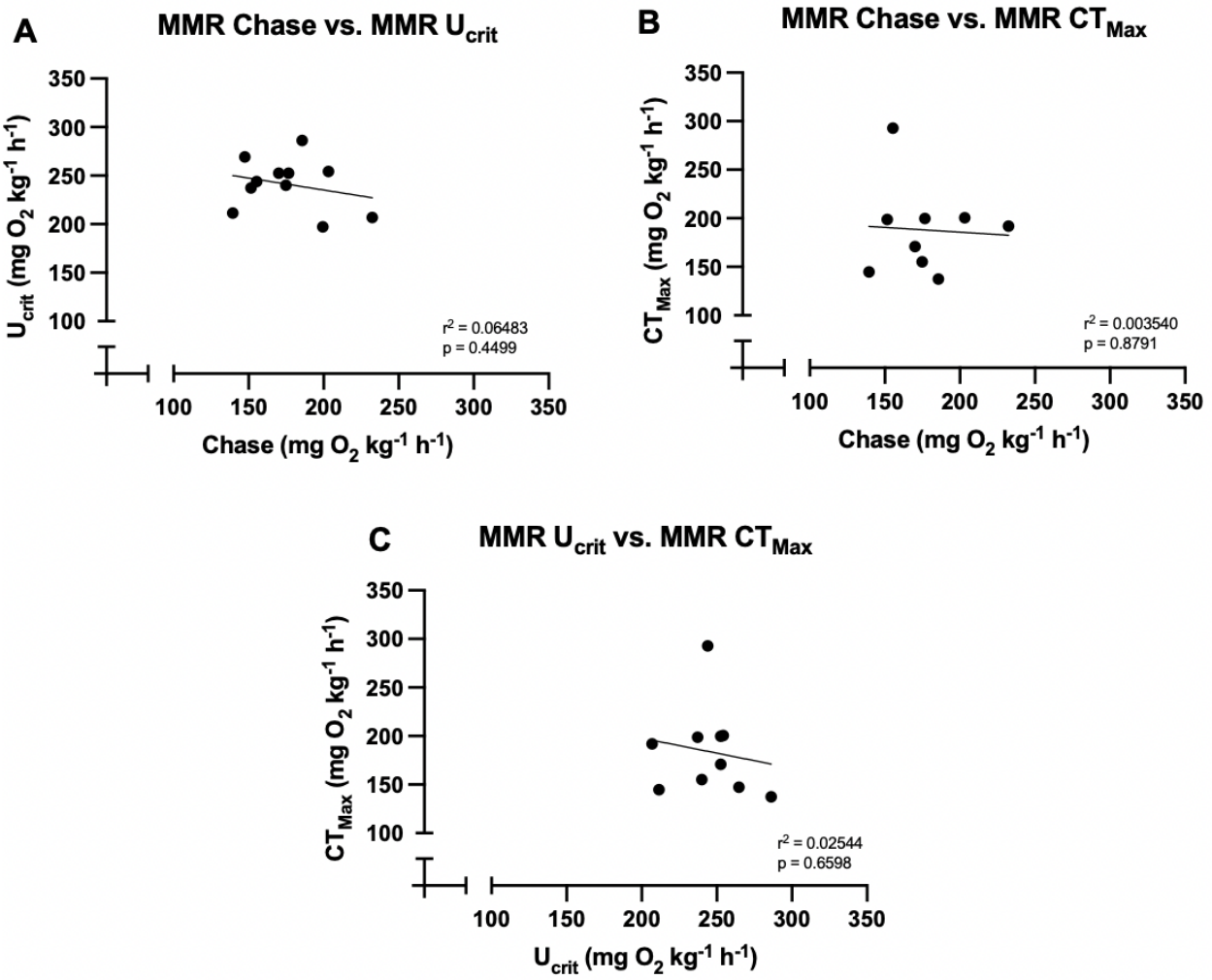

Figure 2.9: Relationships between maximum metabolic rate (MMR) measured in the same individual lumpfish when 'exhausted' using the 3 different methods.

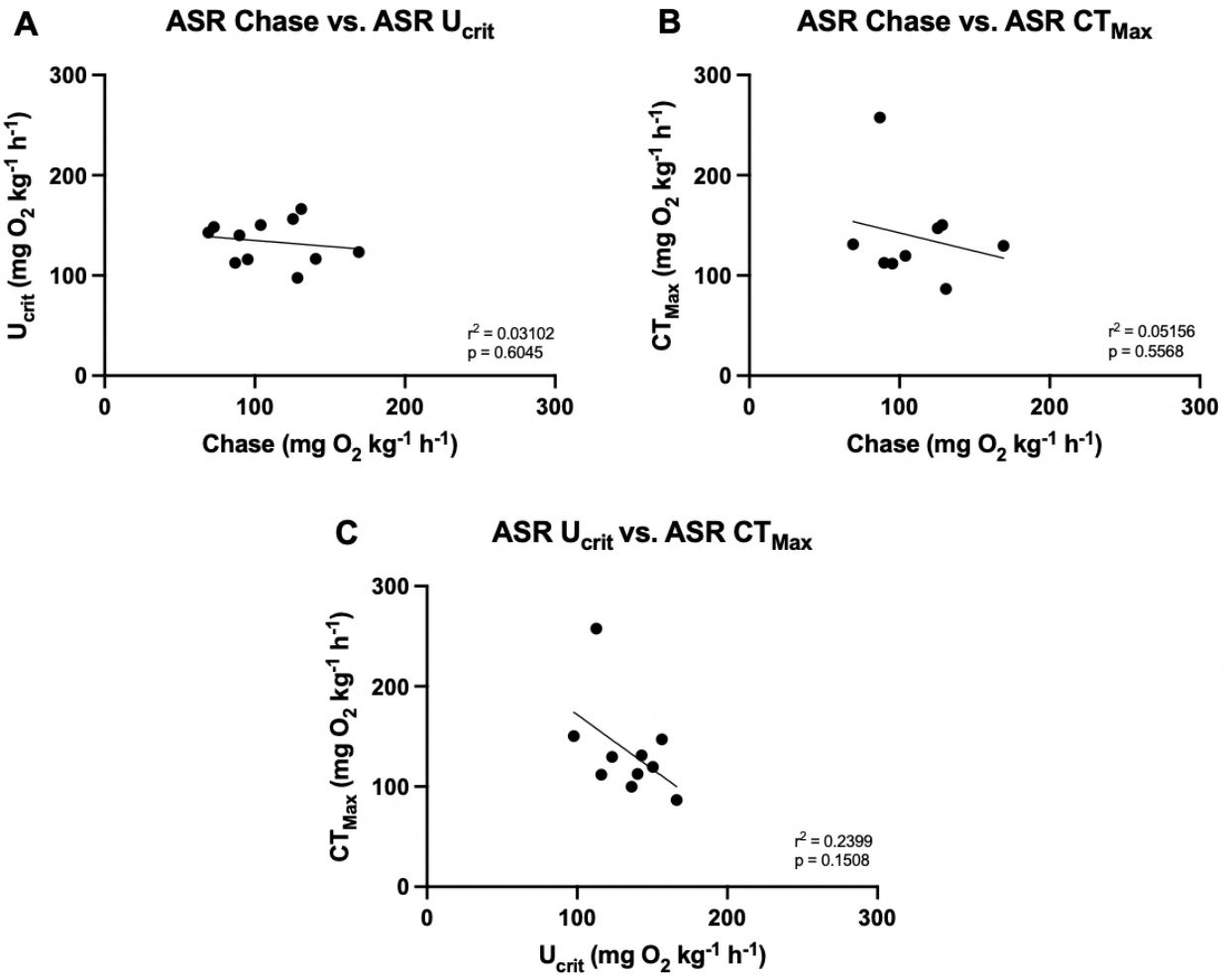

Figure 2.10: Relationships between realistic metabolic scope (ASR) measured in the same individual lumpfish when 'exhausted' using the 3 different methods.

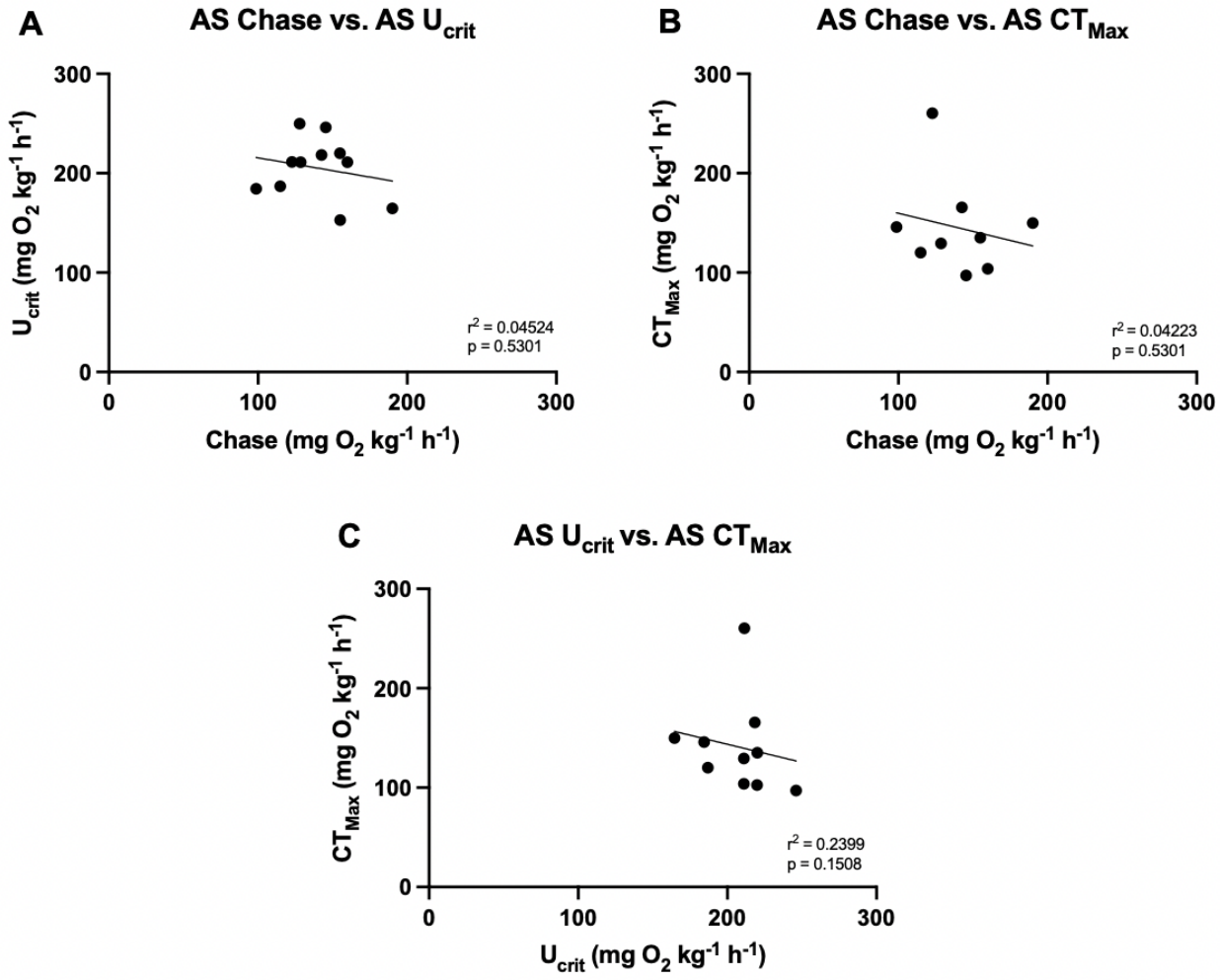

Figure 2.11: Relationships between metabolic scope (AS) measured in the same individual lumpfish when 'exhausted' using the 3 different methods.

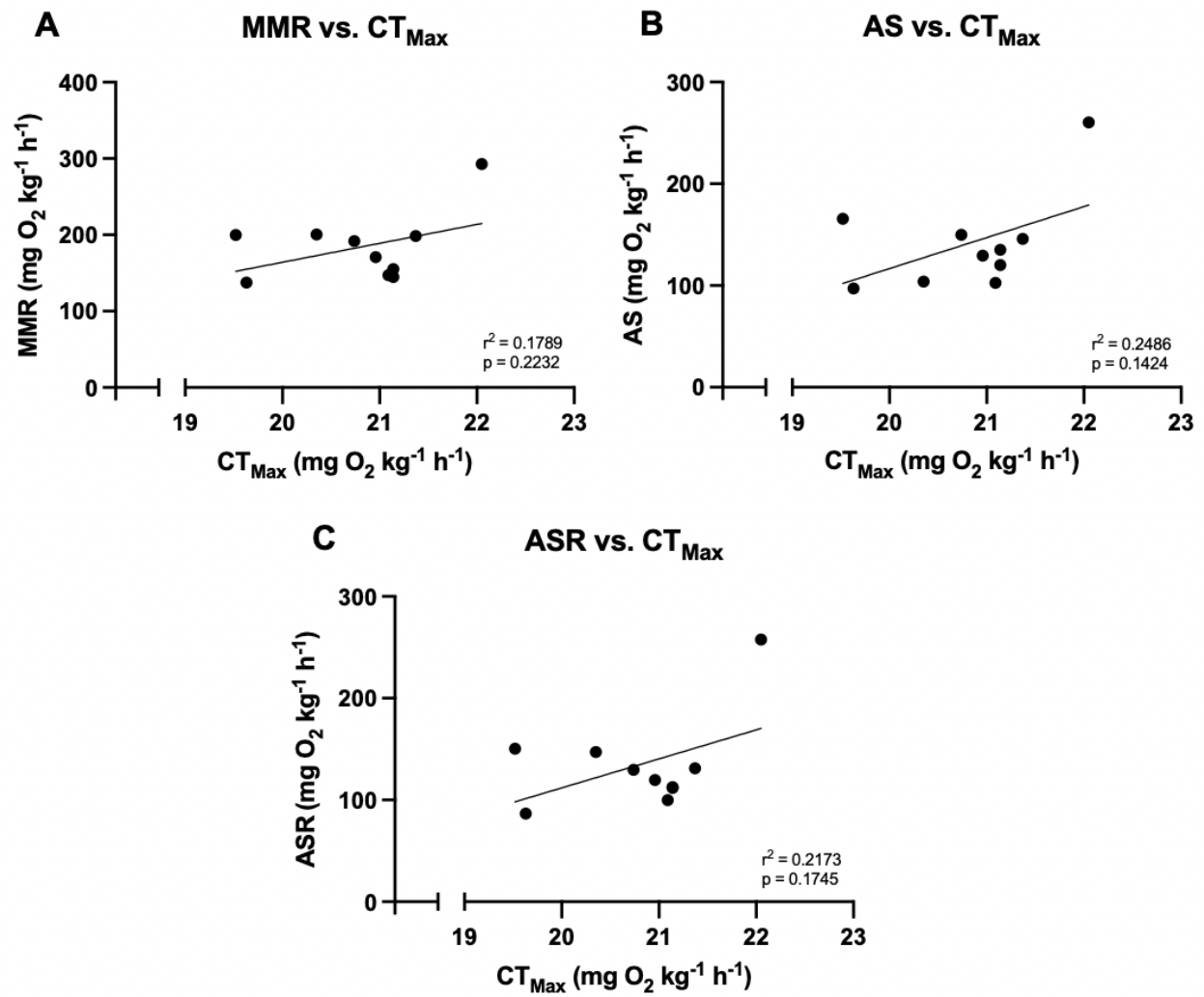

Figure 2.12: Relationships between maximum metabolic rate (MMR; A), absolute aerobic scope (AS; B) and realistic aerobic scope (ASR; C) and each fish's thermal maximum (CT_{Max}).

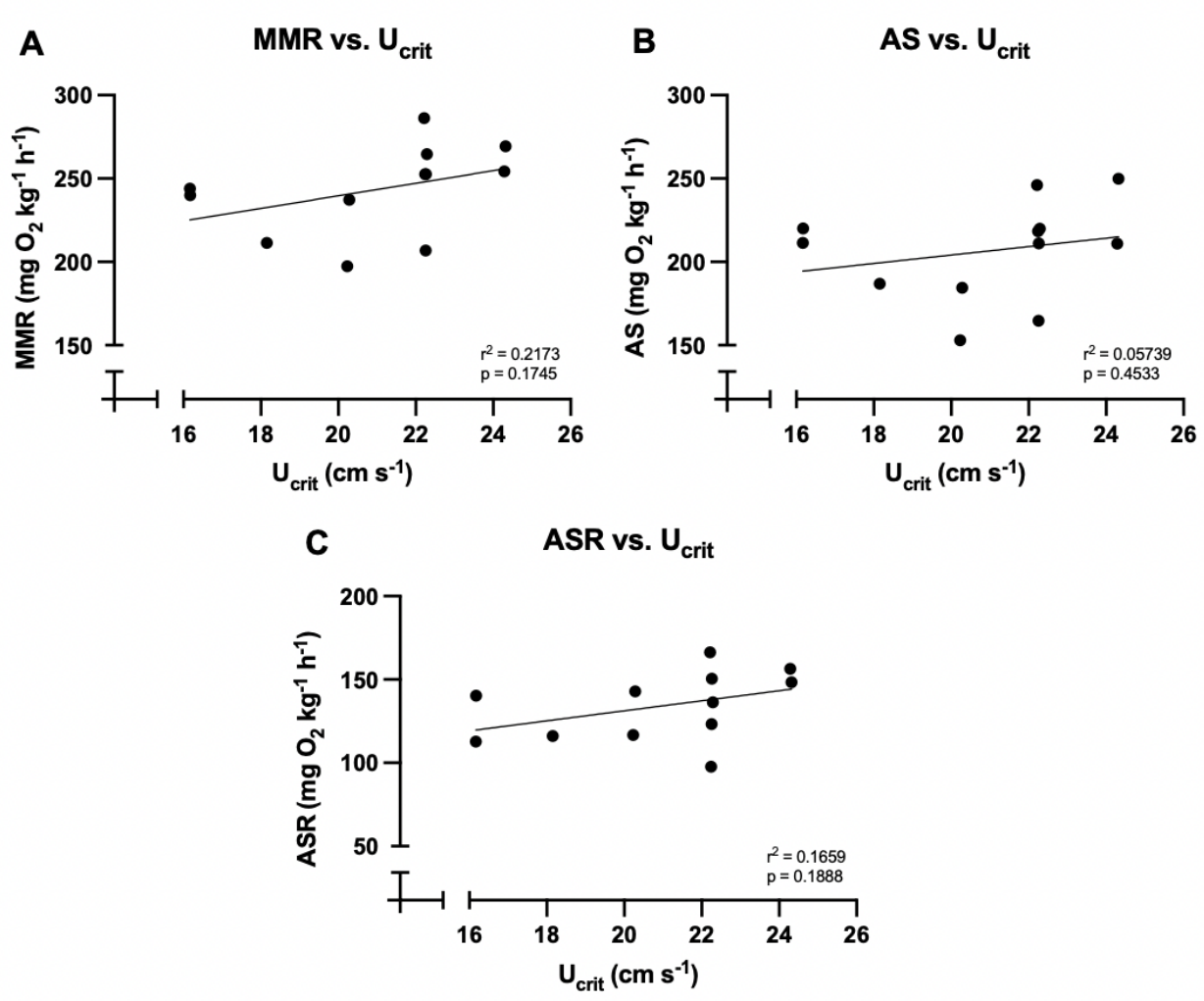

Figure 2.13: Relationships between maximum metabolic rate (MMR; A), absolute aerobic scope (AS; B) and realistic aerobic scope (ASR; C) and each fish's critical swim speed (U_{crit}).

2.4: Discussion

2.4.1: Comparing the 3 Methods of 'Exhaustion'

Few studies have measured the metabolic capacity of lumpfish, and in those that have, the methods and types of stressors have varied considerably (Remen et al., 2022; Hvas et al., 2018; Killen et al., 2007b). In this study, three methods of 'exhaustion'/determining the lumpfish's metabolic capacity were directly compared. Of these methods, the U_{crit} test provided the highest MMR, while the chase and CT_{Max} tests provided similar MMR values (Table 2.1). However, the U_{crit} test also provided the highest RMR of the three tests, which was significantly higher than the RMR obtained from the chase and CT_{Max} tests (Table 2.1). Therefore, the ASR from the U_{crit} and CT_{Max} tests were nearly identical, and the ASR from the chase test was lower, but not significantly different (Table 2.1).

These results are consistent with data from previous studies that have explored the metabolic capacity of lumpfish. When the U_{crit} data from Hvas et al. (2018) are extrapolated to a temperature of 6°C, the ASR they reported (132.0 mg O_2 kg^{-1} h^{-1}) was nearly identical to that found in this study (Table 2.1); although our MMR was approximately 15% higher (236.8 vs. ~205 mg O_2 kg^{-1} h^{-1}). In comparison, after being subjected to a chase test, lumpfish acclimated to 10°C had an AS of 156.6 mg O_2 kg^{-1} h^{-1} (Ern et al., 2016), which is considerably higher than the post-chase AS reported here (124.7 mg O_2 kg^{-1} h^{-1} for fish acclimated to 6°C). Although the AS obtained from this study is lower than that reported by Ern et al. (2016), it is well-established that fish size and acclimation temperature affect the metabolic parameters of fishes (Porter and Gamperl, submitted; Ørsted et al., 2022; Remen et al., 2022; Killen et al., 2021; Zrini et al., 2021; Killen et al., 2016; Schulte 2015; Sandblom et al., 2014; Clark et al., 2013; Clark et al., 2012; Farrell et al., 2009).

There are a few reasons that the MMR and RMR obtained from the U_{crit} test would be significantly higher than that recorded during the CT_{Max} test and after chasing the fish, despite providing similar ASR values. Firstly, the use of a swim tunnel allows fish to achieve a higher MMR (Raby et al., 2020; Clark et al., 2013). However, it is unclear if the increased MMR and AS in a swim tunnel occurs because high water velocity encourages ram ventilation or a higher O_2 demand due to exercise (Raby et al., 2020). For example, the lumpfish had their mouth open while swimming, ram ventilation may result in a ventilation–perfusion ratio which exceeds that required to optimize/maximize gas exchange, and thus, species that utilize this mode of ventilation may only be constrained by the capacity of the circulatory system to deliver oxygen to the tissues (Keen and Gamperl 2012). In this study, the lumpfish's sucker was fitted with a snap to prevent the fish from attaching to the swim tunnel so that swimming performance could be effectively evaluated. In contrast to the chambers where metabolic parameters were measured in the CT_{Max} and chase tests, which have a flat bottom, the swim tunnel is round in cross-section, and there was a small (gentle) current generated in it to mix the water to allow for MO_2 measurements. Thus, the higher RMR in these fish may have reflected an increase in activity to remain upright and in position.

Several recent studies have shown that the U_{crit} test provides consistently higher values for MMR than the traditional chase test, even though they are both methods of exercise-induced exhaustion. For juvenile Atlantic and Chinook salmon, a 3-minute chase followed by the measurement of MO_2 in static respirometry chambers resulted in an MMR for these species that was ~20% lower as compared to measured using a U_{crit} protocol (Raby et al., 2020). Hvas and Oppedal (2019) found that chasing Atlantic salmon post-smolts for three minutes provided an MMR value that was 52% lower than that obtained in a U_{crit} test. Rummer et al. (2016) reported similar results, where all four species of tropical coral reef fishes (*Pterocaesio marri, Caesio teres,*

Acanthochromis polyacanthus and *Chromis atripectoralis*) had higher MMR values when subjected to a traditional U_{crit} test (by 2 to 23%) as compared with a chase test. However, Zhang et al. (2020) recently chased rainbow trout within a respirometer and found that MMR was similar to that measured in a U_{crit} test, and that this value was about 18% higher as compared to the traditional method of chasing fish in a shallow tank and then transferring them to a respirometer. Further, Reidy et al. (1995) compared the MMR of Atlantic cod (*Gadus morhua*) that underwent U_{crit}, U_{burst} and chase protocols, and MMR during the U_{crit} protocol was 35% lower than that determined following a chase test. Thus, differences in MMR determined using U_{crit} and chase tests may be dependent on the species examined and the specific methods utilized.

There are few studies that have directly compared measures of fish metabolic capacity as determined using both CT_{Max} and U_{crit} protocols. In this study, the MMR and AS of lumpfish subjected to a CT_{Max} test were 24 and 32% lower, respectively, than when measured using the U_{crit} test (Table 2.1). This trend is often observed in other studies that compare these methods. Powell and Gamperl (2016) reported that the MMR, RMR and ASR of Atlantic cod measured during a U_{crit} test were 183.2, 90.0 and 96.5 mg O_2 kg^{-1} h^{-1}, respectively, whereas their MMR, RMR and ASR recorded during a CT_{Max} test were 146.2, 73.3 and 72.9 mg O_2 kg^{-1} h^{-1}, respectively. Further, Petersen and Gamperl (2010) conducted a U_{crit} test on Atlantic cod and obtained values for MMR, RMR and ASR of 234.6, 82.5 and 152.1 mg O_2 kg^{-1} h^{-1} respectively, whereas those measured by Gollock et al. (2006) using a CT_{Max} test were 210.8, 82.2 and 128.6 mg O_2 kg^{-1} h^{-1}, respectively. However, lower values for these parameters are not always reported for fish subjected to a CT_{Max} test vs. a U_{crit} test. For example, Norin et al. (2019) found that the MMR and ASR of 12°C acclimated cod and haddock (*Melanogrammus aeglefinus*) were very comparable using these two tests (see Tables 2.2 and 2.3).

