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ENHANCING FISH TOLERANCE TO GLOBAL WARMING THROUGH SWIMMING EXERCISE TRAINING

Cardiac plasticity to swimming exercise training in hatchery-reared fish and its implications for thermal tolerance

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“Per aspera ad astra”

To my beloved grandma, my Angel on the brightest star

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ABSTRACT

Anthropogenic climate change is increasing the magnitude and frequency of marine heatwaves, challenging the physiological limits of ectothermic animals, such as fish, and negatively affecting human activities that depend on them, such as aquaculture. Therefore, there is an urgent need to understand the physiological capacity of fish to cope with rising temperatures and provide solutions to increase the resilience of hatchery fish to heatwaves. Previous studies have suggested that thermal tolerance in fish is strongly related to cardiac function. In this thesis, I studied how phenotypic plasticity of cardiac function can help fish to cope with high temperatures. In study I, I investigated whether acclimation to a mild level of hypoxia can induce functional and molecular adjustments similar to those induced by high temperature acclimation, and whether hypoxia acclimation can induce cross-tolerance protection to high temperatures. By measuring the thermal performance curve for maximal heart rate in European sea bass, I have determined that European sea bass do not show evidence of cross-tolerance protection between hypoxia and high temperature. In studies II, III, and IV, I investigated whether improvements in cardiac performance induced by aerobic exercise training could provide cross-tolerance protection to cope with high temperatures in hatchery-reared rainbow trout and brown trout. By training fish with aerobic exercise training, I demonstrated that trained fish have significantly higher cardiac performance and cardiac thermal tolerance, which might help them to face heatwaves. However, the improvement in thermal tolerance was highly dependent on the intensity of the training. Furthermore, in Study IV, the transgenerational effect of maternal exercise training on the thermal tolerance, survival, and growth rate of the offspring were investigated. The results of this study demonstrated that maternal exercise can improve the survival of embryos and growth of offspring without affecting their thermal tolerance and cardiac performance. This thesis provides deeper insights into the cardiac plasticity of fish and its relationship with thermal tolerance. Taken together, these results suggest that exercise training improves cardiac thermal tolerance in hatchery fish and potentially enhances their ability to cope with heatwaves. In the context of global warming, these findings have significant implications for aquaculture sector and conservation programs, providing a valid aquaculture practice to enhance the resilience of fish to climate warming.

KEYWORDS: Acclimation, Aquaculture, Brown trout, Cardiac Arrhythmia, Cardiac Plasticity, Climate Change, Conservation Program, European Sea bass, Exercise Training, Hatchery-Reared Fish, Heart Rate, High Temperature, Rainbow trout, Salmonid, Swimming Training, Thermal Tolerance, Training intensity, Transgenerational Plasticity, Water flow

TURUN YLIOPISTO
Matemaatis-luonnontieteellinen tiedekunta
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TIIVISTELMÄ