2.4.2: The 3 Methods of 'Exhaustion' Were Not Correlated, and Cannot be Directly Compared

It is essential to determine which method of 'exhaustion' provides the highest MMR, and most accurate AS for the species of interest (Killen et al., 2021; Blasco et al., 2020; Little et al., 2020; Raby et al., 2020; Zhang et al., 2020; Hvas and Oppedal, 2019; Killen et al., 2017; Rummer et al., 2016; Roche et al., 2013; Nelson et al., 2002; Reidy et al., 1995). It has been suggested that strong swimmers reach their MMR during sustainable swimming tests rather than after chasing or feeding (Norin and Clark, 2016; Rummer et al. 2016). In contrast, species that are poor swimmers, have a benthic lifestyle, or are ambush predators may have greater values for MMR after a chase or ingesting a meal (Norin and Clark 2016; Killen et al., 2014). If a method of exhaustion/stressor is selected to be used in an experiment that may not reflect the highest MMR of a species, a correction factor could be applied to the values of MO_2 obtained. For instance, it may be difficult to conduct a U_{crit} test in the field, and in this case, it may be more appropriate to conduct a chase test (Little et al., 2020). However, it is essential that preliminary experiments be conducted to determine the method of exhaustion that provides the highest values for MMR and AS/ASR for the study species and determine if/what correction factor is needed.

An important tenet of this approach should be that there is a significant correlation between measures of metabolic capacity at the individual level, as there is when fish are repeatedly exposed to the same method of exhaustion (Mullen and Rees, 2022; Morgan et al., 2018; Nelson et al., 2007; Kolok, 1992). As shown in Figs. 2.9 – 2.11, none of the measures of metabolic capacity obtained using the three protocols in this study were correlated with each other ($0.25 > r^2 > 0.0035$). For lumpfish, this indicates that the MMR and AS achieved using one method of 'exhaustion' are not comparable or interchangeable with the MMR and AS achieved when exposed to another stressor (e.g., temperature stress as in a CT_{Max} challenge vs. exhaustive exercise). This finding is

consistent with the recent data of Mullen and Rees (2022), where three possible methods of achieving MMR (a chase, a U_{crit} test and feeding to satiation) were compared using Gulf killifish (*Fundulus grandis*). Much like this study, Mullen and Rees (2022) failed to find any significant correlations between MMR obtained in the three tests. A possible reason is that the physiological mechanisms that determine maximum performance, and its metabolic cost, when exposed to various stressors may be different (Ørsted et al., 2022). For instance, larval zebrafish subjected to a CT_{Max} test experience a decline in spontaneous neural activity, and a loss of neural response to visual stimuli, at high temperatures. These data suggest that insufficient oxygen availability during warming caused impaired brain function as zebrafish reached their CT_{Max} (Andreassen et al., 2022). In contrast, the MMR measured during sustainable swimming is largely due to skeletal muscle activity, with contributions from other tissues that are active during exercise (e.g., heart). Finally, the processes underlying MO_2 after an exhaustive chase include residual skeletal muscle activity, replenishment of blood and tissue oxygen stores, and the clearance of products of anaerobic metabolism (Norin and Clark, 2016).

2.4.3: Comparing Lumpfish with Other Species

The CT_{Max} of 6ºC acclimated lumpfish in this study was 20.6°C (Fig. 2.8B). This value is comparable to that reported by other authors for this species when acclimation temperature is taken into consideration (Beitinger and Lutterschmidt, 2011). Ern et al. (2016) and Chapter 3 showed that lumpfish acclimated to 10°C had CT_{Max} values of 22.3°C and 22.9°C, respectively. These values are several degrees lower than measured in various salmonids. For example, the CT_{Max} of 10-12°C acclimated Atlantic salmon ranges from 26.2 to 28.5°C when temperature is increased at 2°C h^{-1} (Table 2.3; Ignatz et al., 2021; Leeuwis et al., 2019; Penney et al., 2014), and that of the rainbow trout ranges from 23.7 to 25.3°C (Table 2.3; Motyka et al, 2017; Keen and Gamperl,

2012). In addition, they are lower than the CT_{Max} measured for haddock (*Melanogrammus aeglefinus*) when acclimated to 12°C (23.9°C; Norin et al., 2019) and sablefish (*Anoplopoma fimbria*) acclimated to 10°C (24.9°C; Leeuwis et al., 2019). However, they are comparable to the values reported for Atlantic cod, another north temperate marine species; 21.4°C when acclimated to 8°C (Norin et al., 2019) and 22.5°C when acclimated 10°C (Zanuzzo et al., 2019).

In this study, the U_{crit} for our cultured lumpfish was 2.39 $\pm$ 0.081 BL s^{-1} and values for MMR, AS and ASR were 243.0 $\pm$ 7.7, 206.4 $\pm$ 8.5 and 134.0 $\pm$ 5.9 mg O_2 kg^{-1} h^{-1}, respectively (Table 2.1). This value for U_{crit} is very comparable to that reported by Hvas et al. (2018) for lumpfish at 9°C when using their fork length vs. U_{crit} relationship (2.55 BL s^{-1}). Further, while their value for MMR (~ 205 mg O_2 kg^{-1} h^{-1} at 6°C) is slightly less than reported here, their values for ASR and factorial AS (FAS: MMR / RMR) are very similar (132 mg O_2 kg^{-1} h^{-1} and 2.6, respectively). Hvas et al. (2018) concluded that the metabolic and swimming performance of lumpfish reflected that of a sluggish and benthic species based on its performance, rather than a species that is pelagic during large parts of its lifecycle (i.e., as adults; Kennedy et al., 2016; Kennedy et al., 2015; Pampoulie et al., 2014; Davenport, 1985). This was largely based on a comparison of its values for U_{crit} and metabolic capacity as compared to Atlantic salmon (e.g., Hvas et al., 2017).

Recently, U_{crit} experiments were conducted on Atlantic salmon acclimated to 1, 4 and 8°C (Porter and Gamperl, submitted), and values for their MMR, ASR and FAS at 8°C were ~ 352.08 mg O_2 kg^{-1} h^{-1}, 293.30 mg O_2 kg^{-1} h^{-1} and 6.0, respectively (Table 2.3). However, Atlantic salmon are known to be athletic fish with a strong swimming ability, and again, values obtained for the Atlantic cod at 8°C (Norin et al., 2019) are not that different to those reported for the lumpfish; the U_{crit} of cod at 12°C was 2.62 BL s^{-1}, while their MMR, ASR and FAS were ~ 315 and 130 mg O_2

kg^{-1} h^{-1} and 2.0, respectively (Table 2.3). While there are limited performance (U_{crit}) values for true benthic species, Joaquim et al. (2004) reported that wild winter flounder only had a U_{crit} of 0.65 BL s^{-1} at 4°C. Thus, while the lumpfish cannot be considered an 'athletic' fish, it appears that they should not be considered 'sluggish' either. Studies on other North Atlantic species are needed before this species' swimming and metabolic capacity can be categorized as compared to other taxa. Further, with respect to the lumpfish's metabolic capacity (MMR, AS and ASR), these values may be underestimates. A significant portion of the lumpfishes body mass is composed of subcutaneous 'gristle' / 'jelly' (e.g., see Fig. 1 in Willora et al. 2020) (especially in their 'hump'), and this tissue likely contributes little to the oxygen consumption of this species. Thus, there is less oxidative tissue in the lumpfish per gram of body mass, and if this tissue's mass were to be deducted from the fish's weight, the lumpfish's mass specific MO_2 would be considerably higher.

Excess post-exercise oxygen consumption (EPOC) is an estimate of the quantity of oxygen required to restore tissue and cellular oxygen stores and high-energy phosphates, biochemical imbalances in metabolites such as lactate and glycogen, and other functions such as ionic and osmotic balance following exhaustive exercise (Lee et al., 2003). The EPOC of juvenile lumpfish (~55 g, acclimated to 6°C) was relatively similar after the chase and U_{crit} tests (42.0 and 38.7 mg O_2 kg^{-1} respectively; Table 2.1) and comparable to that reported for Atlantic cod and some populations of Sockeye salmon [73.1 mg O_2 kg^{-1} (Petersen and Gamperl, 2010); 20.3-107.7 mg O_2 kg^{-1} (Lee et al., 2003)]. However, our post-exercise recovery time (~ 40 minutes) was relatively short (< 1 h, Fig. 2.8A, C) as compared to these two species [e.g., 125 minutes for Atlantic cod (Petersen and Gamperl, 2010); and 50 minutes for Sockeye salmon (Lee et al., 2003)]. In contrast, our values for EPOC are much lower than reported for Atlantic salmon acclimated to 12°C that underwent a 10-minute chase followed by two minutes of air exposure (704.1 mg O_2 kg^{-1}; Zhang

et al., 2016), or for 15°C acclimated Atlantic salmon subjected to a U_{crit} test (525 mg O_2 kg^{-1}; Powell et al., 2009). Clearly, there is considerable variation (almost 10-fold) in what authors are reporting for the EPOC of even closely related taxa (e.g., Sockeye vs. Atlantic salmon), and this makes comparing our data to the literature difficult. This could be related to differences in size and acclimation temperature of the various species. For instance, the Atlantic salmon referenced above were 126 g and acclimated to 15°C (Powell et al., 2009), the Atlantic cod were 550 g and acclimated to 10°C (Lurman et al., 2012), and the Sockeye salmon ranged from 2,690 g – 3,000 g and were acclimated to 12 - 18°C (Lee et al., 2003).

There is one paper that has previously examined the EPOC of lumpfish (~ 300 g), and these authors reported that EPOC was not detected following a U_{crit} test (Hvas et al., 2018). This was likely because of their measurement protocol (i.e., MO_2 measurements starting at 1 h post-exercise). Based on our data, metabolic rate would have been very close to/returned to values of RMR by this point, and it would have been difficult measure any post-exercise increases in metabolic rate. Nonetheless, these authors did report that post-exercise lactate levels were only 0.31 nM right after the U_{crit} test ended. This is very low as compared to what has been reported in more active species, and this supports our finding that the lumpfish has a relatively limited EPOC. In other fish species, plasma lactate concentrations may reach 5-15 mM following exercise stress and take > 6-12 h to return to control levels (Hvas et al., 2017; Wood, 1991; Milligan and McDonald, 1988;).

2.5: Summary and Conclusions

In this study, 12 cultured lumpfish (~55 g, 6°C) were each subjected to three commonly used methods of 'exhaustion' to determine their metabolic capacity: a chase, a critical swim speed (U_{crit}) test and a critical thermal maximum (CT_{Max}) test. As predicted, the U_{crit} test provided the highest MMR compared to the chase and CT_{Max} tests. However, the RMR of the fish used in the U_{crit} test was unexpectedly high, and therefore, the ASR of the U_{crit} and CT_{Max} tests were nearly identical (Table 2.1). Importantly, MMR, AS and ASR measured using the three different methods of 'exhaustion' were not significantly correlated, and this indicates that measurements of metabolic capacity using one method of 'exhaustion' cannot be used to estimate values that would be obtained using another method. This conclusion agrees with the recent findings of Mullen and Rees (2022) and has important implications for the field of fish physiology.

Knowing the metabolic capacity of lumpfish contributes to our understanding of what factors might constrain this species' physiological performance and tolerance limits, and how their ecology (i.e., as a benthopelagic fish) and their performance in cages-sites may relate to their metabolic physiology. For example, while our measures of U_{crit} and metabolic capacity are much lower than more active species such as salmon, they are comparable to that measured for the Atlantic cod, another pelagic North Atlantic species. These data suggest that the lumpfish is not as 'sluggish' or 'unathletic' as previously suggested in the literature. Further, the lumpfish's CT_{Max} surpassed 20°C (20.6°C), even when acclimated to relatively cool temperatures (6°C). Therefore, it is questionable whether high cage-site temperatures (typically 16-18°C) are the primary cause of lumpfish mortalities at salmon sea-cages during the summer.

Table 2.2: Comparisons of CT_{Max}, and the MMR, SMR, AS, RMR and ASR, of various north temperate fish species subjected to a CT_{Max} test in the Gamperl lab. All species experienced similar experimental conditions and rate of heating (2°C h^{-1} until loss of equilibrium). Temperatures indicated are those to which the fish were acclimated, and the mean mass of animals tested is indicated in grams (g)

	Species							
Parameter	Lumpfish *(Cyclopterus lumpus)* *(55 g; 6°C)* *Current Study*	Atlantic cod *(Gadus morhua)* *(73.0 g; 8°C)* *Norin et al. (2019)*	Atlantic salmon *(Salmo salar)* *(624 g; 10°C)* *Penney et al. (2014)*	Arctic char *(Salvelinus alpinus)* *(747 g; 10°C)* *Penney et al. (2014)*	Haddock *(Melanogrammus aeglefinus)* *(64.4 g; 12°C)* *Norin et al. (2019)*	Steelhead trout *(Oncorhynchus mykiss)* *(935 g; 12°C)* *Keen and Gamperl (2012)*	Sablefish *(Anoplopoma fimbria)* *(675 g; 10°C)* *Leeuwis et al. (2019)*	Cunner *(Tautogolabrus adspersus)* *(~100 g; 10°C)* *Kelly et al. (2014)*
CT_{Max} (°C)	20.6	21.4	26.5	23	23.9	23.7	24.9	26.3
MMR (mg O_2 kg^{-1} h^{-1})	183.9 ± 14.4	285.5 ± 7.9	343.1 ± 8.4	223.2 ± 11.4	331.0 ± 6.3	295.5 ± 20.9	234.3 ± 5.9	208.9 ± 12.9
SMR (mg O_2 kg^{-1} h^{-1})	36.6 ± 3.1		83.5 ± 8.3	67.6 ± 7.5			57.6 ± 3.4	
AS (mg O_2 kg^{-1} h^{-1})	141.0 ± 15.0		254.8 ± 16.1	154.8 ± 9.0			176.9 ± 7.0	
RMR (mg O_2 kg^{-1} h^{-1})	48.9 ± 4.0	143.6 ± 15.5			165.5 ± 7.0	111.0 ± 3.5	81.0 ± 4.6	42.3 ± 3.3
ASR (mg O_2 kg^{-1} h^{-1})	134.6 ± 15.0	141.8 ± 18.3			168.0 ± 6.1	184.5 ± 17.4	153.3	166.6 ± 13.0

Table 2.3: Comparison of the U_{crit}, and the MMR, SMR, AS, RMR and ASR, of various species subjected to a U_{crit} test in the Gamperl lab. All species experienced similar protocols and were tested under similar conditions in a lab setting. Temperatures indicated are those to which the fish were acclimated, and the mean body mass of animals tested is indicated in grams (g).

	Species			
Parameter	Lumpfish *(Cyclopterus lumpus)* *(55 g; 6°C)* *Current Study*	Atlantic cod *(Gadus morhua*) *(67.6 g; 12°C)* *Norin et al. (2019)*	Atlantic salmon *(Salmo salar)* *(681.4g; 8°C)* *Porter and Gamperl (in press)*	Haddock *(Melanogrammus aeglefinus)* *(63.0 g; 12°C)* *Norin et al. (2019)*
U_{crit} (BL s^{-1})	2.39 ± 0.81	2.62 ± 0.09	2.08 ± 0.039	3.02 ± 0.09
MMR (mg O_2 kg^{-1} h^{-1})	243.0 ± 7.7	315.0 ± 13.8	352.08 ± 14.85	284.9 ± 6.0
SMR (mg O_2 kg^{-1} h^{-1})	-	156.7 ± 15.2	46.35 ± 3.29 *(Extrapolated)*	84.3 ± 4.2
AS (mg O_2 kg^{-1} h^{-1})	206.4 ± 8.5 (*SMR from CT_{Max})*	130.0 ± 18.6	305.72 ± 13.75	165.5 ± 12.3
RMR (mg O_2 kg^{-1} h^{-1})	109.1 ± 6.4	185.0 ± 15.5	58.78 ± 2.76	119.4 ± 7.1
ASR (mg O_2 kg^{-1} h^{-1})	134.0 ± 5.9	130.0	293.30	165.5

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Chapter 3 - The Thermal Dependent Biology of Cultured Lumpfish (*Cyclopterus lumpus*): Differences Due to Temperatures During Early Life and How Tolerance is Measured

3.1: Introduction

As a result of climate change, global sea surface temperatures are expected to rise considerably, and be more variable. For example, the Intergovernmental Panel on Climate Change (IPCC) predicts that global mean sea surface temperatures will increase by up to 1.5°C by 2050 and could rise by 4.5°C by 2100 (IPCC, 2021), and the frequency, spatial area, duration and intensity of marine heat waves are all increasing (Oliver et al., 2021; Viglione, 2021; Oliver et al., 2019; Smale et al., 2019; Stilman, 2019; Frölicher et al., 2018; Oliver et al., 2018). Such events are having dramatic effects on marine populations/ecosystems but are also impacting marine aquaculture operations (Sánchez-Jerez et al., 2022; Bricknell et al., 2021). This is because many cultured fishes are reared/grown-out in sea-cages that are moored in coastal regions, and thus, susceptible to large changes in temperature (e.g., Gamperl et al., 2021; Burt et al., 2012; Bjornsson et al., 2007). Summer water temperatures have reached ~23°C in Tasmania in recent years, and this is having a variety of impacts on Atlantic salmon (*Salmo salar*) production (Wade et al., 2019; Stehfast et al., 2017). Further, a marine heat wave in 2019 in Newfoundland (Canada) resulted in the death of 2.6 million salmon, and one contributing factor to the loss of fish was reported to be temperatures above 18-20°C for a prolonged period (Burke et al., 2020). However, high temperatures are only one challenge facing salmon aquaculture, and in Atlantic Canada and Norway sea lice (*Lepeophtheirus salmonis*) infestations are a major cause of losses to the industry (Imsland et al., 2021; Geitung et al., 2020; Overton et al., 2019; Powell et al., 2018).

There are a number of strategies/options that can be used within an integrated pest management strategy to control sea lice at Atlantic salmon sea-cages, including the use of skirts, snorkel sea-cage technology, submerged lighting and feeding, and thermolicing and hydrolicing (Østevik et al., 2022; Walde et al., 2022; Parent et al., 2021; Bui et al., 2020; Geitung et al., 2019;

Overton et al., 2019; Grøntvedt et al., 2018; Grøntvedt et al., 2015; Frenzl et al., 2014). The use of cultured lumpfish (*Cyclopterus lumpus*) as a biological control for sea-lice at Atlantic salmon cage-sites has increased greatly over the past decade (Foss and Imsland, 2022; Powell, 2018; Imsland et al., 2016; Imsland et al., 2014a; Imsland et al., 2014b). Lumpfish are an environmentally responsible and efficient way to remove sea lice from salmon in sea-cages as compared to other treatment options (such as hydrogen peroxide, pesticides, mechanical removal and thermocycling; Klakegg et al., 2020; Aaen et al., 2015; Grøntvedt et al., 2015; Helgesen et al., 2015; Van Geest et al., 2014) that are known to stress salmon and/or result in mortalities as high as 15-30%. (Imsland et al., 2019; Overton et al., 2019). In addition, sea lice are becoming increasingly resistant to many common chemical treatments (Imsland et al., 2021; Geitung et al., 2020; Aaen et al. 2015, Helgesen et al., 2015).

However, significant losses of lumpfish at cage-sites bring into question the lumpfish's welfare when co-reared with salmon, and their suitability as a sea lice control strategy in locations that experience warm summer ocean temperatures (Reynolds et al., 2022; Garcia de Leaniz et al., 2021; Rabadan et al., 2021; Staven et al., 2021; Geitung et al., 2020; Imsland et al., 2014a; Imsland et al., 2014b). For instance, the Norwegian Food Safety Authority estimated that the annual mortality rate for lumpfish at surveyed cage-sites in 2018 and 2019 was 40% (Martos-Sitcha et al., 2020), and there is anecdotal data from cage-sites in Atlantic Canada that there are significant lumpfish mortalities in the summer months (pers. comm). This may not be surprising as temperatures in Atlantic salmon sea-cages in Norway and Atlantic Canada often reach 18-20°C in the summer (Gamperl et al., 2021; Burt et al., 2012; Björnsson et al., 2007), and although there is little information on the upper thermal tolerance of cultured lumpfish, Hvas et al. (2018) reported

that they cannot be held at temperatures ≥ 18°C for prolonged periods (weeks) without experiencing significant mortalities.

Clearly, the salmon aquaculture industry must look at addressing/improving the health and welfare of lumpfish when used as a biological control for sea lice. One way to increase the upper temperature tolerance of lumpfish at sea-cages is selective breeding. For example, it has recently been shown that both the critical thermal maximum (CT_{Max}) and incremental thermal maximum (IT_{Max}) of cultured Atlantic salmon are strongly heritable (Benfey et al., 2022). However, such information is not available for lumpfish, there are limited genetic resources for this species, and selective breeding programs could take considerable time before any gains in thermal tolerance are realized. An alternative approach could be to take advantage of the phenotypic plasticity displayed by fish when exposed to challenging environmental conditions. It has been shown that rearing early life stage fishes at higher temperatures increases their upper thermal tolerance (Del Rio et al., 2019; Shaefer and Ryan, 2006). There is evidence that the effects of developmental temperatures on heat tolerance (i.e., 'developmental plasticity') persist throughout the lifetime of the animals, and that this phenomenon may help some ectothermic organisms cope with the more variable temperatures that are expected under future climate-change scenarios (Scott and Johnson, 2012). Finally, while Shaefer and Ryan (2006) reported that acclimation temperature has considerable positive influence on the thermal tolerance of adult zebrafish (*Zebra danio*), these authors also report that exposing fish to varying thermal environments of the same average temperature amplifies this effect.

Given the very limited data on how rearing temperatures influence the early life history stages and production of lumpfish (Pourtney et al., 2020; Imsland et al., 2019; Collins et al., 1978) and on whether, and to what extent, developmental plasticity allows fish to cope with thermal

extremes, I exposed lumpfish eggs (embryos) and larvae/juveniles to varying thermal regimes (incubation temperatures of 6°C, 8.5°C and 6-11°C; rearing temperatures of 9 and 9-11°C). Then I measured a number of production metrics (e.g., survival, incidence of deformities, growth), their acute (CT_{Max}) and incremental (IT_{Max}) thermal tolerance at the size at which they would normally be put into sea-cages (40-60 g), and their metabolic capacity and plasma cortisol levels (as an indicator of temperature-induced stress). I hypothesized that 1): increasing temperatures during incubation/rearing would enhance the upper thermal tolerance of the lumpfish; 2) this effect would be most evident in those groups exposed to a fluctuating (stochastic) thermal environment; and 3) differences in the thermal tolerance of the groups would be reflected in their maximum metabolic rate (MMR) and aerobic scope (AS; Norin and Clark, 2016). The IT_{Max} measurements are particularly informative with regard to evaluating whether such rearing protocols might enhance the thermal tolerance of cultured lumpfish in sea-cages, and for predicting how this species might be impacted in the wild by climate change. As compared to the more commonly used CT_{Max}, IT_{Max} appears to be a more realistic and relevant measure of a fish's tolerance to long-term changes in water temperature (Ignatz et al., 2023; Bartlett et al., 2022; Zanuzzo et al., 2019). I measured plasma cortisol levels in the lumpfish at 10, 16, 18 and 20°C during the IT_{Max} test to assess if this parameter is influenced by early rearing temperatures and is a good indice/biomarker of temperature stress in this species. Plasma cortisol levels are used widely to assess acute and chronic stress in fish (Uren Webster et al., 2020; Sadoul and Geffroy, 2019; Tsalafouta et al., 2014). However, previous work on Atlantic salmon and cod indicates that plasma levels of this corticosteroid are not informative with regards to the effects of incrementally/gradually increasing temperatures up the fish's IT_{Max} (Zanuzzo et al., in prep as cited in Beemelmanns et al., 2021; Perez-Casonova et al., 2008).

3.2: Materials and Methods

3.2.1: Incubator Set-Up

Eight, 9 L, upwelling incubators were assembled as shown in Fig. 3.1A. Three of the incubators were supplied with 6°C seawater directly from the Dr. Joe Brown Aquatic Research Building's (JBARB's) water system at 2 L min^{-1}. The others were supplied with water from an 80 L Rubbermaid® bucket using a Little Giant® submersible pump (Franklin Electric, USA). The temperatures in these reservoirs were controlled by 1000 W and 1500 W immersion heaters with digital controllers (Intelligent Heater, USA). The first reservoir supplied three incubators with 8.5°C water at 2 L min^{-1}, whereas the other was used to achieve stochastic temperature changes (6 – 11°C, depending on the day) in two incubators. Only two incubators were used in this final group as the water could only be heated to 11°C at 4 L min^{-1}. The entire experimental set-up can be viewed in Fig. 3.1B.

3.2.2: Milt Collection

Two adult male lumpfish were selected at a time from the two-year-old broodstock that were maintained in the JBARB. Males were chosen that had a darker yellow-purple-green colour, indicating that they were reproductively mature. Males with a bright red-fuchsia colour, however, were avoided as there is anecdotal evidence that these males are spent. In addition, it has been suggested that the brighter red colour of male lumpfish may indicate higher stress levels than in males that have a more muted colour (Staven et al., 2021). The different colours of male lumpfish in the population are shown in Fig. 3.2.

An anaesthetic bath (0.1 g L^{-1} TMS), and a euthanasia bath (0.5 g L^{-1} TMS) were prepared.

Once the male lumpfish were anaesthetized, fish were selected one at a time and lightly dabbed with a paper towel to remove excess water. The fish were then weighed, and strip spawning was attempted. This was done by holding the fish at a 45° angle to the beaker, pointing the anal pore into the beaker and kneading the fish towards the anal pore with the thumbs. However, strip spawning was not successful for any male fish. Thus, the male lumpfish were then placed in the euthanasia bath for ~1 minute. Next, the lumpfish were removed from the euthanasia bath and laid on their side on a piece of paper towel. The testes were then exposed by dissection, carefully cut out, weighed after being dabbed with a paper towel to remove excess water, and placed in a Petri dish (Fig. 3.3A). The gonads were poured from the Petri dish into an herb grinder that was held over a strainer which prevented pieces of tissue from mixing with the milt. The gonads were then ground, and the milt was collected in a glass container (Fig. 3.3B). The previously tared glass container with the milt was then weighed to determine the volume of milt. Finally, a piece of parafilm was placed over the glass container, and the container was labelled and stored in the fridge until it was required for fertilization (see Section *3.2.4: Fertilization*).