Ilmastonmuutoksen seurauksena merien lämpöaallot voimistuvat ja niiden esiintymistiheys kasvaa. Tämä haastaa vaihtolämpöisten eläinten, kuten kalojen, fysiologiset sietokykyrajat ja voi aiheuttaa ongelmia esimerkiksi vesiviljelylle. Siksi onkin kiireellistä selvittää, miten kalat reagoivat ja pystyvät sopeutumaan kohonneisiin lämpötiloihin. Lisäksi on selvitettävä, voiko kalojen korkeiden lämpötilojen sietoa parantaa kasvatusoloja muuttamalla. Aiemmat tutkimukset ovat osoittaneet, että kalojen lämmönsietokyky liittyy vahvasti sydämen toimintaan. Väitöskirjassani tutkinkin, mikä merkitys sydämen toiminnan fenotyyppisellä plastisuudella on korkeiden lämpötilojen siedossa ja voisiko plastisuutta käyttää hyväksi kalojen kasvatuksessa, kun veden lämpötila nousee. Tutkimuksessa I selvitin, aiheuttaako vähähappisuusaltistus samanlaisia toiminnallisia ja molekyylitason muutoksia kuin altistuminen korkeille lämpötiloille. Päämääränä oli selvittää, voiko altistuminen alhaiseen happipitoisuuteen vaikuttaa korkeiden lämpötilojen sietoon (ristitoleranssi). Meribasseilla tehdyt maksimisykemittaukset kuitenkin viittaavat siihen, että happivajeen ja korkean lämpötilan välillä ei ole ristitoleranssia. Tutkimuksissa II, III ja IV selvitin, miten aerobinen uintiharjoittelu vaikuttaa kalojen sydämen toimintaan ja etenkin voisiko harjoittelu, parantamalla sydämen toimintakykyä, parantaa korkeiden lämpötilojen sietoa. Tutkimukset tehtiin kirjolohilla ja taimenilla. Tutkimuksissa osoitin, että harjoittelu parantaa huomattavasti sydämen toimintakykyä etenkin korkeissa veden lämpötiloissa, mikä saattaa auttaa kaloja selviytymään lämpöaalloista. Lämmönsietokyvyn paraneminen riippui kuitenkin huomattavasti harjoituksen intensiteetistä. Osatyössä IV tutkin myös, miten naaraskalojen harjoittelu vaikuttaa niiden jälkeläisten lämmönsietokykyyn, selviytymiseen ja kasvuun. Tämän tutkimuksen tulokset osoittivat, että naaraiden harjoittelu voi parantaa alkioiden eloonjäämistä ja jälkeläisten kasvua vaikuttamatta kuitenkaan jälkeläisten lämmönsietokykyyn tai sydämen toimintakykyyn. Yhdessä väitöskirjani tulokset viittaavat siihen, että uintiharjoittelu tehostaa kasvatettujen kalojen sydämen toimintaa korkeissa lämpötiloissa ja tätä kautta mahdollisesti auttaa kaloja selviytymään helleaalloista. Näillä tuloksilla on merkittäviä vaikutuksia sekä vesiviljelyyn että kalojen suojeluohjelmiin, sillä ilmastonmuutoksen seurauksena lämpöaallot vääjäämättömästi tulevat lisääntymään. Väitöskirjani tarjoaa uusia mahdollisuuksia ja käytänteitä vesiviljelyyn, mitkä voivat parantaa kalojen sietokykyä ilmaston lämpenemistä vastaan.

ASIASANAT: Acclimation, Aquaculture, Brown trout, Cardiac Arrhythmia, Cardiac Plasticity, Climate Change, Conservation Program, European Sea bass, Exercise Training, Hatchery-Reared Fish, Heart Rate, High Temperature, Rainbow trout, Salmonid, Swimming Training, Thermal Tolerance, Training intensity, Transgenerational Plasticity, Water flow.

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Abbreviations

16H	Sea bass experiment, hypoxia condition: seasonal average temperature of 16°C and with moderate hypoxia of 50% air saturation
16N	Sea bass experiment, control condition: seasonal average temperature of 16°C and normoxia
25N	Sea bass experiment, high temperature condition: temperature of 25°C with normoxia
AS	Aerobic Scope (MMR-SMR)
ATP	Adenosine-Triphosphate
bl s^{-1}	Body length per second
C	Control speed in the training experiments
CaO_2	Arterial oxygen content
CaO_2-CvO_2	Arteriovenous oxygen difference
CS	Citrate synthase
CT_{MAX}	Critical Thermal Maximum
CT_{MIN}	Critical Thermal Minimum
CvO_2	Venous oxygen content
Dpf	Day post fertilization
Dph	Day post hatching
EC-coupling	Excitation Contraction coupling
ECG	Electrocardiogram
$f\mathrm{H}$	Heart rate
$f\mathrm{H}_{MAX}$	Maximum heart rate induced by manipulation of the autonomic nervous system via injections of atropine and isoproterenol in anesthetised fish
$f\mathrm{H}_{PEAK}$	Highest value of $f\mathrm{H}_{MAX}$ measured during acute warming
GAS	General Adaptation Syndrome
GxE	Genotype-by-Environment interactions
GxPE	Genotype-by-Parental-Environment interactions
H	High intensity training group in the study III
h^2	Narrow-sense of Heritability (h^2 = VA/VP)
Hb	Haemoglobin
Hct	Haematocrit