3.2.3: Egg Collection

Females ready to release their eggs were purple-grey in colour, looked swollen, and possessed a large, round, swelling at their urogenital orifice (Fig. 3.4A). The selected females were gently placed into the anaesthetic bath. Once anaesthetized, female lumpfish were retrieved from the anaesthetic bath, and lightly dabbed with a paper towel to remove excess water and weighed. Next, a dry 500 mL container was placed onto a flat surface, and the female lumpfish was held just above the container at a ~45° angle, with her ventral side pointing towards the container. Using the forefinger, the ventral swelling of the lumpfish was gently pressed, being careful not to injure

the lumpfish (Figure 3.5A). This normally resulted in the release of eggs and ovarian fluid (Fig. 3.5B) into the container (Figure 3.5C). Once the flow of eggs from the lumpfish had ceased, the lumpfish was kneaded by gently pushing on the body wall with the thumbs to release the rest of the eggs and ovarian fluid (Fig. 3.5D).

Once the eggs were manually expelled, female lumpfish were taken back to their respective tank, and held in the water until they regained consciousness and swam away, and the weight of the eggs and ovarian fluid was recorded. This procedure was repeated with two more anaesthetized lumpfish, so that three containers of eggs and amniotic fluid, each from a separate fish, were obtained at a time.

3.2.4: Fertilization

Three plastic containers with a large surface area were assembled, and a large strainer was set on top of each container (Fig. 3.6A). One container of eggs (from one female only) was poured into the strainer, to separate the ovarian fluid from the eggs. The 'dry' eggs were carefully emptied from the strainer into a new container and weighed on a tared scale. This was repeated with the eggs and ovarian fluid from each of the other two fish. Eighty grams of 'dry' eggs were then scooped from each of the three 'dry' egg masses using a sterilized spoon, and placed gently into a new container, so that the 'dry' egg mass in fourth container was 240 g; 1/3 of the eggs coming from each of 3 lumpfish (Fig. 3.6B). This process was repeated two more times, so that there were 3 containers each containing 80 g of eggs from each of three female lumpfish.

Next, 30 mL of ovarian fluid from each female was added to each of the containers with eggs, and the eggs were gently mixed. After sufficient mixing, the two containers of milt (one from each male) were retrieved from the fridge, and 1.5 mL of milt from each male was pipetted into

each of the containers of wet eggs so each container contained a total of 3 mL of milt (Fig. 3.6C). The eggs were then gently mixed again and left for eight minutes (Fig. 3.6D). The gentle mixing, and standing of eggs was repeated two more times, to ensure fertilization. Gametes from two males and three females were then poured into a partially filled incubator in a circular fashion, ensuring that the eggs were evenly distributed about the center of the incubators. The gametes from three females and two males were placed into each incubator to ensure genetic diversity (Fig. 3.7). The egg masses were allowed to water-harden for 15 minutes without water flow, and thus, produced an 'egg cake'. Thereafter, the lids were placed on the three upwelling incubators, and water flow was initiated into each incubator at 2 L min^{-1}.

The *Milt Collection, Egg Collection* and *Fertilization* procedures were repeated, so that all incubators were filled with fertilized eggs. Refer to Fig. 3.7 for schematic of the incubator set-up indicating the males and females that contributed gametes to each incubator.

3.2.5: Incubation and Assessment of Egg Viability

The lumpfish eggs were incubated for 250 degree days (dd). The water temperature of all incubators was maintained at 6°C for the first three days. On day four, the water temperature of the 8.5°C incubators and 6-11°C incubators were increased gradually over two days to their respective temperatures. After this point, water temperature was maintained constant for the 6 and 8.5°C incubators. In contrast, the water temperature of the 6-11°C (stochastic) incubators was adjusted and checked twice daily (Fig. 3.8).

Egg viability was assessed at 50 and 100 degree days. A wireless scale, tared with a Petri dish, was placed next to the incubators, and 0.5 g of eggs were collected from each incubator. Using a tally counter and a dissecting scope, the total number of eggs and those that were

developing normally ('viable') [based on a chart from Imsland et al. (2019)] were counted. An egg was considered viable at 50 dd if there were "bubbles" or droplets in the egg (Fig. 3.9A). According to this chart, the eggs reached stage "J" by 50 dd (Fig. 3.9B). At 100 dd (Fig. 3.9C), eggs that had developed past stage J were counted as viable. Egg viability was not checked after 100 dd, as most eggs had reached the last stages of development [Figure 3.9D; stages M - P in Imsland et al. (2019)].

3.2.6: Rearing

At 250 dd, just prior hatching (at ~ 300 dd), the lumpfish eggs were transferred from their incubators to 10, 500 L, rearing tanks (Fig. 3.10A) at 6°C. When the eggs were transferred to these tanks, they were placed into buckets suspended in the tank (Fig. 3.10C). These buckets contained a water inlet and outlet, with water flow set at 6 L min^{-1}. This allowed the hatched larvae to enter the rearing tank, while unhatched eggs and the rest of the 'egg cake' remained behind. When placing eggs from the 6 and 8.5°C incubators into the buckets, 1/3 of the eggs from each incubator (108 g in total) were placed into each rearing tank. For the tanks receiving eggs from the 6-11°C (stochastic) incubation groups, 54 g of eggs from each of the two incubators were placed into each tank. Again, this procedure was followed to ensure genetic diversity in each tank / treatment (Fig. 3.11).

Water temperatures in these rearing tanks were then increased to 9°C over the course of two days, and the tanks containing the eggs and larvae assigned to one of two temperature regimes: either a constant temperature of 9°C (the temperature of the water coming from the seawater system) or 9-11°C, changing stochastically (i.e., the temperature changed each day based on a randomization scheme). Water temperature in the stochastic rearing tanks was also controlled

using immersion heaters that were adjusted manually, twice per day (Fig. 3.10B). The combination of different rearing and incubation temperature regimes produced 5 incubation / rearing temperature groups, each with replicate tanks: 6 / 9°C, 8.5 / 9°C, 8.5 / 9-11°C, 6-11 / 9°C and 6-11 / 9-11°C (Fig. 3.11).

Once the eggs had finished hatching, the white bucket was removed and water flow into the tank was reduced to 3 L min^{-1}. Larvae were then reared in these tanks until they were ~30-40 g juveniles. These rearing tanks had central standpipes with numerous slots to allow water, but not larvae, to exit the tank (the width of these slots increasing from 300 to 500 µm during rearing). In addition, the rearing tanks had air stones to provide a gentle upwelling water current, and a surface skimmer to remove lipids and other material that accumulated at the surface of the tank. The photoperiod during larval rearing was 24h light: 0h dark. During juvenile rearing, the photoperiod was 12 h light: 12 h dark. Light intensity was maintained at 100 - 135 lux. The rearing tanks were siphoned every 1-2 days to remove debris accumulating on the tank bottom, and to allow for the quantification of mortalities.

Both larvae and juveniles were fed according to protocols established at the JBARB for this species. The larvae were initially fed *Artemia nauplii* enriched with ORI-N3 (an algal based DHA enrichment; Skretting, Vancouver, BC. Canada) twice per day at 9:00 am and 3:00 pm for four days; 0.25 million *Artemia* per tank at 9:00 am, and if the *Artemia* were eaten by 3:00 pm, that tank would receive another 0.25 million. At five days post-hatch (dph), the larvae were fed the same amount of *Artemia nauplii* three times per day: at 9:00 am, 3:00 pm and 9:00 pm. At 10 days post-hatch, the larvae were weaned off live feed, and then exclusively fed a marine fish microdiet (Skretting Europa Marine Finfish feed, Nutreco, Gujarat, India). Table 3.1 shows the

approximate ration, feed size and tank water flow during the rearing period. Adjustments to the fish's rearing environment were made following established protocols in the JBARB.

Larvae and juveniles were sampled for weight and length every 100 dd until 500 dd, after which they were sampled every 200 dd (n = 25 per tank; Fig. 3.12A-D). Larvae were measured after euthanization with an overdose of TMS (0.8 g L^{-1}) in a 200 mL beaker (one beaker per tank). Each larva was removed from the beaker with a plastic pipette and placed into a 24-well plate (one fish remained in the beaker). After blotting each larva carefully on paper towel, the fish were carefully transferred, using tweezers, onto a piece of parafilm placed on scale with a precision of 0.1 mg (Sartorius Practicum® Precision Standard Balance, 210 g, PRACTUM213-1S, Goettingen, Germany; Fig. 12A). The larva's mass was then recorded, and the larvae was returned to its well. This process was repeated for all 25 larvae from one tank.

Next, the well-plate and beaker containing the 25 larvae were carried to a dissecting scope, with an attached camera (INFINITY 3-6UR Digital Un-Cooled CCD Research Microscope Camera, Lumenera Corporation, Ottawa, CA) interfaced with a desktop computer running Infinity Capture software (Infinity Capture for Windows, v 6.5.5, Lumenera Corporation). One larva at a time was pipetted into a Petri dish with a few drops of seawater, the Petri dish containing a very small ruler for use in measuring the length of the fish. The fish was gently maneuvered so that it aligned with the ruler, and a photo was taken with the computer. This photo was used to measure the length and height of the larvae using Infinity Analyze software (Infinity Analyze for Windows, v 6.5.5, Lumenera Corporation) (Fig. 3.12C). In addition, any deformities were noted, and a clear image of the deformity was taken. Deformities that were identified/recorded in larvae were scoliosis, sucker deformities, cataracts, missing eyes, and other eye deformities. This analysis was

completed for all 25 fish. Using the mean body mass of the fish in each tank obtained from each sampling, the amount of diet fed to each tank was also adjusted.

Once the fish became juveniles (~ 2.0 g), they no longer needed to be anaesthetized prior to sampling. Twenty five fish from each tank were netted into a bucket containing oxygenated seawater. One at a time, fish were selected, patted dry, and placed upright on a tared scale covered with parafilm to prevent them sticking (Fig. 3.12B). Mass was recorded, and the fish was laid flat on top of a clear ruler (Fig. 12D). Length was recorded, and a photo was taken. If any deformities were observed, the type of deformity was recorded, and a photo of the deformity was taken. The fish was then gently placed back into its tank. This procedure was repeated for all 25 fish per tank, and the mean body mass of the fish was used to adjust the tank's daily ration.

The fish in the tanks were counted, graded (using a grader with 4 mm slots to retain all but the smallest individuals), and the numbers were reduced to 700-800 individuals per tank at 900 dd post-hatch (ddph) following standard JBARB procedures. At this point, the tanks were also drained, cleaned, and refilled. Using this new count of fish, the numbers of mortalities recorded to this point, and the number of eggs placed into the tank (108 eggs per g of egg mass) hatching success and survival during rearing were calculated for each tank/group.

3.2.7: Tagging and Distribution of Fish for the CT_{Max} and IT_{Max} Experiments

Once the lumpfish were 30-40 g sixty fish (n = 12 from each incubation/rearing group; n = 6 from each tank) were anaesthetized with 0.4 g L^{-1} of TMS, weighed, and had a PIT-tagged (Loligo Systems; ISO 11784 certified PIT tags, Viborg, Denmark) after making a small incision in the abdominal cavity with a curved scalpel blade. Next, the fish had coloured thread sutured to the base of their dorsal fin (Figure 3.13A). This was done so that the incubation / rearing group

and tank of each fish could be easily identified (Table 3.2). Once the fish were PIT-tagged and marked, they were transferred to a newly filled 500 L rearing tank in the JBARB, until they regained consciousness. After all the fish were tagged, they were moved to a 500 L tank in the Laboratory for Atlantic Salmon and Climate Change Research LASCCR. The water in the new tank was initially 10°C, and flow into the tank was maintained at 5 L min^{-1}.

Next, the 400 fish that were to be used for the incremental thermal maximum (IT_{Max}) experiments were PIT-tagged and transported to the LASCCR where they were placed into four tanks (100 fish / tank; 20 fish from each incubation / rearing group in each tank). The water temperature in all tanks was initially maintained at 10°C for one week, with flow kept at 5 L min^{-1}, before temperature in these tanks was increased (see below). Finally, another 100 fish were PIT-tagged in the same manner and maintained in a separate 500 L rearing tank in the LASCCR. These fish were used as a time-matched control for the IT_{Max} test. Water temperature in this tank was maintained at 10°C for the duration of the experiment.

The fish used for the CT_{Max} experiments were fed a maintenance diet of 0.5% of the mass of fish in the tank, until they were ready for experimentation. Their ration was adjusted each week, depending on the number of fish remaining in the tank, and their approximate mass. The IT_{Max} fish were fed a maintenance ration during their week of recovery at 10°C, based on the average mass and number of fish in each tank. However, once the experiment began, they were hand fed to satiation on two consecutive days each week. The average amount of feed consumed was then calculated, and the fish were fed this ration for the remaining five days of the week.

3.2.8: Critical Thermal Maximum (CT_{Max}) Test

Two fish at a time were selected to undergo a CT_{Max} test, with the larger fish selected first. This ensured that they would not grow too large for the respirometry chambers. The two fish selected were similar in mass, although they were never from the same incubation/rearing group. The selected fish were held in their 500 L rearing tank in a floating cage, to prevent feeding for 24 hours before the experiment began. The following day, the two fish were transported from their 500 L rearing tank in the LASCCR building, weighed, and placed into 1065 mL custom-made rectangular glass respirometry chambers (14.0 cm length x 8.0 cm width x 8.5 cm high) at 10°C with a 'false' bottom (Fig. 3.14C). These chambers were submersed in a temperature-controlled shallow tank, positioned over top of a multi-position stir plate (IKA RT 15 Magnetic Stirrer, Wilmington, North Carolina, USA), which was located in a blacked-out enclosure with indirect lighting (Fig. 3.14 B). Mixing of water inside the respirometry chambers was accomplished using a stir bar (rotated at 240 rpm) placed underneath the 'false bottom' (Rodgers et al., 2016).

Oxygen consumption (MO_2) was measured overnight (i.e., from 6:00 pm to 8:00 am) using automated intermittent closed respirometry (as per the recommendations of Killen et al., 2021; Rodgers et al., 2016; Svedsen et al., 2016), with 'flush' and 'closed' periods of four minutes and 16 minutes, respectively. This was accomplished by automatically turning on/off an Eheim 5 L min^{-1} submersible pump (Model 1048; Eheim GmbH Co., Deizisau, Germany) using a computer program written by Tommy Norin (Norin et al., 2019); note that the flush rate (~ 440 mL min^{-1}) was selected so that water in the chambers returned to > 95% air saturation between oxygen measurements. Water oxygen partial pressure (PO_2) inside the respirometry chambers was recorded at 0.1 Hz using fibre-optic dipping probes connected to a FireSting optical oxygen meter

(Pyro Science GmbBH, Resenberg, Germany) and a computer running Pyro Oxygen Logger, version 3.314 (2019 by Firmware 2.30, Aachen, Germany).

The following morning, the lights in the respirometry enclosure were slowly turned on over 10 minutes, and the fish were allowed to adjust to the lighting for one hour before the experiment began. An initial measurement of MO_2 was taken for 20 minutes. Then, an immersion heater (Intelligent Heater LLC, QDNTY-1.8-1, 1800 W) was used to increase the water temperature in the tank by 2°C h^{-1}, with MO_2 recorded at every 1°C increase (Fig. 3.14A). The 'flush' and 'closed' periods were controlled manually and adjusted as the experiment progressed so that the O_2 level in the chambers did not fall below 95% saturation even as temperature was increased. Temperature was increased until each fish lost equilibrium (i.e., reached its CT_{Max}) as determined by watching live video feed from a Go-Pro® (model HERO 6.0 Black) camera placed in front of the chambers (Fig. 3.14 D).

After each fish reached its CT_{Max}, it was immediately removed from the respirometer, euthanized with an overdose of TMS (0.8 g L^{-1}), and had its PIT-tag removed. Given that the CT_{Max} of 60 fish (12 per group) were measured, it took approximately one month to complete measurements on all fish.

3.2.9: Calculation of Growth and Metabolic Parameters

After each test was completed, the time and PO_2 data were imported into LoggerPro® 3.1 (Vernier Software & Technology, Beaverton, Oregon, USA). The program created a graph of % air saturation as a function of time, and at each measurement point, the slope of the decline in oxygen (in units of % air s^{-1}) was determined, and this was used to calculate the fish's MO_2:

$$MO_2 \text{ (mg } O_2 \text{ kg}^{-1} \text{ h}^{-1}) = \frac{\text{slope (\% air sat. s}^{-1}) \text{ x 3600 (s h}^{-1}) \text{ x [water oxygen concentration (mg } O_2 \text{ L}^{-1})] \text{ x [volume of chamber (L) – mass of fish (L)]}}{\text{100\% x mass of the fish (kg)}} \quad (1)$$

Note: 1) The value for water oxygen concentration was calculated at each temperature using a 2nd order polynomial. Oxygen in mg / L = $0.0034x^2$ - 0.2723x + 11.575, where x = temperature

2) We assumed that 1 g of fish = 1 mL of seawater.

Standard metabolic rate (SMR) was calculated by taking the average of the lowest 10% of the MO_2 recordings from the overnight measurements. Routine metabolic rate (RMR) was recorded as the MO_2 measurement before the start of the CT_{Max} protocol at 10°C. Maximum metabolic rate (MMR) was recorded as the highest metabolic rate recorded for each individual during the CT_{Max} protocol. To account for background respiration when calculating fish MO_2, blank MO_2 measurements were taken in each respirometry chamber after the fish were removed. However, background respiration was negligible in all cases. The fish's aerobic scope (AS) was calculated by subtracting the SMR of each fish prior to the CT_{Max} test from its respective MMR. This value represents the capacity of fish to increase their metabolic rate beyond the oxygen required for maintaining essential cellular and physiological functions (Halsey et al., 2018).

3.2.10: Incremental Thermal Maximum (IT_{Max}) Test, and Blood, Liver and Ventricle Sampling

In the IT_{Max} test, temperature in the four 500 L tanks in the LASCCR was increased by 0.1-0.2°C day^{-1} (average 1°C wk^{-1}) until all fish lost equilibrium/100% mortality was reached (Fig. 3.15A). At 10°C, eight fish were sampled, n = 2 fish per tank. Whereas, at 16, 18, and 20°C, 50 fish were sampled (n = 10 fish per tank, and n = 10 fish per incubation/rearing group). In addition, at 16, 18, and 20°C, 12 fish were sampled from the time-matched control (10°C) tank (Fig. 3.15A).

All fish in this experiment that were not sampled had their PIT-tag read when they reached their IT_{Max}.

Sampling fish involved drawing blood and weighing the fish's liver and ventricle. The fish had their PIT-tag read so that their incubation / rearing group could be identified, and they were then immediately placed into a 5 L bucket containing seawater with 0.4 g L^{-1} of TMS. Once the fish lost equilibrium (after ~ 30-45 s), they were removed from the bucket and placed ventral-side-up on a sponge, and ~ 0.5-0.8 mL of blood was drawn from their caudal vessels using 1 mL heparinized syringes and ½" (25 gauge) needles (this process took less than three minutes). The fish were then placed into a 5 L euthanasia bath, containing 0.8 g L^{-1} of TMS for ~ two minutes, and the blood was transferred into a 1.5 mL Eppendorf® tube and briefly placed on ice. This process was repeated until 10 fish had been sampled. The Eppendorf® tubes containing the blood were then centrifuged a 1000 *xg* for ~ two minutes, and the plasma was pipetted into several new labelled Eppendorf® tubes. These plasma samples were snap-frozen in a 1 L dewar containing liquid nitrogen before being placed in a -80°C freezer. The weight and length of each fish were then recorded, and each fish's liver and ventricle were removed and weighed (Fig. 3.15D).

Growth parameters, including specific growth rate (SGR), thermal growth coefficient (TGC), and body mass index (BMI) were calculated using the following equations, respectively:

$$SGR = \frac{[\ln(m_2) - \ln(m_1)\ ] * 100}{t} \quad (2)$$

Where m_2 = the final mass of fish the fish, m_1 = the initial mass of the fish, and t = time (in days).

$$TGC = \frac{(m_2^{1/3} - m_1^{1/3}) * 1000}{T * t} \quad (3)$$

Where T = temperature (°C).

$$\text{BMI} = \frac{m}{l^2} \quad (4)$$

Where m = mass of the fish, and l = length. BMI was calculated as a measure of condition of lumpfish to account for their unusual body shape, as used in Rabadan et al., 2021. Hepatosomatic index (HSI) and relative ventricular mass (RVM) were then calculated by dividing each tissue's mass by that of the fish and multiplying by 100. Finally, the presence of cataracts and any deformities were recorded. This procedure was repeated until all fish were sampled.

3.2.11: Cortisol Analysis

The concentration of cortisol in lumpfish plasma was determined using a commercial ELISA kit (Neogen Life Sciences, Cortisol ELISA kit, 402710, Lexington, KY, USA) following the manufacturer's instructions. It was determined in preliminary work that, due to the low cortisol levels, different dilution factors were needed for each time point (dilution factors of 1x for fish held at 10°C, 2x for fish held at 16°C, and 5x for fish held at 18 and 20°C were used. The dilutant used was the extraction buffer provided in the ELISA kit. The samples were randomized on each plate within each sampling point.

3.2.12: Statistical Analyses

For many of the parameters measured (i.e., egg viability and hatching success, survival at 900 ddph, indices of growth, the number of deformities) the unit of replication was the incubator or tank, and thus, independent sample size was only two or three per group. This provided very little/limited statistical power with the types of models that were run (see below), which had up to two fixed factors in addition to nested (tank) and/or random factors. Thus, it was decided that

statistical analyses would not be run on these parameters, and that the reporting of these data would be solely descriptive in nature. However, there were a number of parameters where fish was the sampling unit, and this allowed for statistical analysis of these data in RStudio 4.1.2 (RStudio Team, 2021). For all of the general linear mixed models that were run (see below), tank was initially used as a nested factor. However, it did not explain a significant amount of variation in any of the models. Thus, it was taken out of the models, and they were run again.

Differences in body mass as a function of age (over the age ranges 100 – 400 ddph and 400 – 900 ddph), and parameters measured during the IT_{Max} test (i.e. mass, length, body mass index, HSI and RVM), were identified using linear mixed effects models with age or temperature, respectively, and group as fixed effects. This was followed by least square means post-hoc tests to identify differences in mass between groups at a particular age (temperature), and between ages within a particular group (Fig. 3.17A-B).

A linear mixed effects model was used to examine differences in CT_{Max}, metabolic parameters during the CT_{Max} test, and IT_{Max} between groups, with group as a fixed effect and fish as a random effect. Group did not have an effect on any of these parameters, and thus, no post-hoc tests were performed. In addition, IT_{Max} and CT_{Max} within each group were compared with paired t-tests. Cortisol levels between fish held at the various temperatures were also compared using the fixed factors of group and temperature with fish as a random factor. However, there was no effect of group on cortisol levels, and thus, the data for all groups was combined before running least square means tests to examine the effects of temperature on the levels of this parameter.

A Kaplan-Meier curve (Fig. 3.20; Yang et al., 2021) was used to visualize differences in survival for larvae/early juveniles during the temperature anomaly and during the IT_{Max} tests.

Significance was set at $p < 0.05$, and data in figures, tables and throughout the text are means $\pm$ 1 standard error of the mean.

Figure 3.1: Picture of (A) the components of a 9 L upwelling incubator (9 L base, inlet line with spiral base, and the mesh sheet that was placed on top of the spiral base; lid not shown) and (B) the incubator set-up including the temperature-controlled reservoirs, the incubators, manifolds, and the water table.

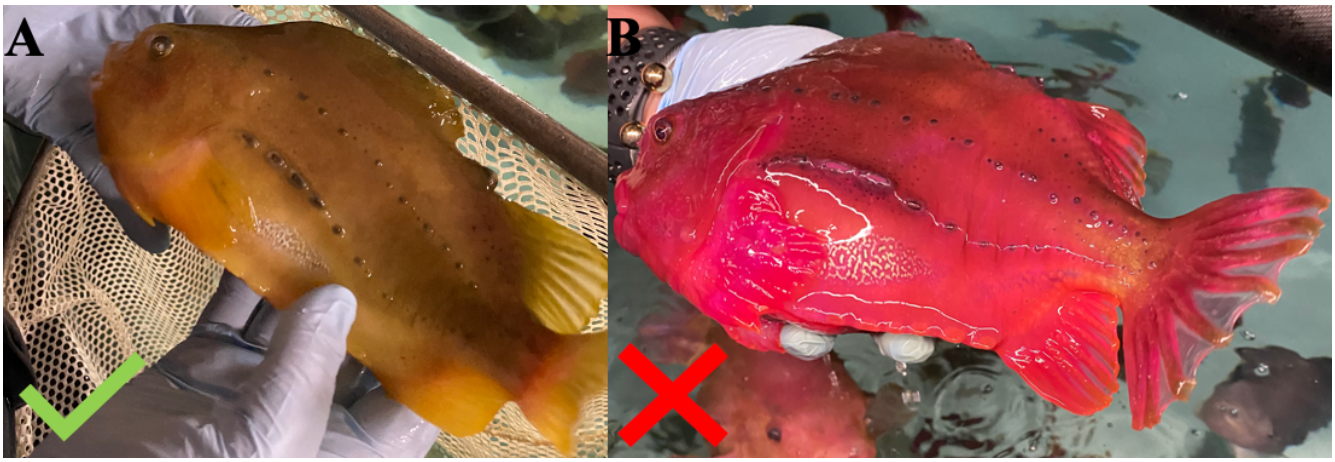

Figure 3.2: Images of two-year-old male broodstock at the JBARB facility. Fish that were used for fertilization were yellow-purple-green in colour (A), and fish that were avoided were bright fuchsia in colour (B).

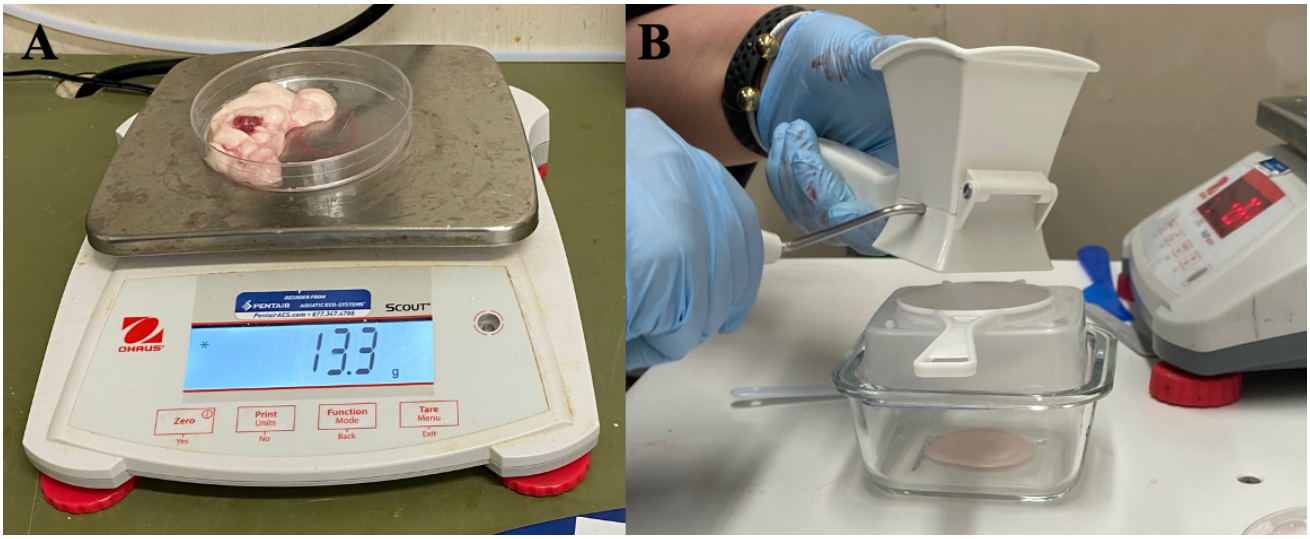

Figure 3.3: Lumpfish gonads in a Petri dish (A), and the process of grinding the gonads to extract the milt (B).

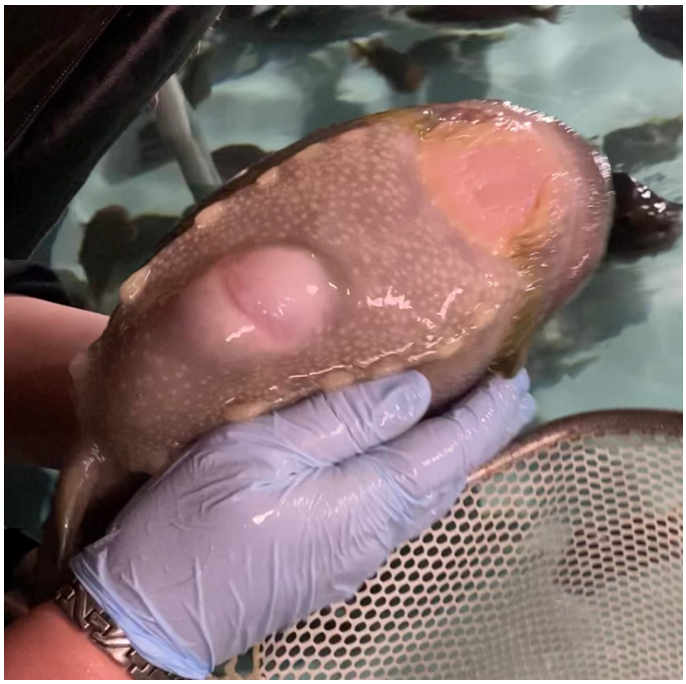

Figure 3.4: A healthy female lumpfish with a swelling on her ventral side, indicating she is ready to release her eggs.

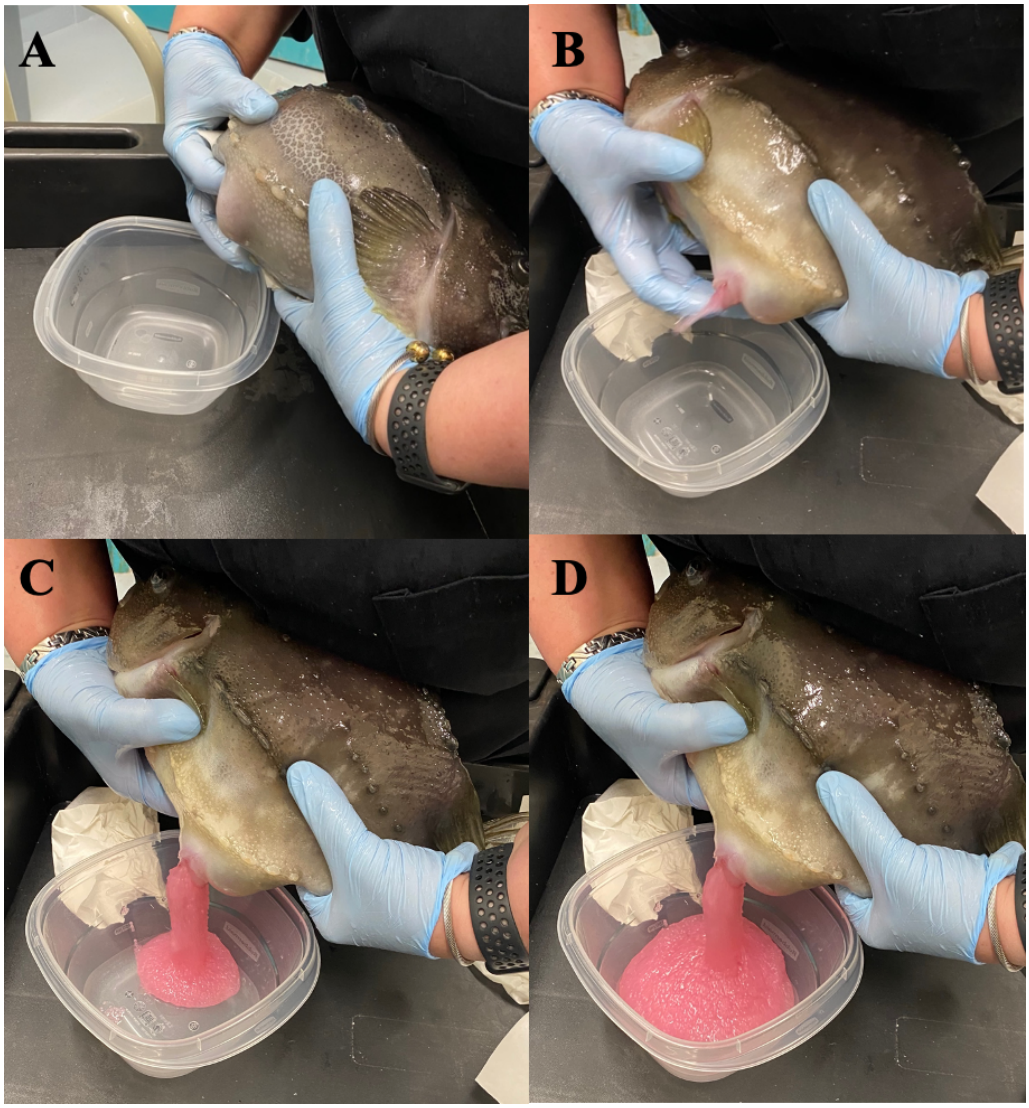

Figure 3.5: The process of collecting eggs from of a female lumpfish. This involved using the index finger to gently press on the ventral swelling (A) and holding the anaesthetized fish at an angle (B) that allowed the eggs to be released into a container (C). To ensure all the eggs were released, the thumbs were then used to gently massage the eggs towards the ventral swelling (D).

Figure 3.6: Combining of egg masses from 3 different female lumpfish and fertilizing them with milt from 2 different male lumpfish. The eggs and ovarian fluid from each female were separated using a fresh container and a strainer (A). The 'dry' egg masses were then weighed, and 80 g of eggs from each female were combined to produce 3 new egg masses (B). The ovarian fluid (1/3rd from each female) was then added to the 'dry' egg masses, and each egg mass was fertilized with 3 mL of milt, 1.5 mL from each of 2 males (C). Lastly, the egg masses were stirred, and poured into new containers (D) prior to being placed in the incubators.

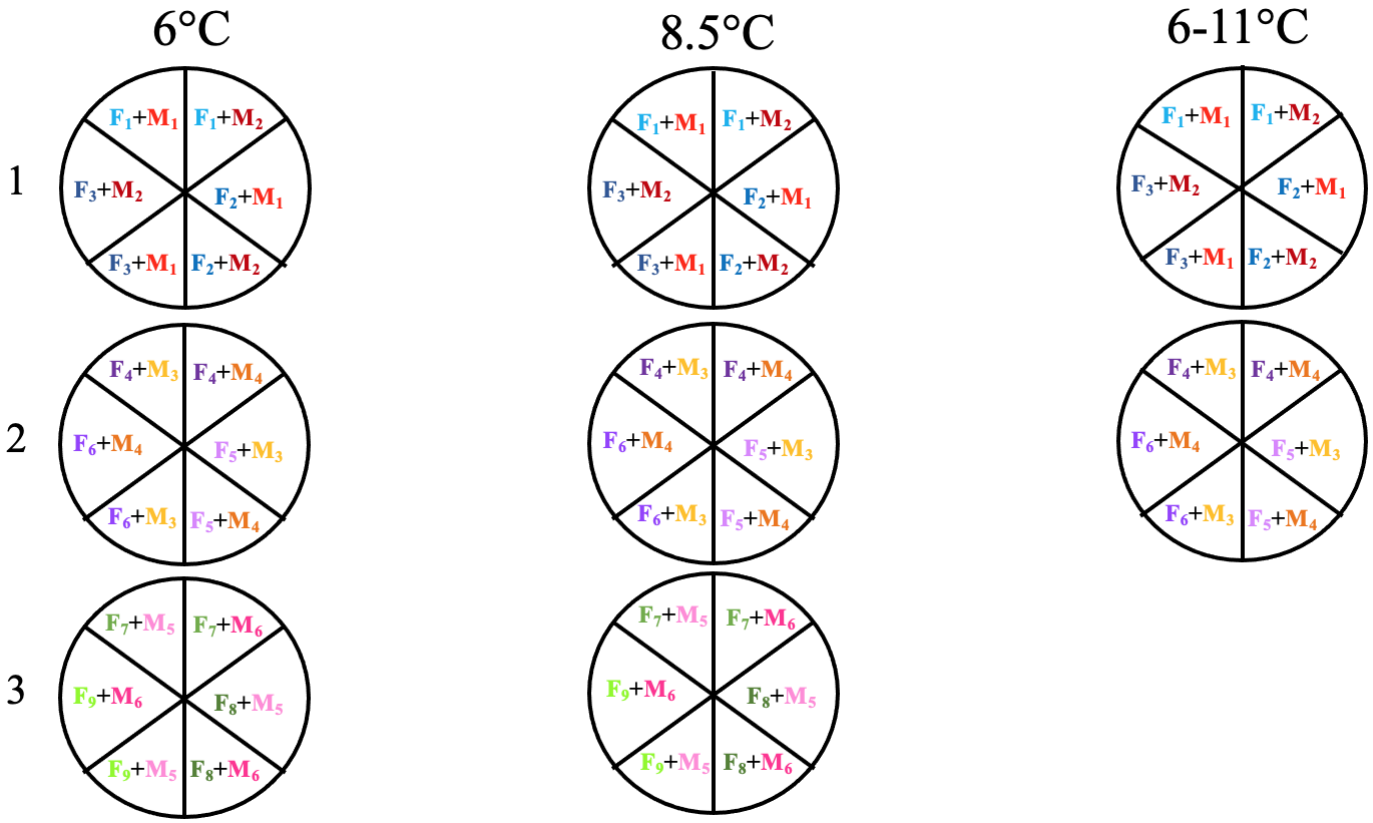

Figure 3.7: Schematic diagram showing the females and males that contributed gametes to each incubator. Each "F_{number}" represents the gametes from a different female lumpfish, and each "M_{number}" represents the gametes from a different male lumpfish. Each incubator contained the gametes from three female and two male lumpfish. Incubator 1 in the 6, 8.5, and 6-11°C groups contained gametes from the same individuals: F_1, F_2, F_3, M_1 and M_2. Incubator 2 from the 6, 8.5, and 6-11°C groups contain gametes from the same individuals as each other, but these were different than the individuals used for incubator 1: F_4, F_5, F_6, M_3 and M_4. Lastly, incubator 3 from the 6 and 8.5°C groups contain gametes from the same individuals as each other, but these were again different than used for incubators 1 and 2: F_7, F_8, F_9, M_5 and M_6. In total, nine female lumpfish and six male lumpfish were used to make the crosses used in this experiment.

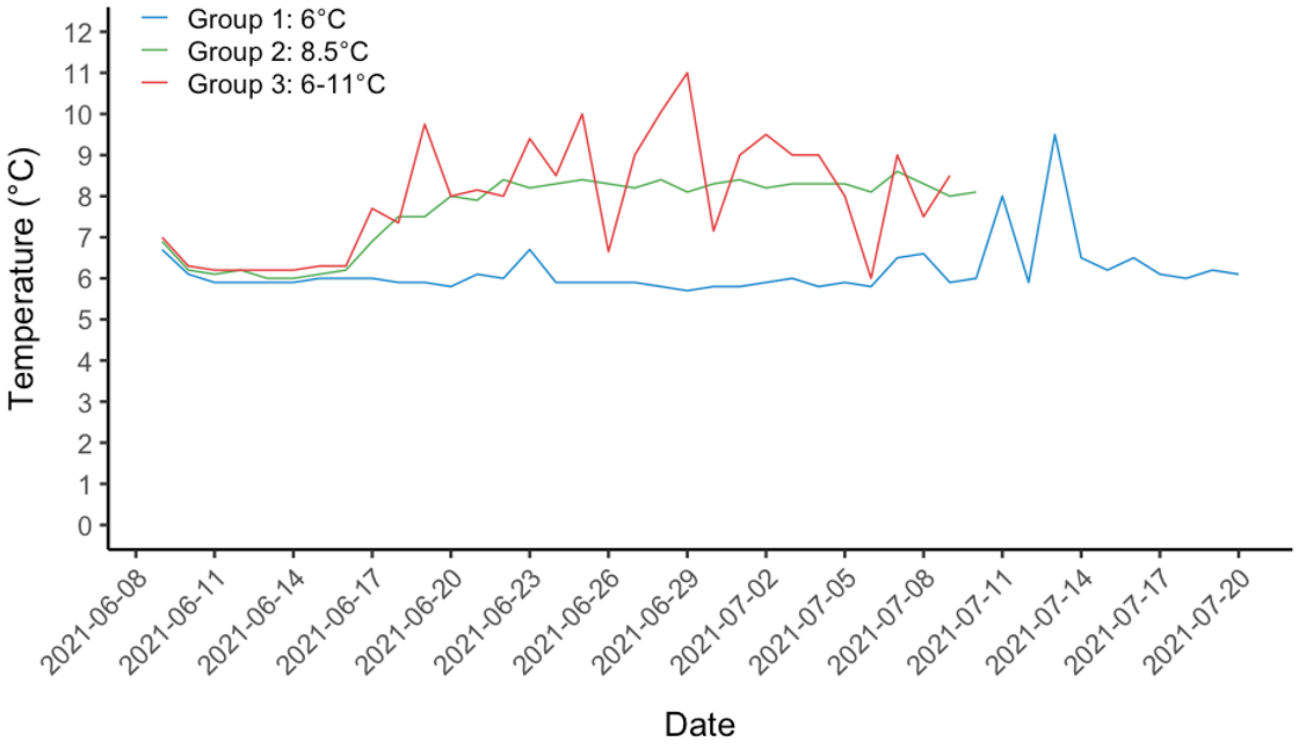

Figure 3.8: Profile of water temperatures experienced by each group of incubators from fertilization until transfer to the rearing tanks. Eggs were transferred to rearing tanks at 250 degree days (dd). As each group of incubators experienced different daily temperatures, the 8.5 and 6-11°C groups reached 250 dd sooner than the 6°C group.

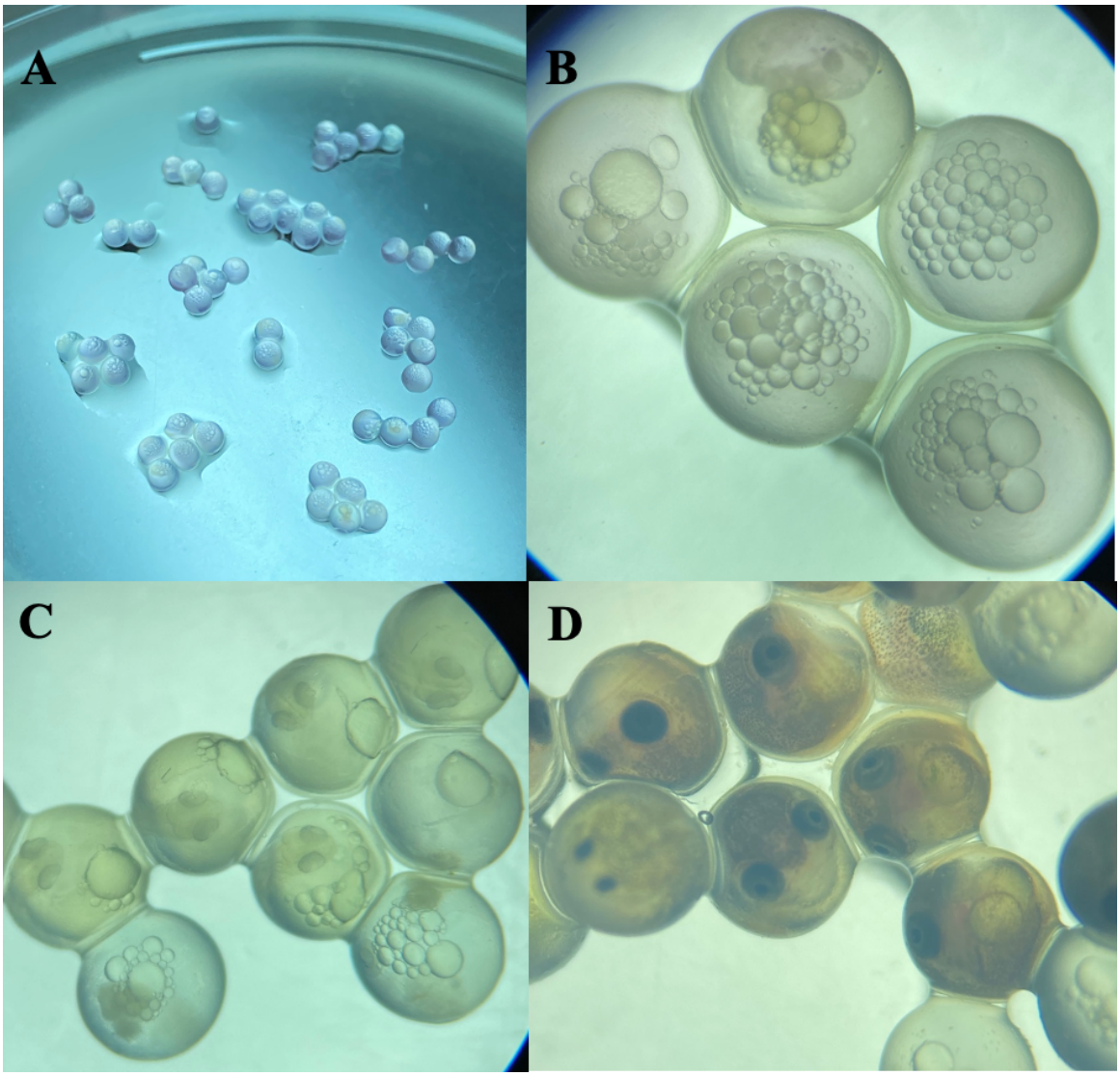

Figure 3.9: Images of lumpfish eggs at various stages of development. To determine egg viability, eggs were placed in a Petri dish under a dissecting scope (A). Eggs were assessed at 50 dd (B) and 100 dd (C) and examined just before their transfer to the rearing tanks at 250 dd (D).

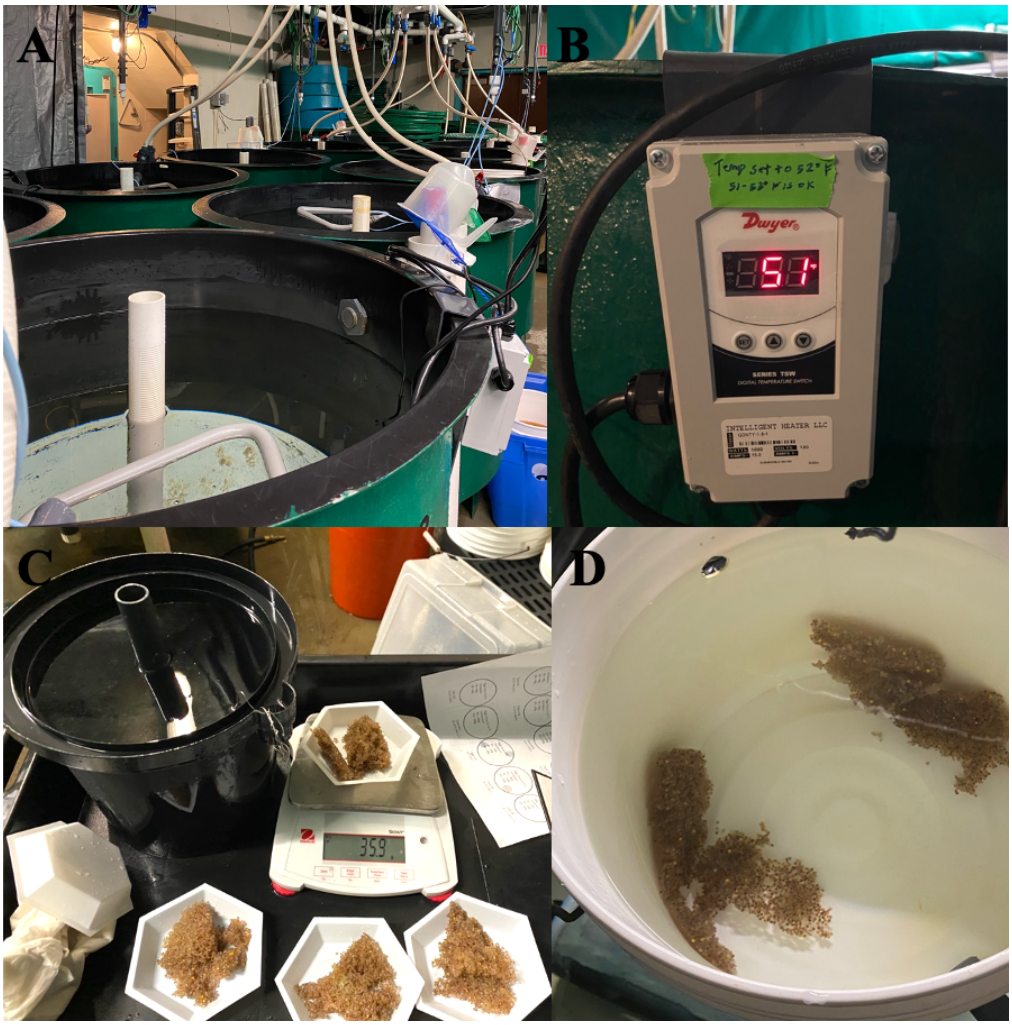

Figure 3.10: The rearing tanks (A), heaters used for the stochastic groups (B), and separation of the eggs (C) just before they were put into a bucket in the rearing tanks (D) suspended near the center drain. See Figure 3.11 for further details.

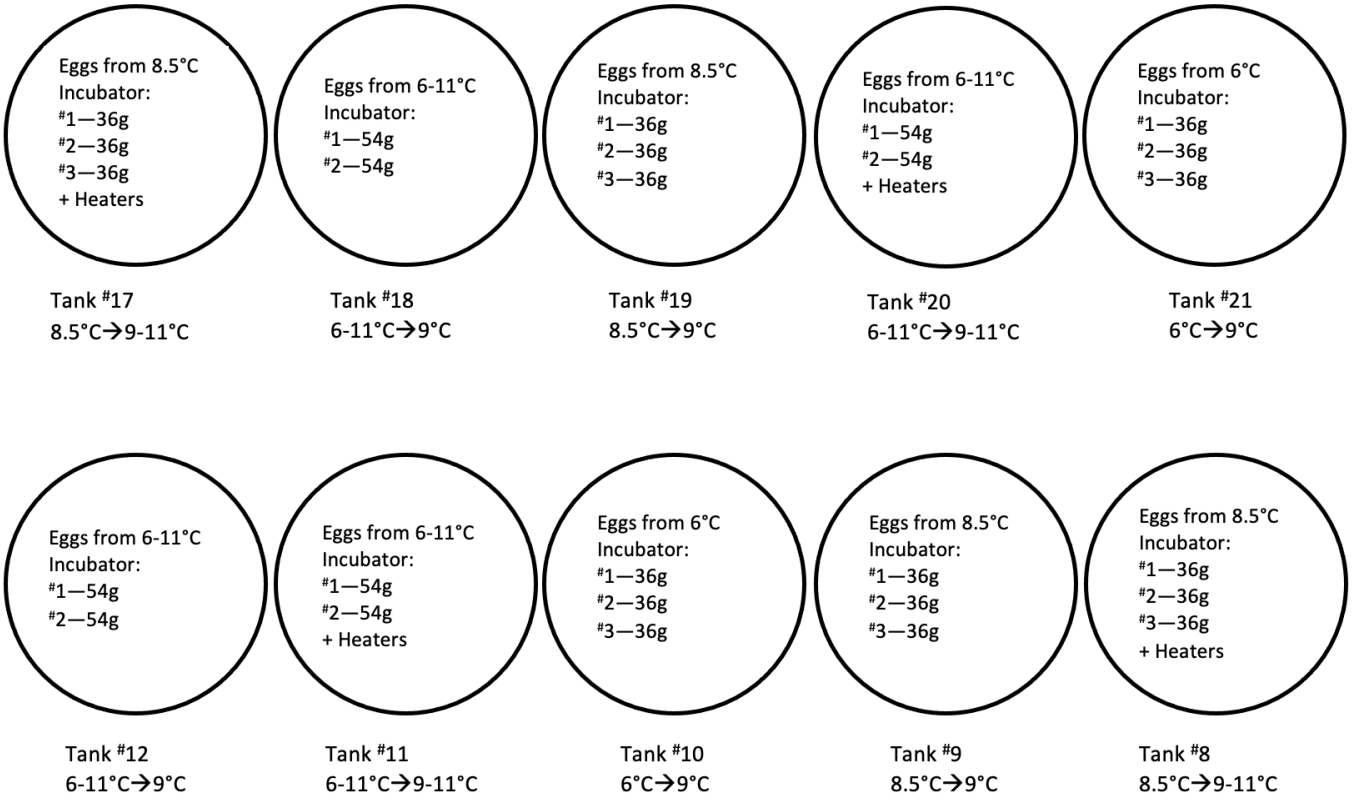

Figure 3.11: Distribution of egg masses among the tanks after transfer from the incubators. Each rearing tank was provided with 108 g of eggs.