HIF	Hypoxia-inducible factor
HSI	Hepatosomatic index
LDH	Lactate dehydrogenase
LOE	Loss of Equilibrium
M	Medium intensity training group in the study III
MMR	Maximum metabolic rate
MO_2	Aerobic metabolic rate
NCX	Na^+/Ca^{2+} exchanger
OCLTT	Oxygen- and capacity-limited thermal tolerance
Q	Cardiac output
RVM	Relative ventricle mass
SERCA	Sarco(endo)plasmic reticulum Ca^{2+-}ATPase
SMR	Standard metabolic rate
T	Training speed in the training experiments
T_{ABP}	Arrhenius break point temperature for TPC of $f\mathrm{H}_{MAX}$, measured during acute warming
T_{ARR}	Arrhythmia temperature (Cardiac thermal tolerance) for TPC of $f\mathrm{H}_{MAX}$, measured during acute warming
TDEE	Temperature dependent deterioration of electrical excitability
TGP	Transgenerational Plasticity
T_{OPT}	Thermal optimum for AS
TPC	Thermal performance curve
T_{PEAK}	Temperature corresponding to $f\mathrm{H}_{PEAK}$
TS	Thermal Scope for $f\mathrm{H}_{MAX}$ (T_{ARR}- T_{ABP})
U_{CRIT}	Critical Swimming Speed
VA	Genetic variance
VEGF	Vascular endothelial growth factor
VP	Total phenotypic variance
V_S	Stroke volume

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

- I Luca Pettinau, Frédéric Lancien, Yangfan Zhang, Florian Mauduit, Hélène Ollivier, Anthony P Farrell, Guy Claireaux, Katja Anttila. Warm, but not hypoxic acclimation, prolongs ventricular diastole and decreases the protein level of Na^+/Ca^{2+} exchanger to enhance cardiac thermal tolerance in European sea bass. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*; 2022; 272: 111266. https://doi.org/10.1016/j.cbpa.2022.111266
- II Anna Papadopoulou, Luca Pettinau, Eila Seppänen, Asko Sikanen, Katja Anttila. (2022). The interactive effects of exercise training and functional feeds on the cardiovascular performance of rainbow trout (Oncorhynchus mykiss) at high temperatures. *Current Research in Physiology*, 5: 142–150. https://doi.org/10.1016/j.crphys.2022.02.005
- III Luca Pettinau, Eila Seppänen, Asko Sikanen, Katja Anttila. (2022). Aerobic Exercise Training With Optimal Intensity Increases Cardiac Thermal Tolerance in Juvenile Rainbow Trout. *Frontiers in Marine Science*; 9: 963. https://doi.org/10.3389/fmars.2022.912720
- IV Luca Pettinau, Tytti-Maria Uurasmaa, Eila Seppänen, Asko Sikanen, Miika Raitakivi, Jenni M. Prokkola, Amelie Crespel, Katja Anttila. Maternal swimming exercise training improves survival and growth and increases the heritability of thermal tolerance in offspring of brown trout. (manuscript).

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Authors contribution to the original publications:

	I	II	III	IV
Research design	L.P., H.O., F.M., Y.F., F.L., A.P.F., G.C., K.A.	A.P., L.P., K.A.	L.P., K.A.	L.P, K.A.
Animal husbandry	H.O., F.L., F.M., Y.F., G.C.	A.S., E.S.	A.S., E.S.	E.S., A.S., M.S.
Practical/laboratory work and data collection	L.P., K.A., F.L.	A.P., L.P., K.A.	L.P., K.A.	L.P, T.U., E.S., M.S., J.M.P., K.A.
Data processing and Statistical analysis	L.P.	A.P., L.P.,	L.P.	L.P., A.C,
Writing first draft manuscript	L.P.	A.P.	L.P.	L.P.
Writing and reviewing the final draft manuscript	L.P., H.O., F.M., Y.F., F.L., A.P.F., G.C., K.A.	A.P., L.P., E.S., A.S., K.A.	L.P., E.S., K.A.	L.P, T.U., E.S., J.M.P., A.C., K.A.