Table 3.1: The quantities and sizes of dry feed fed to the lumpfish during rearing, and when seawater flow rates were changed.

Age (days post-hatch)	Dry Feed (% body mass day^{-1})	Feed size (mm)	Flow rate (L min^{-1})
10	10	0.2	5
15	10	0.2/0.3	5
20	10	0.2/0.3	5
25	10	0.3	6
30	8	0.3	6
35	8	0.3	7
41	8	0.3	7
46	8	0.3	7
50	8	03	10
55	8	0.3/0.5	10
60	8	0.3/0.5	10
65	8	0.3/0.5	10
~70	4	0.5/0.8	10
~85	1.5	1.2/1.5	10
~100	1.5	1.2/1.5	10
~110	1.5	1.8/2.0	10
~125	1	3.0	10
~145	0.75	3.0	10
~165	0.5	3.0/4.0	10
~185	0.5	3.0/4.0	10

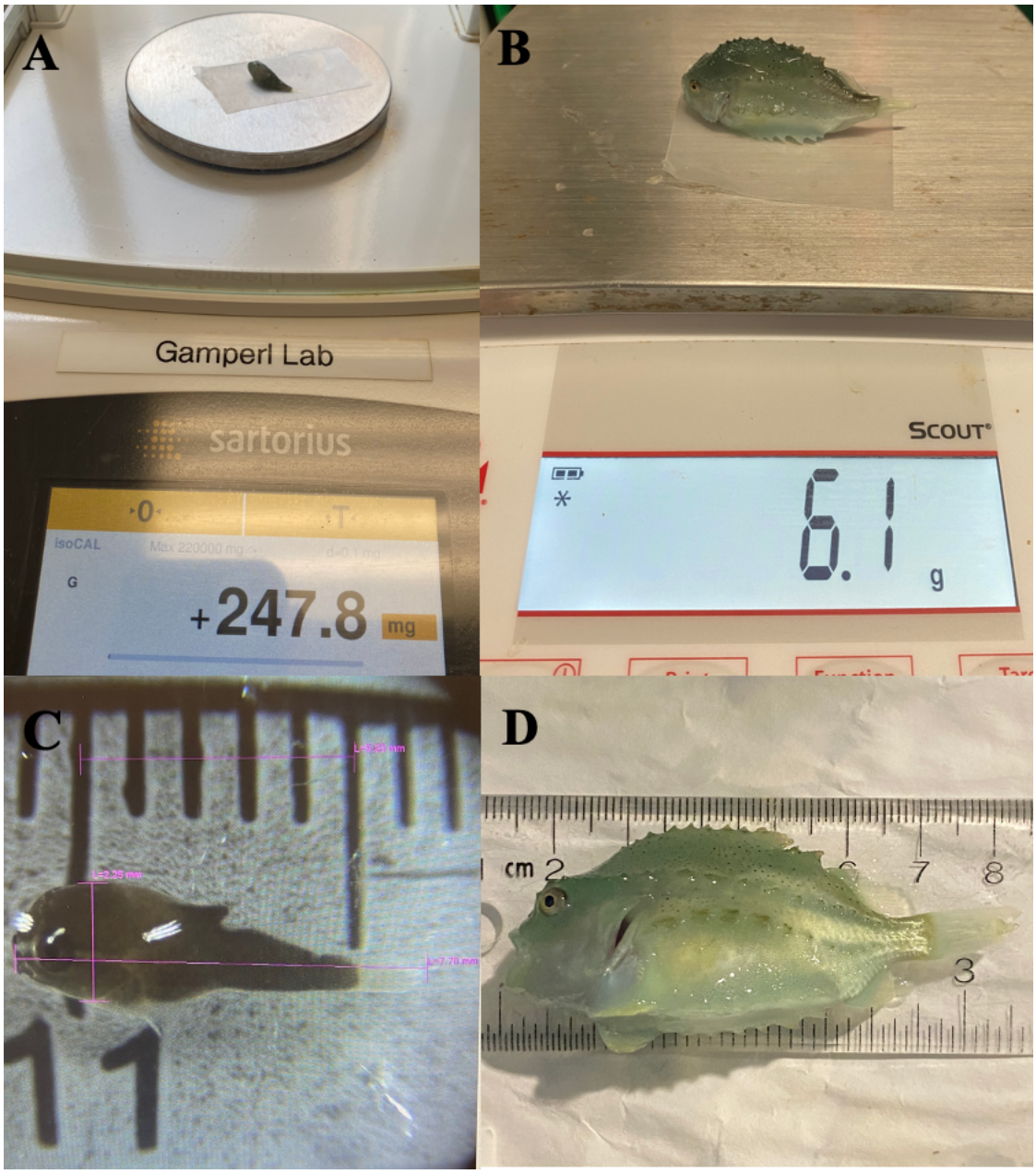

Figure 3.12: Weighing (A, B) and measuring the length (C, D) of larval and juvenile lumpfish, respectively.

Table 3.2: Number of sutures, location, and the colour of sutures used to mark fish from each incubation / rearing group used for the CT_{Max} experiment.

Incubation / Rearing Group	Tank	Number of Sutures	Side of Fish	Colour of Suture
6 / 9°C	1	1	Left	Blue
6 / 9°C	2	2	Right	Blue
8.5 / 9°C	1	1	Left	Yellow
8.5 / 9°C	2	2	Right	Yellow
8.5 / 9-11 °C	1	1	Left	Red
8.5 / 9-11 °C	2	2	Right	Red
6-11 / 9°C	1	1	Left	Black
6-11 / 9°C	2	2	Right	Black
6-11 / 9-11°C	1	1	Left	Orange
6-11 / 9-11°C	2	2	Right	Orange

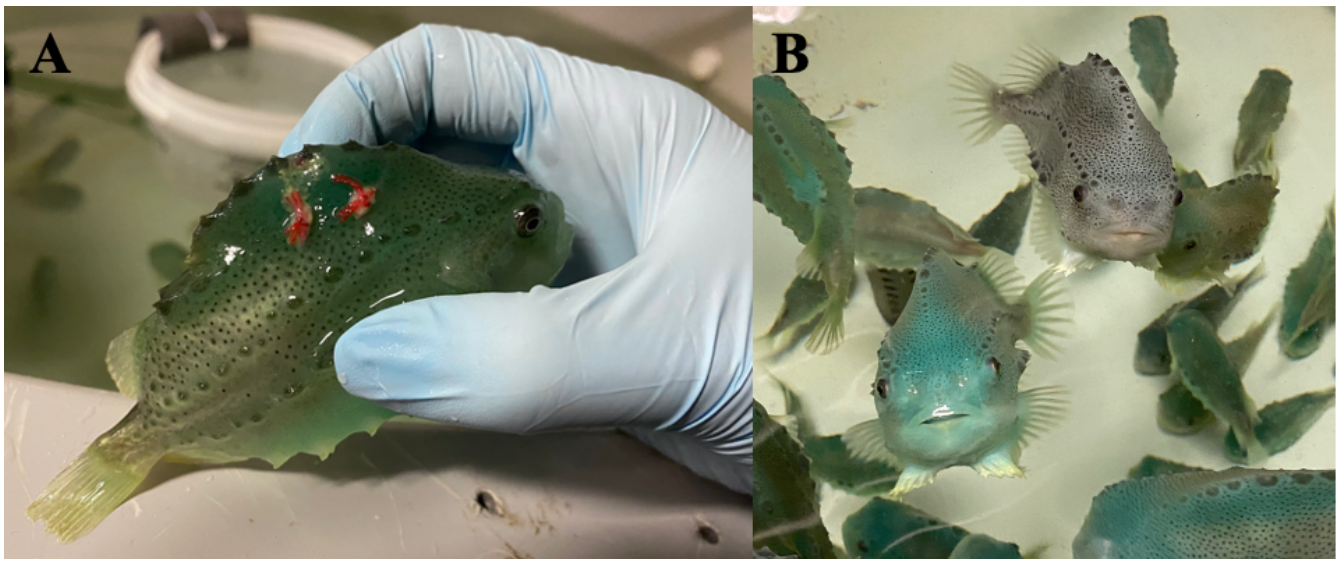

Figure 3.13: Lumpfish used for the CT_{Max} tests with two red sutures (A), and lumpfish used for the IT_{Max} test with no sutures (B).

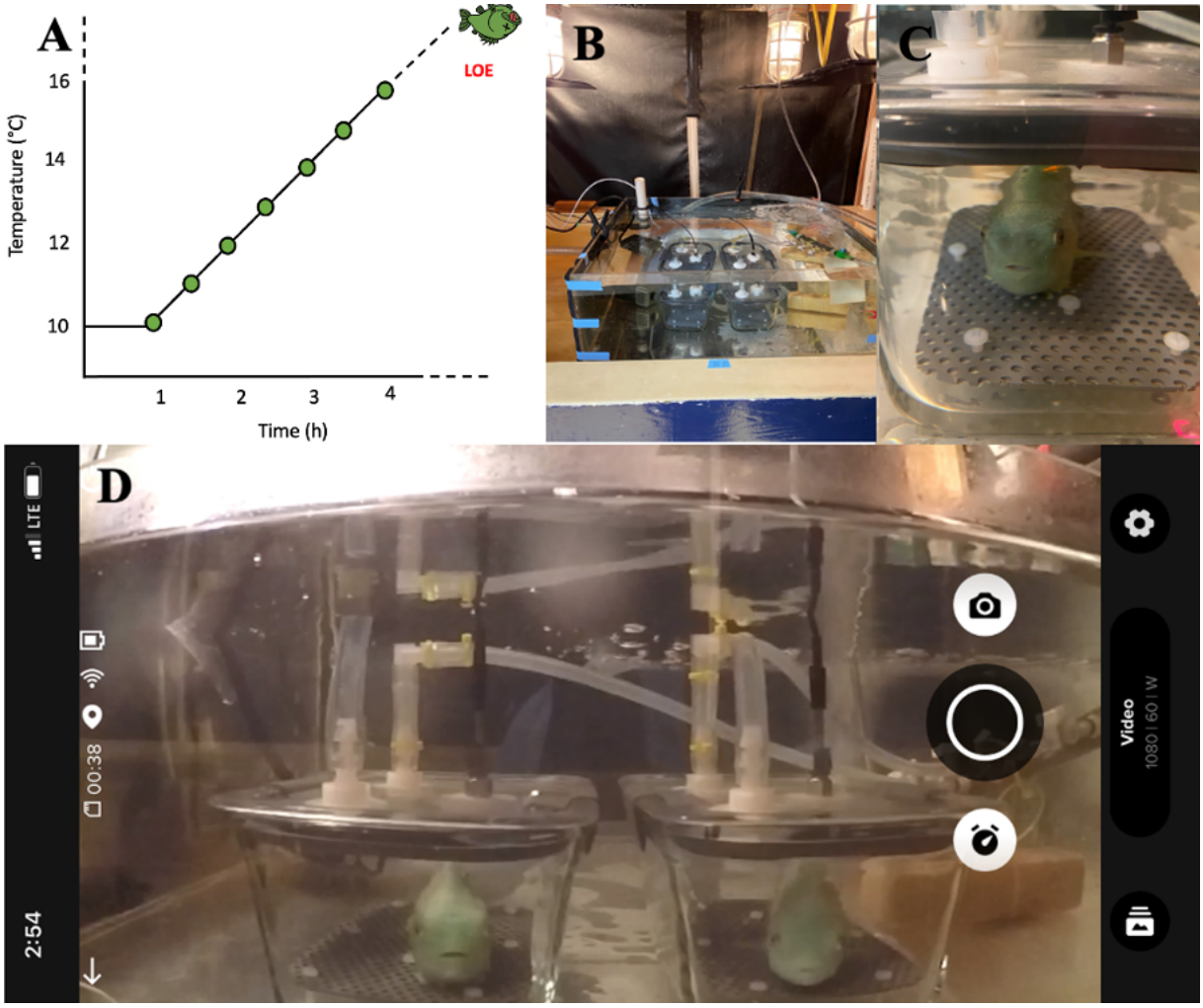

Figure 3.14: A schematic diagram depicting the rate of increase in temperature during the CT_{Max} tests (A). The dotted lines indicate an indeterminate number of steps. The respirometry setup in the tarped enclosure (B), a lumpfish in the respirometry chamber, showing the false bottom of the chamber (C), and the view of the lumpfish from the GoPro® camera during the CT_{Max} test.

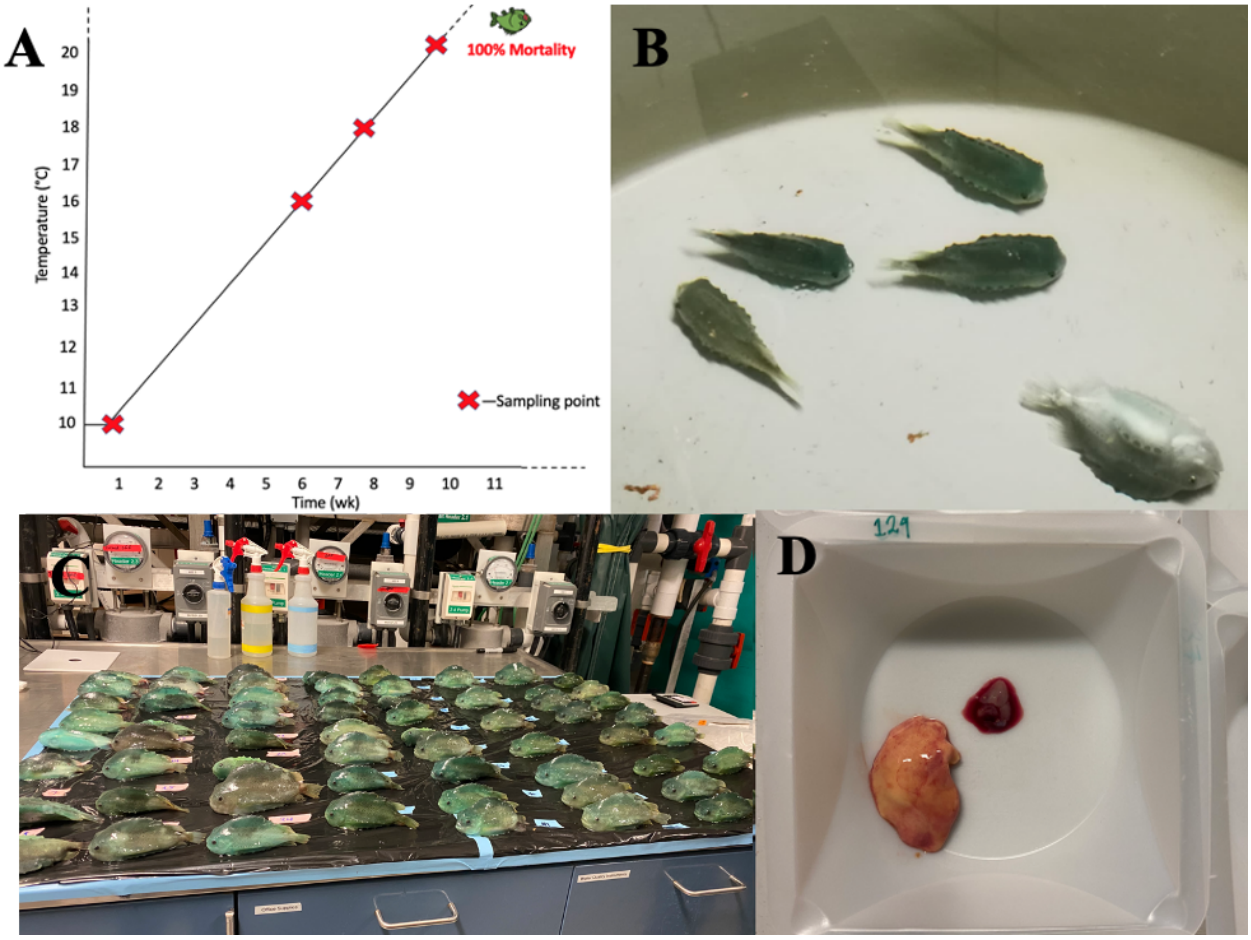

Figure 3.15: A schematic diagram depicting the rate of increase in temperature, and time points of sampling for the IT_{Max} test (10, 16, 18 and 20°C) (A). The dotted lines indicate an indeterminate number of steps. A photograph of lumpfish undergoing the IT_{Max} test (B). Fish that were sampled for blood, liver weight and ventricle weight were euthanized after they were bled, then numbered, and organized before further sampling (C). The liver and ventricle (D) were dissected from the fish, placed in a numbered weigh boat, and their weight recorded.

3.3: Results

3.3.1: Rearing

Egg viability at 50 dd post-fertilization (ddpf) was ~100% in all treatment groups. This value was lower at 100 ddpf (ranging from 84.8% to 92.9%), but again, was not influenced by incubation temperature. In contrast, hatching success was very different between the incubation groups. Eggs incubated at 6°C had the highest proportion of individuals hatch (61%), whereas eggs incubated at 8.5°C and 6-11°C only had hatching success rates of 46.8 and 26.5%, respectively (Table 3.3).

The eggs that were incubated at 8.5°C and 6-11°C began hatching at 40 dpf (340 ddpf), 11 days earlier than larvae incubated at 6°C, which began hatching at 51 dpf / 307 ddpf. Eggs incubated at 8.5°C and 6-11°C also required between 4-7 days to completely hatch out, whereas eggs incubated at 6°C took 1-2 days to completely hatch. At the beginning of the larval rearing period, the water temperatures were controlled effectively in the JBARB. However, a temperature anomaly occurred between 41 days post-hatch (dph) and 88 dph for larvae incubated at 8.5°C and 6-11°C, and between 34 dph and 81 dph for larvae incubated at 6°C, where ambient water temperatures fluctuated between 10 and 15°C (Fig. 3.16A). These temperatures were much warmer than normally delivered by our deep sea-water pumps (Han et al., 2015; Colbourne and Anderson, 2003) at this time of year (Aug. 29, 2021 – Oct. 13, 2021) and the JBARB does not have the capacity to cool/chill seawater. This issue, which occurred at the end of weaning, limits our ability to interpret the data with respect to the effects of rearing temperature on the latter measurements that were performed (i.e., CT_{Max}, IT_{Max} and resting cortisol levels). However, this anomaly did present a substantial thermal challenge for the larvae/early juveniles, and thus, provided an opportunity to examine how incubation/early rearing temperatures affected the thermal tolerance

of this life stage (Fig. 3.16B). From the data and the survival curves, it is clear that: 1) fish reared at 6°C had the highest survival (64%); 2) there was no clear pattern of how incubation temperature affected survival. Although survivorship in all the 8.5 and 6-11°C incubated groups started to fall not long after the temperature anomaly began, survival was lowest in Group 5 (6-11°C/9-11°C ; 34% at 900 ddph), and 2nd lowest in Group 3 (8.5°C/9-11°C, at 52% 900 ddph). differences in the survival of the groups as the warm temperatures persisted. Survival was lowest in Group 5 (6-11°C/9-11°C; 34% at 900 ddph), whereas Group 1 (6/9°C) had the highest survival rate of all groups (64% at 900 ddph).

The groups did not differ significantly in body mass during the period of larval / juvenile rearing (Fig. 3.17A, B); however, their growth was considerable. They gained an average of 0.043 g from 100 – 400 ddph and an additional 1.38 g from 400 – 900 ddph; with the specific growth rate (SGR) of the groups ranging from 10.3 to 12.0 % day^{-1} and 5.5 to 7.3% day^{-1} during these two periods, respectively (Table 3.4). The largest number/frequency of deformities was observed at 100 ddph, and their presence decreased over time (Table 3.5). The most frequently observed deformity was scoliosis. This deformity was very prevalent at 100 ddph (ranging in occurrence from 16 to 40% of the fish examined), less prevalent at 400 ddph (present in between 6 and 24% of the fish) and was almost non-existent at 900 ddph. As this deformity is related to curvature of the spine, a trait that would continue to be observed as the fish grew, it is assumed that fish with this condition did not reach 900 ddph. Four of the five groups also had fish that were missing an eye at 100 dph (with this condition being highest in fish sampled from Groups 1 and 4; 16 and 8%, respectively). Although most groups had very few fish with cataracts at 100 dph, this deformity was present in 38% of the larvae sampled from Group 2. There were no fish found with other eye deformities (e.g., shrunken pupil, dysfunctional eye) at 100 dph, and this deformity was limited to

very low levels (i.e., 2%) at the other two sampling points. Sucker deformities were also very rare, with only 4% of fish in Group 2 at 100 ddph, and 2% of fish in Group 5 at 900 ddph, having this deformity.

3.3.2: CT_{Max} and IT_{Max} Values

There were no significant differences in CT_{Max}, SMR, RMR, MMR, AS or ASR between the groups (Figures 3.18 – 3.19). Overnight, SMR averaged 23.78 $\pm$ 1.40 mg O_2 kg^{-1} h^{-1}, and prior to the CT_{Max} test RMR was 51.86 $\pm$ 3.08 mg O_2 kg^{-1} h^{-1}. The lumpfish's metabolic rate increased in a linear fashion as temperature was increased from 10 to 20°C, reaching approx. 160 mg O_2 kg^{-1} h^{-1} at the latter temperature. However, it decreased thereafter, and was only approx. 100 mg O_2 kg^{-1} h^{-1} prior to the fish reaching their CT_{Max} (22.85 $\pm$ 0.12°C; Table 3.6, n = 45). Mean values for MMR, AS and ASR were 189.87 $\pm$ 6.63, 166.08 $\pm$ 6.52 and 138.01 $\pm$ 6.64 mg O_2 kg^{-1} h^{-1}, respectively (Table 3.6; n = 45), and the lumpfish's factorial metabolic scope (MMR/SMR) was 7.98.

There were no significant differences in survival between the groups as temperature was increased or in IT_{Max} (Fig. 3.20), and all fish succumbed over a very narrow temperature range (20.1 to 21.6°C). The mean IT_{Max} of these lumpfish, acclimated to 10°C, was 20.62 $\pm$ 0.03°C (Table 3.7). Although there were no significant differences between the groups in CT_{Max} or IT_{Max}, IT_{Max} was on average 2.22°C lower than CT_{Max} (Table 3.7).

There were no differences between the body mass of the groups during the IT_{Max} experiment, with all groups gaining between 72.0 and 107. 5 g during the 77-day long experiment (Table 3.8). However, the incremental increase in temperature appeared to have an effect on the lumpfish's growth rate. In the 10°C (control) group SGR only decreased by ~ 0.1% body mass day^{-1} over the study, whereas the decrease in this parameter in all IT_{Max} groups ranged from 0.57

to 1.17 % body mass day^{-1} and was concomitant with a temperature-dependent reduction in the feed consumed by the fish (Figure. S2B). Feed consumption was ~ 0.8 - 0.9% body mass day^{-1} at 10°C, ~ 0.5 body mass day^{-1} at 16°C, and had decreased to 0.1% body mass day^{-1} by 20°C; i.e. they had essentially stopped feeding at this temperature (Figure. S2B). Interestingly, despite the decrease in SGR with temperature, the body mass index (BMI) of the fish increased over the study, from ~ 0.35 at 10°C to 0.61 at 20°C, and this change was similar to that observed in the control group.

Hepatosomatic index (HSI) and relative ventricular mass (RVM) were relatively constant in the in the control group, and averaged ~ 1.37 and 0.077, respectively (Table 3.9). In contrast, both HSI and RVM decreased in all experimental groups (1-5) over the course of the IT_{Max} test. The average decrease in HSI for the experimental groups was 0.29 between 18 and 20°C, whereas RVM decreased from ~ 0.086 to 0.050 as the fish were warmed from 16 to 20°C (Table 3.9)

3.3.3: Cortisol

Plasma concentrations of cortisol were very low in these lumpfish (< 1 ng mL^{-1}, mean 0.70 $\pm$ 0.18 ng mL^{-1}) at 10°C. There were no statistically significant differences in plasma cortisol between the groups. However, plasma [cortisol] was higher in 18 (2.3 $\pm$ 0.29 ng mL^{-1}) and 20°C fish (2.08 $\pm$ 0.25 ng mL^{-1}) as compared to fish constantly held at 10°C (Figure 3.21; Table 3.10).

Table 3.3 Egg viability and hatching success in lumpfish incubated at different temperatures. At 100 degree-days (dd) post-fertilization, the developing lumpfish had reached the final stages of development (stage J; Imsland et al., 2019). Values are the mean of THREE (6 and 8.5°C) or two (6-11°C) incubators.

		Incubation Temperature		
		6°C	**8.5°C**	**6-11°C**
Egg Viability (%)	**50 ddpf**	100.0	100.0	99.3
	100 ddpf	84.8	92.9	92.7
Hatching Success (%)		61.0	46.8	26.5

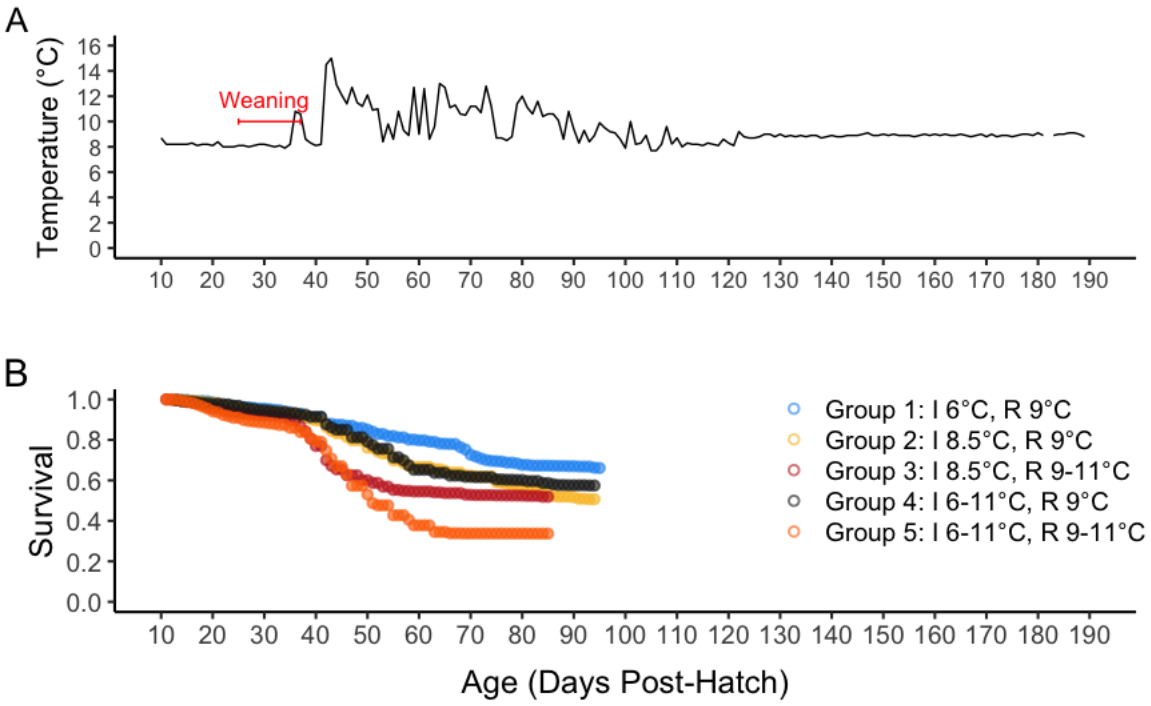

Figure 3.16: Temperatures experienced by all groups of lumpfish during the larval and juvenile stages (i.e., until 900 ddph) (A) and survival curves (B) with colours indicating the different incubation / rearing groups. The temperature profile (A) was collected by a Star Oddi® data logger placed in one of the tanks. The period when the fish were weaned off live feed (*Artemia*) is indicated in (A). The irregular nature of the temperature profile between 41 and 88 days post-hatch indicates an anomaly, where ambient ocean temperatures fluctuated between 10-15°C. These temperatures were higher than the intended temperatures (9°C and 9-11°C).

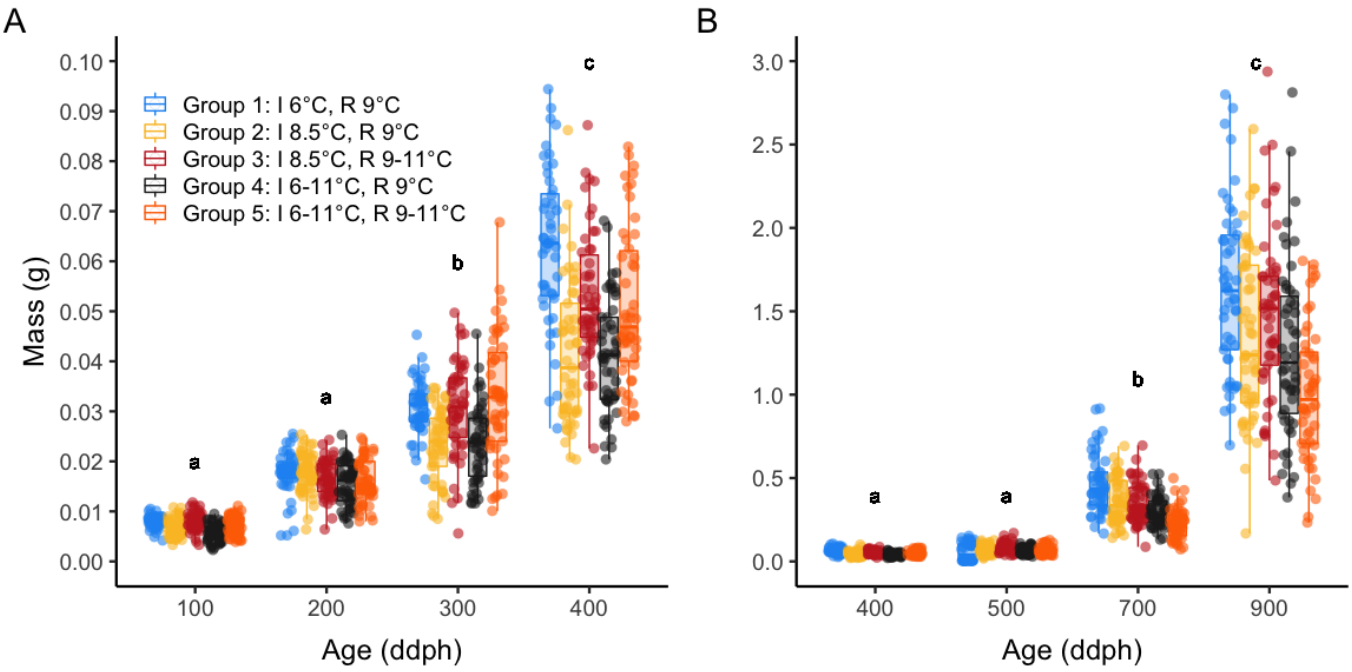

Figure 3.17: Mass of larval / juvenile lumpfish before the temperature anomaly (100–400 degree days post-hatch, ddph; A), and during the temperature anomaly (400–900 ddph; B). Colours indicate the different incubation / rearing groups. After 900 ddph, water temperatures returned to 9°C, and the fish were graded. There were no significant differences in fish mass between the groups at any time point during rearing. Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively. The verticle lines indicate the 10th and 90th percentiles, respectively. Each circle represents the mass of an individual fish sampled at its respective age, in each group (n = 25). Dissimilar letters indicate significant differences ($P < 0.05$) in mass as the lumpfish grew.

Table 3.4: Weight gained, specific growth rate (SGR) and thermal growth coefficients (TGC) between incubation / rearing groups, and between time periods. One hundred to 400-degree days post-hatch is before the temperature anomaly, and 400-900 degree days post-hatch is during the temperature anomaly. No statistical analysis was performed on this data as n= 2 (tanks) per group.

Growth Parameter	Degree Days Post-Hatch	Incubation / Rearing Group				
		1 (6°C → 9°C)	**2 (8.5°C → 9°C)**	**3 (8.5°C → 9-11°C)**	**4 (6-11°C → 9°C)**	**5 (6-11°C → 9-11°C)**
Weight Gained (g)	100-400	0.0570	0.0358	0.0450	0.0465	0.0331
	400-900	1.60	1.31	1.79	1.22	0.968
SGR (% Day^{-1})	100-400	10.7	10.3	11.7	10.7	12.0
	400-900	5.48	6.86	6.69	6.42	7.31
TGC	100-400	0.0488	0.0373	0.0440	0.0456	0.0362
	400-900	0.156	0.160	0.170	0.150	0.133

Table 3.5: Frequency and types of deformities observed in a sample of 50 lumpfish per group (**1** - incubated at 6°C, reared at 9°C; **2** - incubated at 8.5°C, reared at 9°C; **3** – incubated at 8.5°C, reared at 9-11°C; **4** – incubated at 6-11°C, reared at 9°C; and **5** – incubated at 6-11°C, reared at 9-11°C). n = 2 (tanks) per group. ddph = degree days post-hatch; dph = days post-hatch.

	Age (ddph)	**100**					**400**					**900**				
	Group Age (dph)	**1** *11*	**2** *13*	**3** *13*	**4** *13*	**5** *13*	**1** *45*	**2** *48*	**3** *41*	**4** *48*	**5** *41*	**1** *95*	**2** *94*	**3** *89*	**4** *94*	**5** *89*
Percentage of deformities	Scoliosis	30	30	16	40	20	6	22	10	24	16	0	0	0	0	2
	Missing Eye	16	2	2	8	0	0	0	2	0	4	0	0	0	0	0
	Eye Deformity	0	0	0	0	0	0	2	0	0	0	0	2	0	0	0
	Sucker Deformity	0	4	0	0	0	0	0	0	0	0	0	0	0	0	2
	Cataract	2	38	0	8	0	0	0	0	0	0	0	0	0	0	0

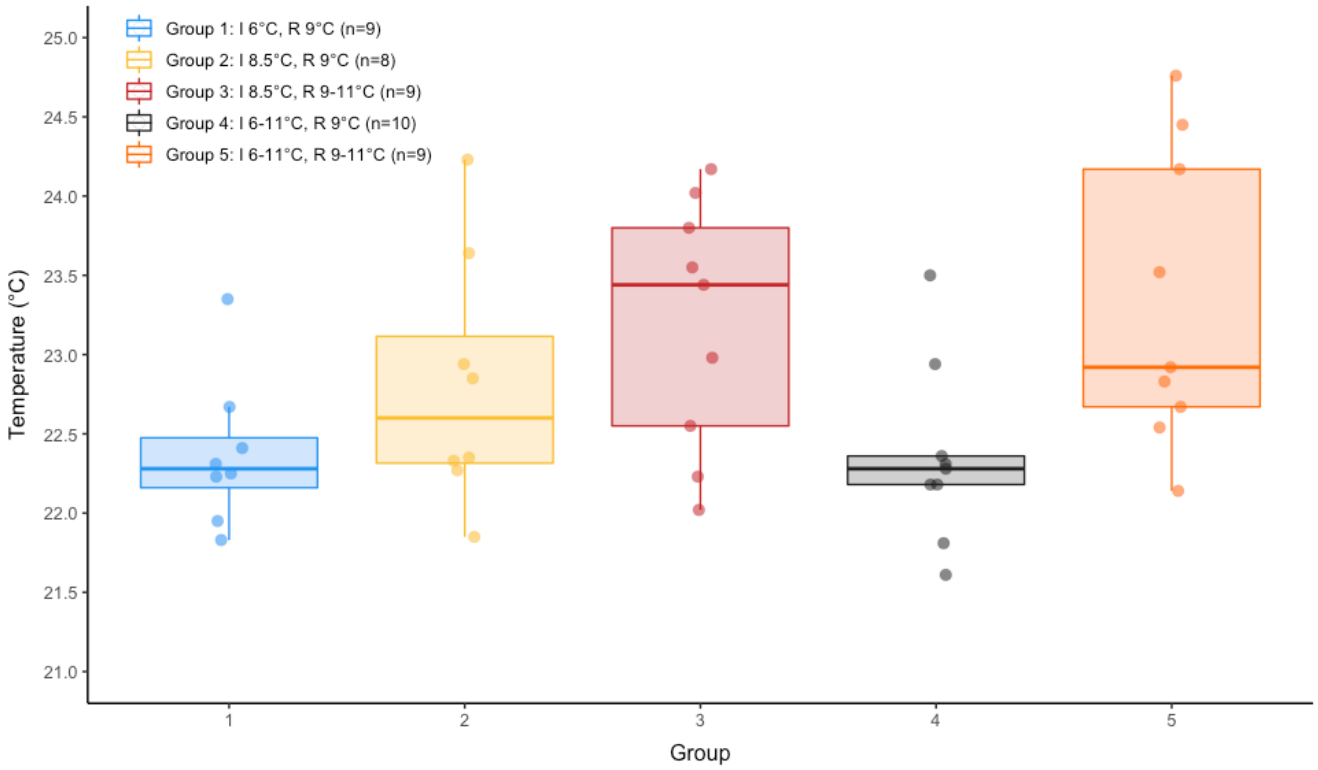

Figure 3.18: CT_{Max} for each incubation / rearing group. There were no statistical differences between the groups ($p = 0.103$). Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively. The horizontal line inside each box represents the median value. The verticle lines indicate the 10th and 90th percentiles, respectively. Each circle represents the mass of an individual fish sampled at its respective age, in each group (n = 8-10).

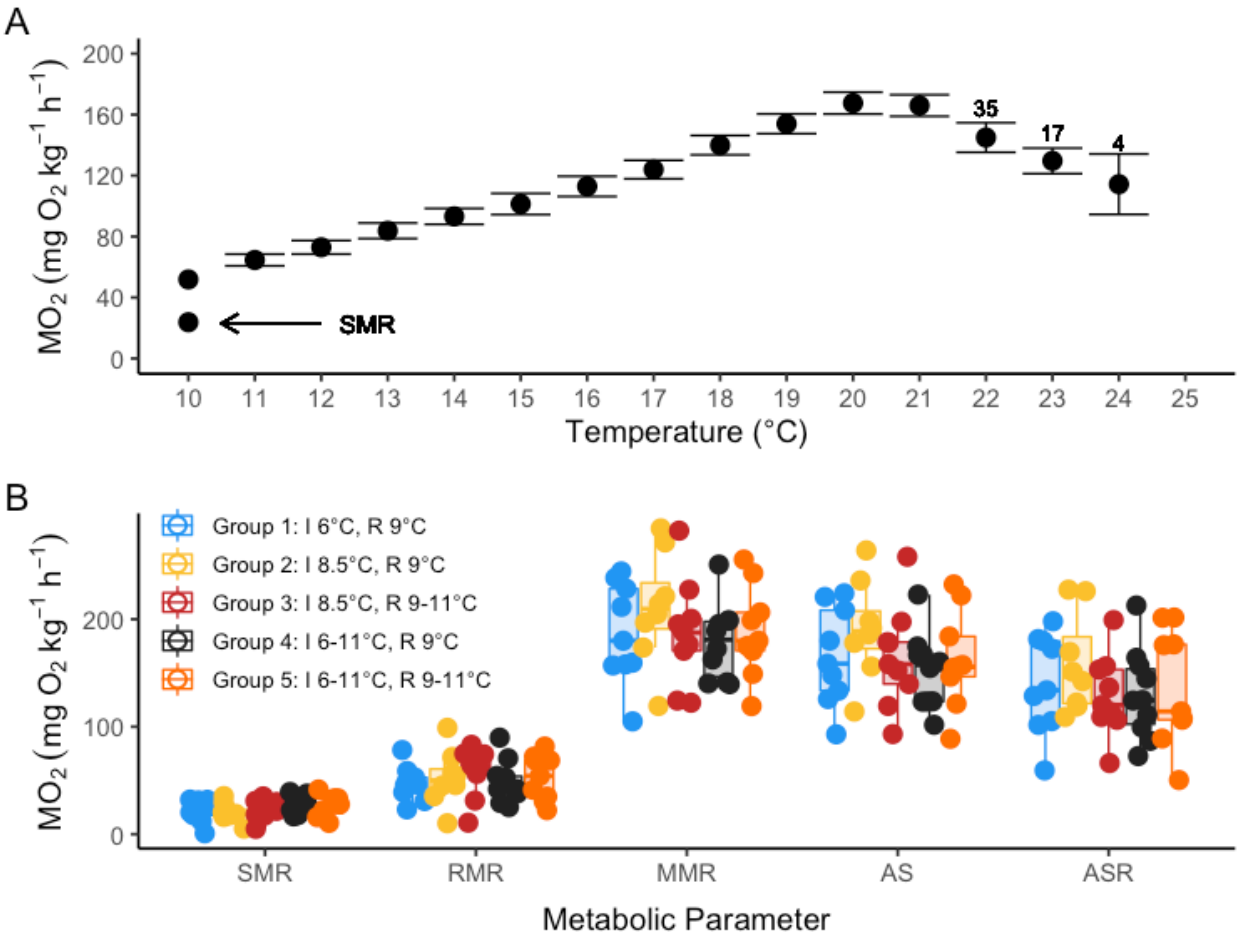

Figure 3.19: A: The mean oxygen consumption (MO_2) of all fish tested during the CT_{Max} trials (n = 45), measured at each 1°C increase in temperature. The numbers above the points beginning at 22°C indicate the number of fish that represent that point (i.e., n < 45). B: Comparisons of metabolic parameters between groups, during the CT_{Max} test. There were no significant ($p < 0.05$) differences in metabolic parameters between groups (SMR - standard metabolic rate; RMR - routine metabolic rate; MMR - maximum metabolic rate; AS - aerobic scope; ASR - realistic aerobic scope). Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively. The verticle lines indicate the 10th and 90th percentiles, respectively. Each circle represents the mass of an individual fish sampled at its respective age, in each group (n = 25).

Table 3.6: Mean CT_{Max} and metabolic parameters of lumpfish (n = 45) acclimated to 10°C. As there were no significant ($p < 0.05$) differences between incubation/rearing groups, the data from all groups were pooled.

Metabolic Parameter	**Mean Value (± SEM)**
CT_{Max} (°C)	22.85 ± 0.12
SMR (mg O_2 kg^{-1} h^{-1})	23.78 ± 1.40
RMR (mg O_2 kg^{-1} h^{-1})	51.86 ± 3.08
MMR (mg O_2 kg^{-1} h^{-1})	189.87 ± 6.63
AS (mg O_2 kg^{-1} h^{-1})	166.08 ± 6.52
ASR (mg O_2 kg^{-1} h^{-1})	138.01 ± 6.64

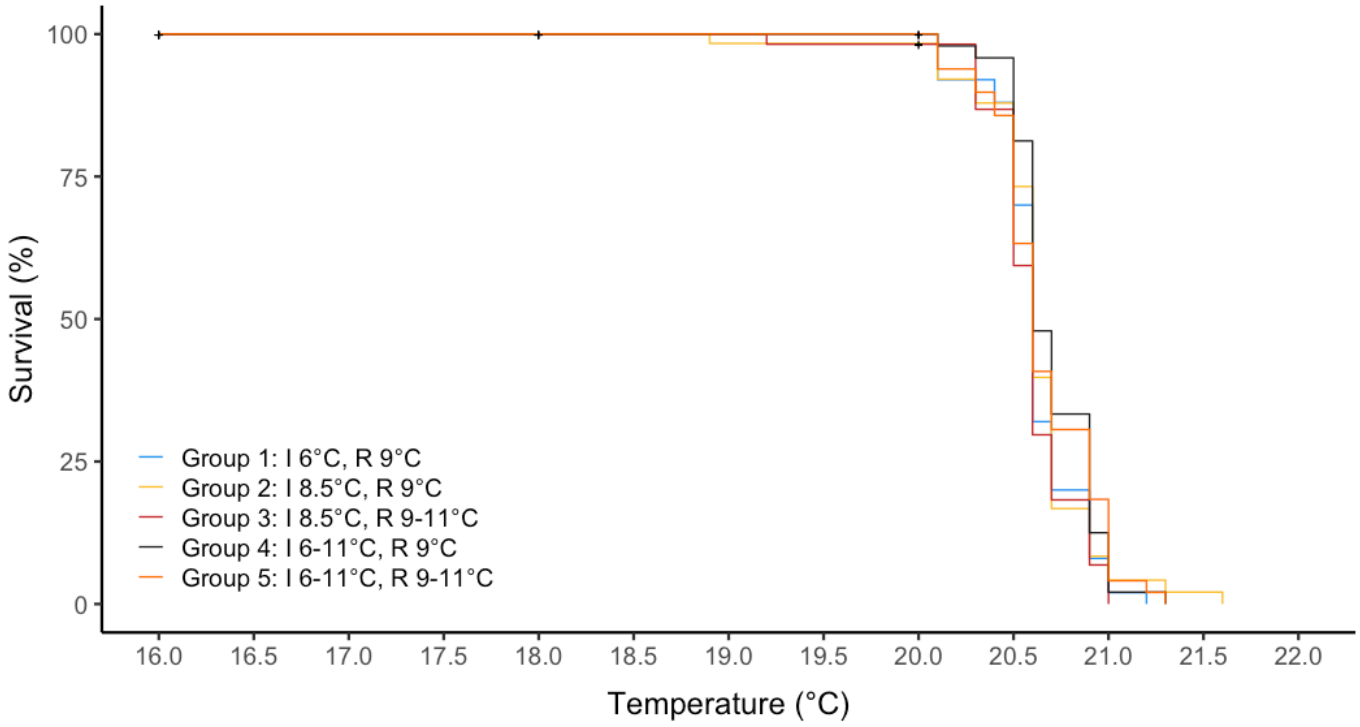

Figure 3.20: Kaplan-Meier survival curves for lumpfish (n = 400) undergoing an IT_{Max} (+ 1°C wk^{-1}) test. There was no statistical difference in IT_{Max} between the groups (p = 0.77).

Table 3.7: Comparisons between the IT_{Max} and CT_{Max} of each incubation / rearing group. There were no significant differences between groups; however, there was a statistically significant ($p < 0.05$) difference between the IT_{Max} and CT_{Max} of the lumpfish (see lower case letters). Mean CT_{Max} was ~2.2°C higher than IT_{Max}.

Group	IT_{Max} (°C; n = 37-40)	CT_{Max} (°C; n = 8-10)
1 (6 → 9°C)	20.62 ± 0.03^{a}	22.40 ± 0.17^{b}
2 (8.5 → 9°C)	20.61 ± 0.06^{a}	22.81 ± 0.28^{b}
3 (8.5 → 9-11°C)	20.58 ± 0.04^{a}	23.17 ± 0.26^{b}
4 (6-11 → 9°C)	20.70 ± 0.03^{a}	22.54 ± 0.28^{b}
5 (6-11 → 9-11°C)	20.66 ± 0.04^{a}	23.33 ± 0.31^{b}
Mean	20.63 ± 0.02^{a}	22.85 ± 0.12^{b}

Table 3.8: Differences in mass, length, body mass index and specific growth rate (SGR) of lumpfish between incubation / rearing groups, and between sampling points during the IT_{Max} test. Lowercase letters indicate statistical ($p < 0.05$) differences between sampling points, and uppercase letters indicate statistical differences between groups. Those that do not share a letter in common are statistically different. Statistical analysises were not performed on SGR as there were only 2 tanks (n = 2) per group. Body mass index was calculated as: (mass/length^2). SGR was calculated from 10°C.

Incubation / Rearing Group	Temperature at Sampling Point (°C)	Mass (g)	Length (cm)	Body Mass Index	Specific Growth Rate (SGR; % day^{-1})
1 (6 - 9°C)	10	24.13 ± 0.97^{a}	8.47 ± 0.12^{Aa}	0.33 ± 0.0064^{Aa}	-
	16	89.35 ± 6.09^{b}	13.21 ± 0.29^{ABb}	0.51 ± 0.021^{ABb}	2.71 ± 0.14
	18	94.21 ± 6.76^{c}	13.06 ± 0.34^{Ab}	0.55 ± 0.023^{Abc}	2.14 ± 0.14
	20	107.54 ± 8.32^{d}	13.28 ± 0.37^{Ab}	0.59 ± 0.054^{Ac}	1.54 ± 0.27
2 (8.5 - 9°C)	10	27.42 ± 1.17^{a}	8.82 ± 0.14^{ABa}	0.35 ± 0.0059^{ABa}	-
	16	97.20 ± 6.09^{b}	13.36 ± 0.30^{ABb}	0.54 ± 0.024^{ABb}	2.57 ± 0.11
	18	112.95 ± 11.10^{c}	13.78 ± 0.47^{ABbc}	0.58 ± 0.023^{Ab}	2.31 ± 0.15
	20	134.90 ± 8.78^{d}	14.03 ± 0.28^{ABc}	0.69 ± 0.054^{Bc}	1.94 ± 0.099
3 (8.5 - 9-11°C)	10	36.06 ± 2.11^{a}	9.60 ± 0.18^{Ca}	0.38 ± 0.011^{Ba}	-
	16	108.50 ± 8.82^{b}	14.02 ± 0.36^{Ab}	0.55 ± 0.019^{Bb}	2.19 ± 0.12
	18	101.90 ± 8.09^{c}	13.54 ± 0.39^{ABb}	0.55 ± 0.019^{Ab}	1.86 ± 0.10
	20	128.60 ± 10.86^{d}	14.32 ± 0.30^{Bb}	0.60 ± 0.020^{Ab}	1.54 ± 0.068
4 (6-11 - 9°C)	10	28.78 ± 1.91^{a}	9.10 ± 0.20^{BCa}	0.34 ± 0.0082^{Aa}	-
	16	97.90 ± 7.82^{b}	13.59 ± 0.35^{ABb}	0.52 ± 0.023^{ABb}	2.17 ± 0.16
	18	122.90 ± 10.11^{c}	14.26 ± 0.29^{Bb}	0.59 ± 0.026^{Ac}	2.18 ± 0.068
	20	101.70 ± 8.00^{d}	13.35 ± 0.34^{Ab}	0.61 ± 0.022^{Abc}	1.87 ± 0.10
5 (6-11 - 9-11°C)	10	29.52 ± 1.80^{a}	8.98 ± 0.19^{Ba}	0.36 ± 0.0090^{ABa}	-
	16	80.35 ± 8.06^{b}	12.97 ± 0.35^{BCb}	0.48 ± 0.027^{Ab}	2.28 ± 0.13
	18	102.00 ± 9.81^{c}	13.34 ± 0.42^{ABb}	0.58 ± 0.022^{Ac}	1.83 ± 0.11
	20	111.30 ± 7.75^{d}	13.42 ± 0.27^{Ab}	0.61 ± 0.022^{Ac}	1.71 ± 0.097
Time-Matched Controls (maintained at 10°C)	10	31.06 ± 1.36^{a}	9.14 ± 0.14^{BCa}	0.36 ± 0.013^{ABa}	-
	10	70.21 ± 3.96^{b}	12.07 ± 0.33^{Cb}	0.44 ± 0.032^{ABb}	1.75 ± 0.088
	10	88.79 ± 5.44^{c}	13.0 ± 0.25^{Abc}	0.53 ± 0.015^{Ac}	1.71 ± 0.086
	10	108.00 ± 5.94^{d}	13.85 ± 0.27^{ABc}	0.56 ± 0.012^{Ac}	1.66 ± 0.072

Table 3.9: Differences in hepatosomatic index (HSI) and relative ventricular mass (RVM) between incubation / rearing groups and sampling points during the IT_{Max} test. Values without a lower-case letter in common are significantly ($p < 0.05$) different between sampling points, whereas upper case letters indicate differences between groups at a particular temperature.

Incubation / Rearing Group	Temperature at Sampling Point (°C)	HSI	RVM
1 (6 - 9°C)	16	1.41 ± 0.15^{ab}	0.082 ± 0.0079^{a}
	18	1.65 ± 0.16^{a}	0.071 ± 0.0062^{b}
	20	1.29 ± 0.050^{b}	0.054 ± 0.0032^{c}
2 (8.5 - 9°C)	16	1.55 ± 0.18^{a}	0.096 ± 0.0067^{a}
	18	1.48 ± 0.14^{a}	0.061 ± 0.0042^{b}
	20	1.22 ± 0.13^{b}	0.045 ± 0.0032^{c}
3 (8.5 - 9-11°C)	16	1.37 ± 0.066^{a}	0.083 ± 0.010^{a}
	18	1.42 ± 0.12^{a}	0.066 ± 0.0049^{b}
	20	1.30 ± 0.078^{a}	0.049 ± 0.0033^{c}
4 (6-11 - 9°C)	16	1.59 ± 0.13^{ab}	0.078 ± 0.0073^{a}
	18	1.76 ± 0.20^{a}	0.069 ± 0.0045^{b}
	20	1.41 ± 0.091^{b}	0.052 ± 0.0040^{c}
5 (6-11 - 9-11°C)	16	1.55 ± 0.12^{a}	0.089 ± 0.0083^{a}
	18	1.59 ± 0.092^{a}	0.068 ± 0.0057^{b}
	20	1.25 ± 0.083^{b}	0.052 ± 0.0047^{c}
Time-Matched Controls (maintained at 10°C)	10	1.22 ± 0.069^{a}	0.071 ± 0.0072^{a}
	10	1.50 ± 0.11^{b}	0.088 ± 0.0031^{b}
	10	1.41 ± 0.080^{b}	0.073 ± 0.0055^{c}

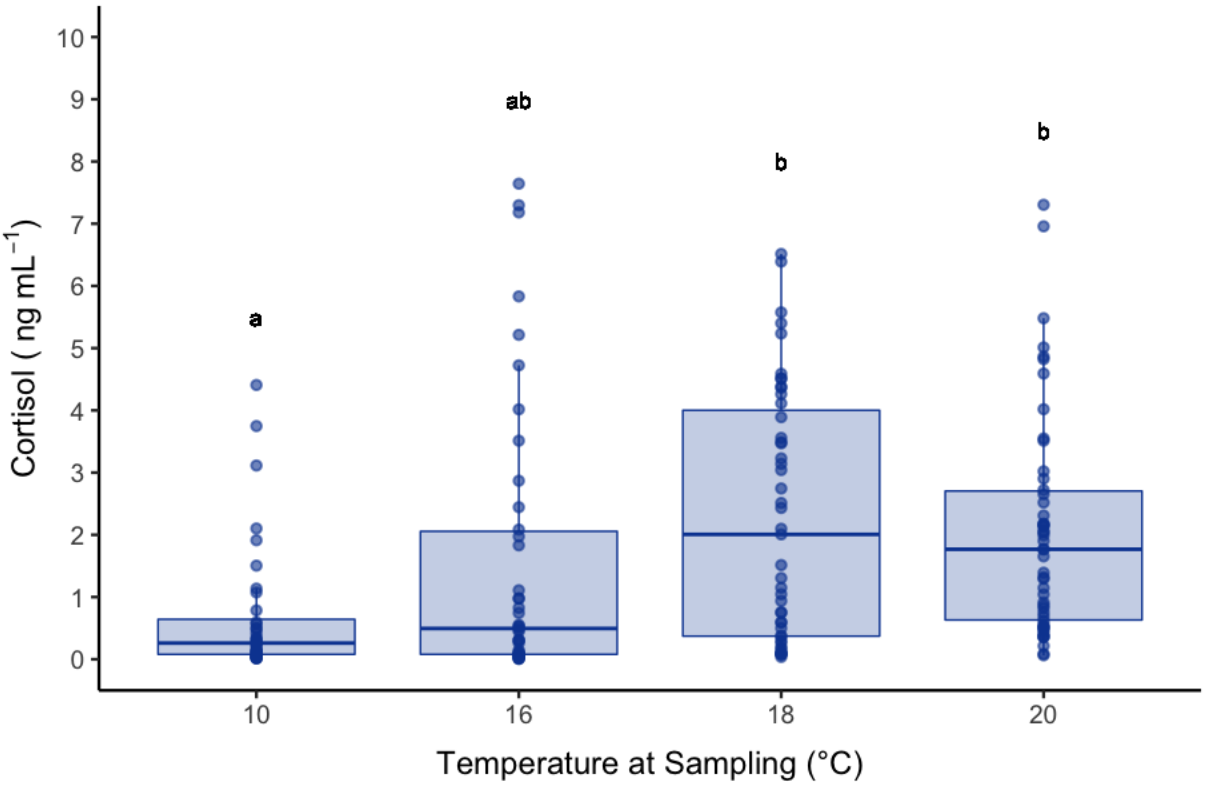

Figure 3.21: Lumpfish plasma cortisol levels as temperature was increased during the IT_{Max} test (n = 50). The 10°C sampling point represents cortisol in the time-match control fish that remained at 10°C for the duration of the IT_{Max} test (n = 36). Lowercase letters indicate statistically significant differences ($p < 0.05$) between sampling points.

Table 3.10: Plasma cortisol levels (ng mL^{-1}) in lumpfish at each sampling point. Lowercase letters indicate significant differences ($p < 0.05$) between sampling points.

10°C Time-Matched Controls, n = 36	**16°C n = 50**	**18°C n = 50**	**20°C n = 50**
0.703 ± 0.180^{a}	1.57 ± 0.34^{ab}	2.27 ± 0.29^{b}	2.07 ± 0.25^{b}

3.4: Discussion

As temperatures rise in the summer, lumpfish mortalities have been reported at Atlantic salmon cage-sites in some locations (Reynolds et al., 2022; Nytrø et al, 2014). In previous studies, rearing mosquitofish (*Gambusia holbrooki*) and zebrafish (*Danio rerio*) at temperatures higher than their optimum values was effective in increasing the thermal tolerance/performance of these species (Seebacher et al., 2014; Scott and Johnston, 2012; Schaefer and Ryan, 2006). If lumpfish are also phenotypically plastic, then adjustments to temperature during embryonic development/larval rearing (so called 'development plasticity'; Beaman et al., 2016) could potentially reduce or mitigate this mortality. This would improve the welfare of lumpfish at cage-sites in the summer and reduce the numbers of lumpfish needed by the aquaculture industry to serve as cleaner fish in Atlantic salmon sea-cages.

3.4.1: Rearing

3.4.1.1: Hatching Success and Time to Hatching

This study showed that higher temperatures during incubation (i.e., 8.5 vs. 6°C) significantly reduced lumpfish hatching success, and that the stochastic temperature regime (6-11°C) reduced this parameter even further (Table 3.3). The former finding is consistent with Geffen et al. (2006) who found that the mortality of Irish Sea cod eggs tended to increase with incubation temperature, and with Chen et al. (2013) who showed that sockeye salmon (*Oncorhynchus nerka*) eggs incubated at 10°C had a hatching success of 45-94%, 14°C incubated eggs had a hatching success of 46-85%, and 16°C incubated eggs had a hatching success of 14-56%. However, it differs from that of Imsland et al. (2019) who reported that incubating lumpfish eggs at ambient temperature (4-6°C) resulted in lower survival as compared to those incubated at

10°C. Nonetheless, the ambient eggs in Imsland's (2019) study were briefly exposed to temperatures as low as 3.8°C, and Collins (1978) reported that lumpfish eggs incubated at an average temperature of 3.8°C failed to hatch at all. Thus, the reported mortality in the ambient group in Imsland et al. (2019) may have been unusually high, therefore, using these data for comparison is questionable/problematic. Imsland et al. (2019) also found that exposing lumpfish eggs to an increasing temperature gradient (4-6°C to 10°C) improved egg survival as compared to those incubated at a constant temperature of 10°C. This contrasts with our data for the stochastic group where temperatures were adjusted daily between 6 and 11°C, and with that of Eme et al. (2018) who showed that variable temperatures (2-8°C, daily changes of 2°C) reduced the hatching success of lake whitefish (*Coregonus clupeaformis*). Collectively, these data suggest that the temporal nature of changes in temperature has a strong influence on embryonic development and hatching success.

Increased temperature appeared to impact the hatching window of lumpfish eggs. The duration of the hatching window for eggs incubated at 8.5°C and 6-11°C was 4-7 days, whereas eggs incubated at 6°C completed hatching in 1-2 days. The results for eggs held at 6°C in this study differ with the findings of Imsland et al. (2019), where most eggs incubated at 4-6°C took 3 days to hatch, but the total duration of the hatching window was 13 days. However, their gradient group (4-6°C, then increasing to 10°C) took 7 days to completely hatch, and this is similar to the 8.5°C and the 6-11°C stochastic groups from this study. It is important to consider that in Imsland et al. (2019) eggs held at a mean of 4-6°C did experience temperatures below 4°C, which are known to be detrimental to hatching success (Collins, 1978). Therefore, some eggs may have been stressed during this critical period, and this may have delayed hatching and reduced hatching success. When lake whitefish embryos experienced an increase in temperature at the end of

primordial organogenesis, their survival increased (Mueller et al., 2015). It is possible that this premise applies to the gradient group in Imsland et al. (2019) and may also explain: why the gradient group had a higher hatching success than the 4-6°C group; and the differences in the duration of the hatching window, and hatching success, between Imsland et al. (2019) and this study.

In comparison to Imsland et al. (2019), the degree days required for the lumpfish eggs to hatch in this study was greater, and those incubated at cold temperatures required fewer ddpf to hatch. In this study, both 8.5°C and 6-11°C incubated eggs hatched at 340 ddpf / 40 dpf, whereas eggs incubated at 6°C hatched in 307 ddpf / 51 dpf. In contrast, in Imsland et al. (2019), eggs incubated at 10°C and those exposed to the gradient regime (4-6°C for 10 days, then an increase over 4 days until 10°C was reached) hatched at 279 ddpf / 28 dpf and 280 ddpf / 35 dpf, respectively, whereas the cold incubated eggs (4-6°C) required 285 ddpf / 63 dpf to hatch. There are a number of factors that could account for the overall greater number of ddpf required for the lumpfish eggs used in this study to hatch (e.g., incubator design). However, the most likely explanation is the broodstock that were the source of the eggs/milt. In this study, the broodstock were of Newfoundland origin, whereas the broodstock used in Imsland et al. (2019) were from Troms County, Norway. Further, while the broodstock used in this study were 2-year-old domesticated (F1) fish that had been reared at the JBARB and fed a commercially available pelleted diet, wild-caught broodstock were used by Imsland et al. (2019). As such, these fish had markedly different thermal and nutritional histories, and genetic lineages. With respect to the cold group in Imsland et al. (2019) taking more ddpf to hatch as compared to the warmer incubation groups, this again could be related to the ambient water dropping below 4°C. This can be stressful for lumpfish eggs, as mentioned earlier (Collins, 1978).

3.4.1.2: Survival

Variations in post-hatch (larval / early juvenile) survival between the groups was also observed during the temperature anomaly where values fluctuated between ~ 10 and 15°C. Group 1 [incubated (I) at 6°C] had the highest survival (66.1 %). Whereas Group 5 (I, 6-11ºC) had the lowest survival (33.7%) and Group 3 (I, 8.5°C) had the second lowest survival (51.5%) (Figure 3.16B). Scott and Johnson (2012) incubated zebrafish eggs at 22, 27 and 32°C and found that embryonic (incubation) temperature had considerable effects on the thermal sensitivity of zebrafish swimming performance after 8-9 months of post-embryonic development. Fish incubated at 22 and 32°C had significantly better performance at their incubation temperature as compared to those tested at the 27°C. These data suggests that lumpfish eggs incubated at higher temperatures would be less sensitive to the effects of elevated temperature during the larval/early juvenile period, i.e.; there would be persistent effects of temperature conditions during the embryonic stage on later life-history stages (Beaman et al., 2016). However, the data in this area are far from consistent. Illing et al. (2020) showed that incubating several species of tropical fish at 28.5 vs. 30.5°C had no effect on their CT_{Max} when tested from 7-21 days post-hatch. Further, Pottier et al. (2022) performed a meta-analysis of how the embryonic and juvenile thermal environment affects the temperature tolerance of a number of ectothermic vertebrates. These authors found that embryos expressed weaker, but more heterogeneous, plasticity and that a number of these responses appeared to be non-adaptive. Based on this, Pottier et al. (2022) concluded that higher incubation temperatures reduce the heat tolerance of later life stages.

While my results agree with these latter authors, one must be careful in using these data to suggest or conclude that warm incubation temperatures limit the thermal tolerance of lumpfish during the entire larval/juvenile period. The temperature anomaly began at the beginning of

weaning the fish off live food (*Artemia* napulii) and onto microdiet (Fig. 3.16A), and this is a particularly sensitive period in the rearing of marine fishes where mortalities can be quite high (Imsland and Reynolds, 2022; Rian, 2019). Thus, the level of mortality, and the magnitude of the effect of incubation temperature, were likely amplified during this period. With regard to the stochastic temperature group having higher mortalities than the 6°C group, this was somewhat unexpected. Schaefer and Ryan (2006) showed that zebrafish reared at varying and stochastic (± 6°C) temperatures had greater thermal tolerance than individuals reared under non-variable environments.

3.4.1.3: Deformities

Although the data in this study are only observational in nature (i.e., no statistical analyses were performed), it is still important information for lumpfish hatcheries and the industry. The most common deformities in the groups were scoliosis and missing eyes, with other eye deformities and sucker deformities relatively rare, and cataracts only recorded at high numbers (38%) in one of the 8.5°C incubated groups at 100 ddph. The first two deformities were prevalent at 100 ddph, and although few fish had missing eyes or other deformities at 400 ddph, the frequency of fish with scoliosis still ranged from 6-16% when all groups were considered (Table 3.5). However, by 900 ddph very few lumpfish (i.e., 6 out of the 250 fish measured) had any deformities. These data suggest that many lumpfish that hatched with a deformity did not survive to the juvenile stage.

Only one study to date has reported rates of spinal deformities in farmed lumpfish, noting that between 2 and 16% of the fish (n = 36-129 per hatchery, n = 6 hatcheries) developed at least 1 spinal deformity (Fjelldal et al., 2021). With regard to the prevalence of cataracts, Rabadan et al.

(2021) reported that cataracts were seen in 5% of juveniles at hatcheries and 17% of fish at sea-cages. The above data on hatchery lumpfish agree with the majority of findings from this study, where four out of the five groups had less than 8% of fish with cataracts at 100 dph. However, in Group 2, the incidence of cataracts at this life stage was 38%, a result for which we do not currently have an explanation. Eye damage and cataracts increase the risk of emaciation as lumpfish rely on their sight to locate their feed and/or prey (Rabadan et al., 2021). In this same study, 37% of fish possessed a sucker deformity, both in the hatchery and at cage-sites. This is detrimental for lumpfish, as the lack of a functioning sucker prevents lumpfish from sticking to surfaces to rest and can exhaust the fish, often to the point of mortality (Reynolds et al., 2022; Rabadan et al., 2021). Clearly, understanding the conditions under which lumpfish are likely to either hatch with, or develop, deformities is crucial to increasing survivorship at the hatchery, reducing mortalities at cage-sites, and ensuring that lumpfish are effective at delousing.

3.4.1.4: Growth Rate

During rearing, the lumpfish had specific growth rates (SGR) that ranged between 10.3-12.0% day^{-1} from 100-400 ddph, and between 5.48-7.31% day^{-1} from 400-900 ddph. The corresponding weight gains were 0.033-0.057 g day^{-1} and 0.97-1.79 g day^{-1}, respectively (Table 3.4). When the growth of the lumpfish reared at the JBARB facility from 2019-2021 are compared with the data reported in this thesis, the growth of the fish are very similar (Fig. S1). This was unexpected, as larvae/juveniles from this study and those from JBARB's 2021 year-class experienced the temperature anomaly (Fig. 3.16). However, while the temperature changes during the temperature anomaly were variable and unpredictable, the fish only spent 12 days at a mean daily temperature > 11°C. Further, it is possible that the temperature anomaly may have selected

for fish that could perform better at these elevated temperatures, and that this resulted in growth rates that were similar to that reported in previous years where a rearing temperature of 9°C was considered optimal. Consistent with this hypothesis, Nytrø et al. (2014) found that juvenile lumpfish had significantly higher SGR values when reared at consistently high temperatures, including 10, 13 and 16°C. Thus, it is possible that the anomaly in this study selected for individuals that were more tolerant of high temperatures, and that this was reflected in their growth. However, few studies have investigated the effects of temperature on the SGR of lumpfish during rearing, and this is an area that warrants further research.

3.4.2: Thermal Tolerance

3.4.2.1: Critical Thermal Maximum (CT_{Max})

The mean CT_{Max} of fish in this study when acclimated to 10°C was 22.85°C, and no statistically significant differences were observed between the incubation/rearing groups (Fig. 3.18). The CT_{Max} of lumpfish in this study is slightly higher than the 22.3°C reported by Ern et al. (2016) for fish acclimated to 10°C, and approx. 2°C higher than that reported in Chapter 2 for lumpfish acclimated to 6°C. The difference in CT_{Max} values between this study and Ern et al. (2016) could be attributed to the different populations/stocks of fish used in the two studies. However, the fish used in both studies were reared at the JBARB, and so this is unlikley. Thus, it is conceivable that the temperature anomaly selected for lumpfish that had a higher upper thermal tolerance. This would fit with the analysis performed by Pottier et al. (2022), which concluded that while the exposure of larvae/juveniles to increased temperatures results in an increase in thermal tolerance later in life, the benefit is relatively small (0.13°C per 1°C increase in rearing temperature). If one considers that maximum temperatures experienced during the temperature

anomaly averaged 13°C (as compared to the typical rearing temperature of 9°C), then based on the analysis performed by Pottier et al. (2022) we might have expected an increase in CT_{Max} as compared to Ern et al. (2016) of 0.52°C. This is very close to the difference in CT_{Max} of 0.55°C reported between this study and Ern et al. (2016). The suggestion that the temperature anomaly had an effect on the lumpfish's CT_{Max} when assessed at ~55 g, would also be consistent with the work of Schaefer and Ryan (2006). These authors report that while the thermal history of zebrafish causes irreversible changes in this species' thermal tolerance, the changes in CT_{Max} are relatively minor; $\leq$ 2°C when both the effects of acclimation temperature and varying temperature regimes are included.

3.4.2.2: Metabolic Parameters

One advantage of performing a CT_{Max} experiment, instead of a longer-term thermal tolerance test (e.g., an IT_{Max} test) is that it permits measurements of the metabolic capacity of fish to be performed. There were no statistically significant differences between the incubation/rearing groups with regard to any of the metabolic parameters (Fig. 3.19B). In particular, there were no differences in MMR or the two measures of metabolic scope (AS and ASR), and this might be expected given the lack of a difference in thermal tolerance between the groups and the main tenets of the OCCLT hypothesis (Pörtner, 2021; Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Pörtner, 2001). However, strong evidence is emerging that oxygen supply and delivery are not the sole/primary factors that limit a fish's thermal tolerance (Ern et al., 2023; Nati et al., 2016; Seebacher et al., 2015). For instance, Andreassen et al. (2022) suggest that a fish's upper thermal tolerance is specifically related to its ability to maintain brain/nervous function, and not whole-body metabolic demand.

With regard to the MMR, AS and ASR of lumpfish, we see only minor differences between the values in this study and those reported in Chapter 2 for 6°C acclimated fish. The lumpfish in this study, acclimated to 10°C, had values for MMR, AS and ASR of 189.9 $\pm$ 6.6, 166.1 $\pm$ 6.5 and 138.0 $\pm$ 6.6 mg O_2 kg^{-1} h^{-1}, respectively (Table 3.6). In comparison, lumpfish acclimated to 6°C and subjected to the same CT_{Max} protocol (Chapter 2) had MMR, AS and ASR values of 183.9 $\pm$ 14.4, 141.0 $\pm$ 15 and 134.6 $\pm$ 15 mg O_2 kg^{-1} h^{-1} respectively. These data suggest that the metabolic capacity of lumpfish is relatively temperature insensitive over this thermal range. There are few studies with which these data can be compared. However. Hvas et al. (2018) report that the MMR of lumpfish acclimated to 10°C was only ~ 25% greater than that of 3°C-acclimated fish, and there were no significant differences in ASR, or its factorial scope, between the two groups.

3.4.2.3: Incremental Thermal Maximum (IT_{Max}) of Lumpfish, and Comparison with Other Studies and Species

The IT_{Max} of lumpfish in this study was 20.63°C, and the lumpfish reached 100% mortality within a very narrow temperature range (Fig. 3.20). Thus, this study strongly suggests that the stock of lumpfish used in this study can be held and maintained at $\leq$ 20°C, without the risk of mass mortality. Although no other researchers have performed an IT_{Max} study on lumpfish to date, a comparison with Hvas et al. (2018) suggests that our stocks are more thermally tolerant than those used in their study. In their study, mortalities began shortly after holding temperature was increased to 18°C, and mortalities reached 15% after 3 weeks at this temperature. In addition, there appear to be other differences between the two studies/populations. Aggressive and erratic behaviour, and injuries, were reported among the lumpfish in Hvas et al. (2018) at 18°C, whereas near the end of the IT_{Max} test the main behaviours observed were lethargy, a large ventilatory effort,

difficulty maintaining buoyancy, and an increased amount of time sticking to the bottom of the tank.

There are limited studies with which the IT_{Max} of the lumpfish can be compared. However, the lumpfish's IT_{Max} measured in this study (20.63°C) is approximately 1°C less than that of 10°C-acclimated Atlantic cod (21.7°C; Zanuzzo et al., 2019), and ~ 2- 4°C less than measured in Atlantic salmon acclimated to temperatures between 8.5 and 12°C (~ 24°C, Ignatz et al. 2023; 22.8°C, Bartlett at al., 2022; 25.1°C, Ignatz et al., 2021). The difference in IT_{Max} between Atlantic salmon and lumpfish in this study is not surprising given that the former species is a eurythermal fish that can encounter temperatures as high as 28-30°C in the wild (Caissie et al. 2012). Further, the more comparable, and lower, values for the cod and lumpfish were not unexpected as both are marine species that are widely distributed throughout the North Atlantic, and cod typically do not experience temperatures greater than ~ 19°C (Righton et al., 2010).

The CT_{Max} and IT_{Max} tests assess different aspects of a fish's thermal tolerance. CT_{Max} is an estimate of a fish's (population's) upper thermal tolerance when acclimated to a particular temperature and exposed to an acute increase in temperature; whereas IT_{Max} is representative of their tolerance to chronic thermal stressors that might be seen in the wild (i.e., seasonal changes in water temperature and those associated with climate change). Further, IT_{Max} may better represent the thermal stressors that fish face when confined in sea-cages (Bartlett et al., 2022; Brauner and Richards, 2020). With regards to the difference between these two metrics of thermal tolerance, it appears that the lumpfish is intermediate between the Atlantic cod and salmon. The lumpfish's CT_{Max} was ~2.2°C higher than their IT_{Max} (22.85 vs. 20.63°C respectively; Table 3.7), whereas this difference was 0.8°C for Atlantic cod (Zanuzzo et al., 2019) and differences between IT_{Max}

and CT_{Max} for Atlantic salmon range from 3.5 to 4.7°C (Ignatz et al., 2023; Bartlett at al., 2022; Ignatz et al., 2021).

Importantly, given the above values for the lumpfish's CT_{Max} and IT_{Max} (22.85°C and 20.63°C, respectively), and that Gamperl et al. (2021) showed that even during a heat wave cage-site temperatures in Newfoundland do not exceed ~19.5°C, it is not likely that high temperatures were directly responsible for the lumpfish mortalities that have been observed at some cage-sites in Newfoundland during the summer months. Other factors alone, or in combination with high temperatures, that could be related to lumpfish mortalities are an insufficient water exchange rate in sea-cages, low dissolved oxygen, increased stress in the presence of Atlantic salmon, disease, lack of sufficient hides, and welfare or other issues (Boissonnot et al., 2022; Foss and Imsland, 2022; Remen et al., 2022; Reynolds et al., 2022; Rabadan et al., 2021; Staven et al., 2021; Klakegg et al., 2020; Powell et al., 2018; Jørgensen et al., 2017).

3.4.3: Hepatosomatic Index (HSI) and Relative Ventricular Mass (RVM) During the IT_{Max} Test

Both the HSI and RVM of lumpfish decreased during the IT_{Max} test. By the time the lumpfish reached 20°C, average HSI and RVM values had decreased by 13.4% and 42.1%, respectively. The former is consistent with data on Atlantic salmon, where HSI was ~28% less than in control fish prior to the salmon reaching their IT_{Max} (Gamperl et al., 2020). As the lumpfish decreased their feed consumption once temperatures reached 16°C (Fig. S2), it is likely that energy reserves (e.g., lipids, glycogen) in the liver were used to sustain the lumpfish at higher temperatures, and that this resulted in a decrease in HSI. In contrast to the lumpfish in this study, the RVM of Atlantic salmon has been shown to increase during an IT_{Max} test (Ignatz et al., 2023; Ignatz et al., 2021). Further, although no such relationship existed in Ignatz et al. (in press), Barlett

et al. (2022) reported a positive relationship between RVM and IT_{Max} in Atlantic salmon. Given the above data, and that heart function is thought to be a key/primary determinant of a fish's thermal tolerance (Eliason et al., 2011; Farrell et al., 2009; Wang and Overgaard, 2007), the fact that the ventricles of the lumpfish got smaller as temperatures rose suggests that this phenomenon might explain / have contributed to this species' lower thermal tolerance (IT_{Max}).

3.4.4: Plasma Cortisol During the IT_{Max} Test

At 10, 16, 18 and 20°C, the lumpfish in this study had plasma cortisol levels of 0.70, 1.57, 2.27 and 2.07 ng mL^{-1}, respectively (Table 3.10), and the values at 18 and 20°C were significantly higher than at 10°C (Fig. 3.21, Table 3.10). In other studies on lumpfish, resting cortisol values have ranged from undetectable to 5.5 ng mL^{-1} (Foss and Imsland, 2022; Patel et al., 2022; Hvas et al., 2018; Jørgensen et al., 2017; Haatuft, 2015). However, when subjected to various stressors, cortisol levels in lumpfish have been reported to be much greater than the values obtained during the IT_{Max} test. For instance, lumpfish plasma cortisol levels were ~ 23-50 ng mL^{-1} when subjected to a hypoxia stressor (Remen et al., 2022; Jorgensen et al., 2017; Haatuft, 2015), ~ 76 ng mL^{-1} following transport stress (Foss and Imsland, 2022), ~ 30-80 ng mL^{-1} when faced with repeated overcrowding (Patel et al., 2022), ~ 44-150 ng mL^{-1} following encounters with Atlantic salmon (Staven et al., 2021; Staven et al., 2019), and up to ~55 ng mL^{-1} after an exercise stressor (Hvas et al., 2018). Collectively, these data suggest that plasma cortisol levels are not greatly elevated in lumpfish as they approach their upper thermal tolerance, and thus, that this parameter is not a good indicator/biomarker of thermal stress in this species. This conclusion would be consistent with the findings for other fish species. Perez-Casanova et al. (2008) reported that plasma cortisol levels in Atlantic cod acclimated to 10°C were not different as compared to those given a long-term (1°C

every 5 days) increase in temperature to 19°C, and Zanuzzo et al. (in prep.; as cited in Beemelmanns et al., 2021) found that plasma cortisol levels were not elevated until Atlantic salmon experienced water temperatures ($\geq$ 22°C) where mortalities were beginning to occur. There has been no work to identify appropriate biomarkers of thermal stress in lumpfish, however, work on other fishes has identified a number of heat stress proteins and other cellular markers that should be examined in this species (e.g., see Beemelmanns et al., 2021; Sopinka et al., 2016). This represents an area for future work.

3.5: Summary and Conclusions

The goal of this research was to examine if elevated temperatures and/or a stochastic temperature regime during embryonic development and larval rearing would improve the lumpfish's acute and long-term thermal tolerance (CT_{Max} and IT_{Max}, respectively), and thus, potentially reduce losses during the summer months at Atlantic salmon cage-sites. This research did reveal that higher and stochastic (8.5 and 6-11°C, respectively) temperatures decreased the proportion of lumpfish eggs that hatched (i.e., hatching success), and thus, that such changes to incubation protocols would be detrimental, not beneficial, to lumpfish production. However, a high temperature anomaly during larval/early juvenile rearing prevented me from examining the main hypotheses to be tested in this thesis chapter. This was unfortunate, yet provided an opportunity to investigate how incubation temperatures affected the survival of lumpfish larvae / juveniles exposed to such phenomena. While the findings of this research are not particularly relevant to the lumpfish aquaculture industry as rearing temperatures are normally strictly controlled, the data show that in addition to their negative effects on hatching success, higher and stochastic temperatures during embryonic development can result in increased mortality during prolonged periods of elevated water temperatures during early life. Clearly, this is a concern for in shore/coastal spawning marine fish species given climate change driven increases in mean global ocean temperatures (IPCC, 2022; Garcia-Soto et al., 2021), and in the frequency, severity and duration of heat waves (IPCC 2021; Oliver et al., 2021; Viglione, 2021; Laufkötter et al., 2020; Oliver et al., 2019; Smale et al., 2019; Stilman, 2019; Frölicher et al., 2018; Oliver et al., 2018).

With respect to the reported values for the CT_{Max} and IT_{Max} for lumpfish of the size normally stocked into salmon sea-cages (~ 50 g), the values for these parameters were 22.85 and 20.63°C, respectively. These values are considerably above the maximum temperatures that have

been reported at salmon cage-sites during the summer in Newfoundland (< 19.5°C, Gamperl et al., 2021), and strongly suggest that high temperatures are not the primary factor leading to the loss of fish during this season. Thus, while this research does not point the industry towards a solution to this important challenge, it strongly implies that the industry should focus elsewhere with regard to reducing summer mortalities at Atlantic Canadian cage-sites. That said, this study does suggest additional work that should be performed related to the thermal biology of lumpfish. For example, as in the Atlantic salmon and cod (Zanuzzo et al., in prep.; Perez-Casanova et al. 2008), it appears that plasma cortisol is not a valuable/sensitive indicator of the stress induced by elevated temperatures. Thus, it would be valuable to identify, and to develop methods for the measurement of, biomarkers of thermal stress in this species. These markers could be used to determine what temperatures are optimal for this species versus those that are sub-optimal and/or induce sub-lethal stress. Further, a novel finding in this study was that the lumpfish's heart (RVM) got smaller, not larger, as temperatures approached this species' upper temperature tolerance. This research suggests that the pumping capacity of the lumpfish heart responds differently to environmental challenges as compared to other fishes (e.g., salmon), and that this may affect their tolerance limits. Interestingly, the lumpfish is one of the few teleost species to lack cardiac myoglobin (Gerber et al., 2021), and this important physiological difference, in combination with a smaller heart, may influence the capacity of the heart to perform under suboptimal conditions and limit this species' capacity to tolerate warm temperatures.

Finally, several findings of this research contribute to our understanding of whether, and to what extent, 'developmental plasticity' influences the thermal tolerance of fishes. In this regard there were two notable results: 1) that high incubation temperatures reduced the ability of larvae/early juveniles to survive prolonged exposure to elevated temperatures; 2) and that the

CT_{Max} of lumpfish in this study was the highest so far recorded for this species [e.g., ~ 0.55°C higher than in Ern et al (2016) who tested JBARB-produced fish of the same size acclimated to 10°C]. The former agrees with Pottier et al. (2022) who performed a meta-analysis using a variety of ectothermic species and reported that higher incubation temperatures persistently reduced the heat tolerance of later life history stages. Why this occurs is not known, but these authors suggest that the high costs of developmental processes may constrain the allocation of energy stores to functions such as plastic responses to temperatures (Pottier et al., 2022). With regard to the second finding, our results suggest that rearing larvae/juveniles at high temperatures has a significant, but weak, overall positive effect on the heat tolerance of ectotherms. This conclusion agrees with the analysis performed by Pottier et al. (2022; i.e., that a 0.13°C increase in thermal tolerance is achieved with each 1°C increase in developmental temperature) and with the results of Schaeffer and Ryan (2006). These authors showed that the effects of increased acclimation temperatures, and exposure to cycling and stochastic temperature regimes, on adult zebrafish thermal tolerance were at most 1°C.

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Chapter 4 - Summary, Research Limitations and Perspectives

4.1: Methods of Measuring Metabolic Capacity and Their Relevance

There are many methods that can be used to measure the oxygen consumption and metabolic capacity of fishes, each of which can provide varying results (i.e., Raby et al., 2020; Zhang et al., 2020; Hvas and Oppedal, 2019; Norin et al., 2019; Roche et al., 2013; Reidy et al., 1995). Further, the effectiveness of any one method can differ between species and between individuals, depending on their specific life history (Clark et al., 2013; Metcalfe et al., 2016; Norin and Clark, 2016; Killen et al., 2017; Neubauer and Andersen, 2019; Norin et al., 2019). In Chapter 2, I showed that the three commonly used methods of 'exhaustion' [the chase test, the critical swimming speed (U_{crit}) test, and the critical thermal maximum (CT_{Max}) test] do not provide equivalent estimates of metabolic capacity in lumpfish (i.e., they vary by 4 to 28 %; Table 2.1), and importantly, that the magnitude of a particular parameter (e.g., MMR, AS) in one test cannot be predicted from the other; i.e., the parameter as measured in one test does not correlate significantly with that measured using another (Figs. 2.9-2.13). These findings agree with the recent work of Mullen and Rees (2022) on Gulf killifish, and collectively, this research: highlights the importance of choosing the most appropriate method of exhaustion to suit the research question, and species, when measuring metabolic parameters; and by extension, strongly encourages that preliminary experiments be performed to choose a method of 'exhaustion' that ensures that the fish/species of interest is utilizing its full metabolic capacity when tested. Such experimental validation is particularly important when the data generated are intended to be compared between fish species.

4.2: The Thermal Tolerance of Lumpfish

In this thesis, the thermal tolerance of cultured/domesticated lumpfish was measured in a laboratory/intensive aquaculture setting where many environmental conditions (e.g., temperature, photoperiod, diet) were controlled. I showed that the incubation of lumpfish eggs at warmer and stochastic (8.5°C and 6-11°C, respectively) temperatures resulted in a significantly lower hatching success as compared to when eggs/embryos were incubated at 6°C (Table 3.3). Further, I determined that the CT_{Max} of lumpfish acclimated to 6°C was 20.6°C (Chapter 2), and that the CT_{Max} and IT_{Max} of fish acclimated to 10°C were 22.85°C and 20.63°C, respectively (Chapter 3). While it is likely that the values reported in Chapter 3 were slightly higher due to the temperature anomaly that occurred during larval/juvenile rearing (i.e., more thermally tolerant fish may have been selected for), when our data are combined with that of Ern et al. (2016; CT_{Max} 22.3°C) and Zrini et al. (2021; CT_{Max} > 22°C) for 10°C-acclimated fish, it is apparent that the upper lethal temperature of lumpfish produced for the industry by the JBARB is $\geq$ 20.5°C. In Newfoundland cage-site temperatures are currently unlikely to exceed 19.5°C even for brief periods during warm summers (Gamperl et al., 2021), and it has been reported that lumpfish mortalities at salmon cage-sites occur at temperatures $\leq$ 12°C in Norway and that temperature was only a contributing factor to ~ 3% of these mortalities (Reynolds et al., 2022). These data strongly suggest that high temperatures alone are not a major contributor to lumpfish mortalities experienced at cage-sites in Atlantic Canada, and thus, that selection for increased temperature tolerance may not be successful in improving lumpfish welfare and survival under cage-site conditions. However, conditions at cage-sites are often much more stressful than lab conditions for fish, and lumpfish are faced with many stressors at cage-sites that may contribute to their vulnerability to temperatures far below their maximum thermal tolerance: these include poor welfare, handling stress, transport stressors,

disease, and other stressors (Boissonnot et al., 2023; Garcia de Leaniz et al., 2022; Imsland et al., 2022a; Reynolds et al., 2022; Rabadan et al., 2021; Geitung et al., 2020; Imsland et al., 2020). Some efforts are being made to improve conditions at cage-sites for lumpfish, including the development and use of operational welfare indices (OWIs; Garcia de Leaniz et al., 2022; Rabadan et al., 2021; Imsland et al., 2020). Further, evidence suggests that pre-exposure to Atlantic salmon, or waters that previously contained Atlantic salmon prior to deployment to the cage-sites, reduces stress levels in lumpfish at cage-sites (Staven et al., 2021). This may be a useful practice that could help mitigate the stress experienced by lumpfish at cage-sites and reduce mortalities.

With respect to the monitoring of stress levels in lumpfish, it is apparent from Chapter 3 that cortisol is a poor indicator of heat stress in this species (Table 3.10). Lumpfish have relatively low cortisol values at rest (this study; Foss and Imsland, 2022; Hvas et al., 2018;) and exposure to very high temperatures only resulted in an ~ 2-fold increase in circulating levels during the IT_{Max} test (i.e., to values < 3.0 ng mL^{-1}). These levels are much lower than those reported following other aquaculture-relevant stressors in this species (< 10 to 75 ng mL^{-1}; Foss and Imsland, 2022; Patel et al., 2022; Remen et al., 2022; Staven et al., 2021; Staven et al., 2019; Jørgensen et al., 2017), and suggest that it will be very difficult to assess heat stress in lumpfish using circulating cortisol levels. That plasma cortisol levels are not a good indicator of long-term heat stress in fishes agrees with Zannuzo et al. (in prep; as cited in Beemelmanns et al., 2021) and Perez-Casanova et al. (2008) who performed IT_{Max} experiments on Atlantic salmon and cod, respectively. Many fish species upregulate heat shock proteins when subjected to a thermal stressor (e.g., Manzon et al., 2022; Beemelmanns et al., 2021; Pandey et al., 2021; Healy et al., 2010; Hori et al., 2010; Fangue et al., 2006), and qPCR and/or enzyme-linked immunosorbent assays (ELISAs) could be developed for the measurement of temperature and stress biomarkers in lumpfish. For example,

heat shock protein 70 (*hsp70*), *hsp*47 (also known as serpinh1), *hsp90aa1*, cold inducible ribosomal binding protein (*cirbp*) and uncoupling protein 2 (*ucp2*) have been identified as key biomarkers of thermal stress in Atlantic salmon (Beemelmanns et al., 2021). Such work, however, will require improved genomic resources for lumpfish. For example, while a chromosome-level genome assembly was recently performed on the lumpfish to allow for identification of a sex determining locus (Holburn et al., 2022), to date, the lumpfish's genome has not yet been sequenced, and annotation of this genome would be extremely helpful (critical) with regards to developing such biomarkers. Clearly, these areas should be priorities for the salmon aquaculture industry with regard to understanding this species' thermal biology and improving its welfare and survival in the sea-cage environment.

4.3: Developmental Plasticity in Fishes

While there are several studies that have examined the influence of transgenerational effects and/or developmental plasticity on the thermal tolerance of fishes (Einum and Burton, 2023; Pottier et al., 2022; Moyano et al., 2017; Seebacher et al., 2014; Scott and Johnston, 2012; Schaefer and Ryan, 2006), these studies are very limited and provide divergent results, and thus, we have a poor understanding of how exposure to different temperatures / temperature regimes during embryonic and/or larval development influence the thermal tolerance and biology of fishes. Unfortunately, a temperature anomaly was experienced during larval/early juvenile rearing in Chapter 3, and this greatly limits the interpretation of the results in this regard. However, this research does provide several potential insights into how temperatures during early life influence the thermal tolerance of older lumpfish (fishes). For example: 1) it was apparent that elevated incubation temperatures and exposure to stochastic temperature changes during this critical life

history stage resulted in reduced survival during the anomaly (i.e., as larvae/early juveniles); and 2) the CT_{Max} for the lumpfish in Chapter 3 was slightly higher as compared to other studies where lumpfish were acclimated to the same temperature (Zrini et al., 2021; Ern et al., 2016), and this suggests that exposure to the temperature anomaly increased the lumpfish's thermal tolerance as subadults. Interesting both these results agree with the conclusions of the meta-analysis performed by Pottier et al. (2021), who reported that higher incubation temperatures reduce the heat tolerance of ectotherms later in life, and that that the larvae's / juvenile's thermal environment has a significant, but weak, positive effect on upper temperature tolerance.

Importantly, there is another caveat with regards to the experiment that was performed in Chapter 3. In this experiment, hatching success was relatively low (i.e., only 61% in the 6^{o}C incubated group as compared to typical values of > 90%; D. Boyce, pers. comm.), and it is possible that egg quality influenced the results. This may be particularly true as it has been suggested that the reliance of embryos solely on stored energy reserves may constrain energy allocation towards diverse functions, including plastic responses to temperature (Pottier et al., 2021). Further, there is one study on fish which has reported that thermal acclimation capacity is not fixed and can be modified by temperature during the embryonic stage (Scott and Johnson, 2012). Additional studies using egg batches from a number of males and females, and where the energy stores/levels are measured and/or manipulated, might prove extremely valuable in clarifying how embryonic temperature influences larval/juvenile thermal tolerance and the role of 'egg quality'.

4.4: Future Research

In addition to the research questions posed above that specifically relate to my thesis topic, there are two main areas of investigation that could have tangible benefits for the Atlantic salmon aquaculture industry.

4.4.1: Influence of Multiple Stressors

Given that lumpfish mortalities are being reported at salmon cage-sites during the summer months, and that fish can experience a number of stressors concurrently, it is crucial that the susceptibility of lumpfish to multiple stressors is further explored. For instance, some studies suggest that hypoxia can impact the metabolic response of some fish species to increasing temperature, thus limiting their thermal tolerance (e.g. sablefish; Leeuwis et al., 2021, Atlantic salmon; Remen et al., 2013). However, hypoxia does not always directly impact a fish's metabolic response and CT_{Max} (e.g., lumpfish and red drum; Ern et al., 2016), the presence of several stressors simultaneously could stress the fish via a number of different physiological mechanisms (Ern et al., 2023), and further research is required to identify these mechanisms. In addition, higher temperatures could impact a number of aspects of the lumpfish's biology, as has been reported for other fish species. These could include swimming capacity (Blasco et al., 2020) and cleaning efficacy (Boissonnot et al., 2022). Lumpfish also experience a wide variety of other stressors at cage-sites, including handling stresses, grading and delousing treatments (Reynolds et al., 2022). As such, it would be beneficial to determine how high temperatures influence the lumpfish's vulnerability to these types of cage-site specific stressors, and how these stressors influence the lumpfish's temperature tolerance.

4.4.2: Welfare and Husbandry Practices

Many concerns regarding lumpfish welfare at Atlantic salmon sea cage-sites have been identified, including their lower temperature preference, poor water quality, insufficient hides, insufficient feeding / nutrition, domestic breeding resulting in deformities, a mismatch in swimming depths, and others (Garcia de Leaniz et al., 2022; Geitung et al., 2020). Efforts are being made to categorize and monitor lumpfish health under culture conditions (i.e., at sea-cages) based on a number of visual indicators, including eye damage and cataracts, sucker deformities, body mass index, fin erosion and skin damage; these being used to determine an Operational Welfare Score Index (OWI; Rabadan et al., 2021). However, many of these OWIs (like sucker deformities and cataracts) may develop before the fish reach the sea-cage and are irreversible. Further, many of these OWIs may have multiple causes. For instance, there are many reasons lumpfish could develop cataracts, including diet, frequency of feeding, genetics, and high water temperatures (Paradis et al., 2019; Imsland et al., 2018a; Imsland et al., 2018b; Jonassen et al., 2017). Clearly, more research effort should be put into preventing lumpfish health/welfare issues that develop in the hatchery from impacting their performance and survival in sea-cages where they accomplish the important function of removing sea lice from Atlantic salmon (i.e., act a 'cleaner fish'). In addition, there is accumulating evidence that liver colour may a good indicator of lumpfish welfare (Imsland et al., 2022; Eliasen et al., 2020). However, what factors specifically influence this trait are unknown, as is the relationship between liver colour and the fish's capacity to survive exposure to increasing temperatures or other stressors. Thus, it is crucial that research continues towards monitoring and improving lumpfish welfare and health, and to identifying and developing more effective welfare indicators, if the industry is to reduce mortality rates and to guarantee a high delousing efficiency.

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Appendix

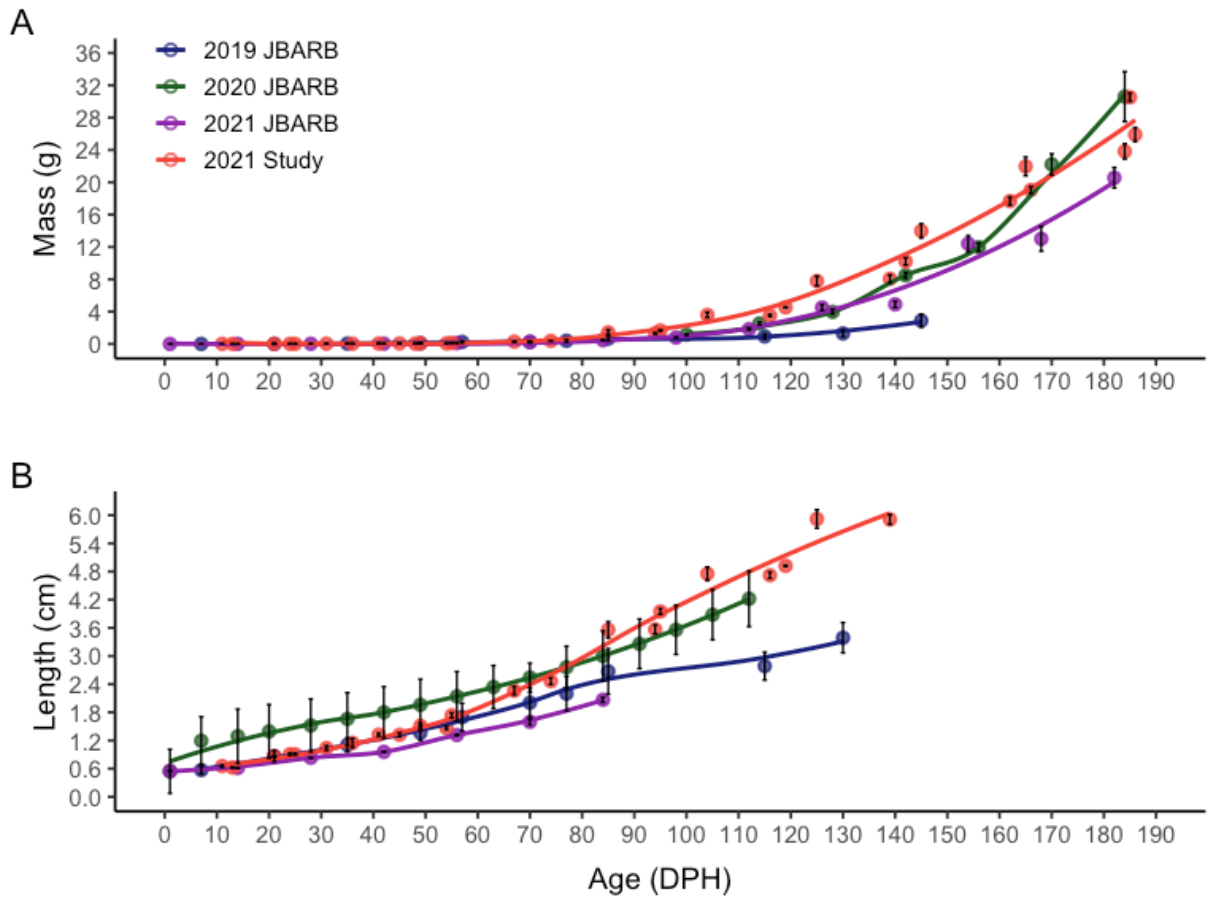

Figure S1: Comparison of lumpfish mass (g; A) from 0 to 186 dph, and length (cm; B) from 0 to 139 days post-hatch between lumpfish reared in this study (all groups combined) and lumpfish reared by the Joe Brown Aquatic Research Building (JBARB) from 2019 - 2021. A second order polynomial function was used to best fit the curve for each year class. There is some mass data missing from the 2019 production run and length data missing from 2019-and 2020, due to COVID-19 related restrictions and limitations on staffing.

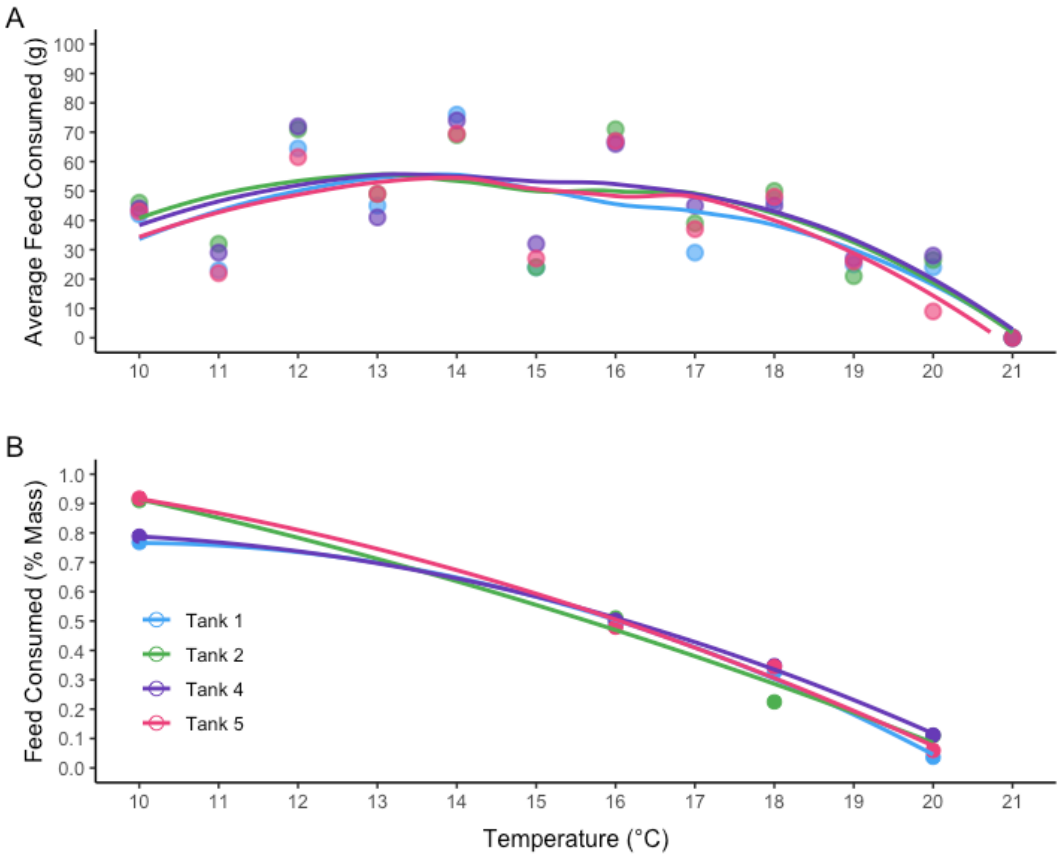

Figure S2: The average daily amount of feed consumed by lumpfish when fed to satiation (g; A) and the amount of feed consumed by lumpfish as a proportion of body mass (%; B) in each of the four tanks (n = 100 fish in each tank) as temperature was increased during the IT_{Max} test (+1°C wk^{-1}). A second order polynomial function was used to best fit the curve for each tank. In the IT_{Max} test, fish were fed to satiation on two consecutive days each week, and these values were averaged.