1 Introduction

1.1 Background

Anthropogenic climate change is negatively affecting natural environments with serious consequences to both ecosystems and human society. The average sea temperature has increased by approximately 1°C over the past 140 years. The warming is especially intense in high-latitude regions, such as the Baltic Sea area, where the water temperature has increased by 1.4°C in 20 years (Belkin, 2009). Climate change is also increasing the magnitude and frequency of extreme climate events all around the world, such as heatwaves and water hypoxia events. Stochastic high temperatures and heatwaves have an important impact on ectothermic animals, such as fishes, compromising their physiological performance (e.g. reducing fecundity, compromising cardiorespiratory and swimming performance), and ultimately causing mass mortality events when temperatures surpass the regulatory capacity of fish. These fish mass mortality events threat not only aquatic ecosystems but also human activities, such as fisheries and aquaculture. Besides the environmental challenges of climate change, human food industries are facing an exponential growth in animal-based protein demand, such as fish. Aquaculture is one of the fastest growing sectors in the food production industry (FAO, 2020) and is predicted to continue to expand to meet the world's increasing demand for animal-based proteins. Therefore, fishery stocks and the aquaculture sector are caught in a crossfire by the threat of climate change and, on the other hand, by the increased demand of fish. For these reasons, urgent solutions are needed to face these challenges and ensure a sustainable future for ecosystems and human food supply.

Mass mortality events in fish, linked to high water temperatures, occur primarily because fish reach their physiological limits to tolerate environmental changes. At supra-optimal temperatures, fish show a collapse in heart rate, impairment in oxygen uptake, and cardiac arrhythmias, which might lead to the death of the fish if warming continues (Casselman et al., 2012; Eliason and Anttila, 2017; Ferreira et al., 2014; Gilbert and Farrell, 2021). The cardiac functions of fish are strongly related to their capacity to handle and acclimate to increasing temperatures (Anttila et al., 2013a; Farrell, 2009; Farrell et al., 2009a; Verhille et al., 2013) and hypoxic events (Anttila et al., 2013b; Joyce et al., 2016). Indeed, mass mortality events, concomitants to heatwaves, have been associated with compromised cardiac system and impaired robustness of farmed fish in response to stressors (Brijs et al., 2020; Castro et al., 2011, 2013a; Ekström et al., 2019; Eliason et al., 2013; Farrell, 2009; Gamperl and Farrell, 2004). Farmed fish are especially vulnerable to environmental stressors because, contrary to wild fish, they cannot migrate to habitats with optimal conditions. Farmed fishes live in floating sea cages or inland tanks, therefore their survival relies only on their capacity to adjust physiologically. Furthermore, previous studies have shown that selective breeding, which focuses on rapid growth and common rearing conditions in aquaculture facilities, impairs several physiological functions in fish. For example, hatchery fish have lower aerobic capacity (Zhang et al., 2016), lower swimming capacity, lower Ca^{2+} handling and oxidative capacity, lower mitochondrial density, and lower aerobic enzyme activities in their swimming muscles (Anttila and Mänttäri, 2009; Anttila et al., 2008b) and cardiac performance, as well as a higher incidence of cardiac deformities as compared to wild fish (Brijs et al., 2020; Brun et al., 2003; Frisk et al., 2020; Gamperl and Farrell, 2004; Poppe and Taksdal, 2000; Poppe et al., 2003).

In this context, aerobic exercise training has been suggested to be a useful tool to improve aquaculture production by improving fish welfare and mitigating the detrimental effects of intensive farming (McKenzie et al., 2021a; Palstra and Planas, 2013). Several studies have shown that aerobic exercise training can improve various physiological traits, such as growth rate, muscle contractility, oxidative capacity, composition of skeletal muscle (Anttila et al., 2006, 2008a, 2011), swimming capacity (Davison, 1989; Lu et al., 2020; Nadler et al., 2018), brain plasticity (Mes et al., 2020), immunity (Castro et al., 2013a), and cardiovascular function (Dindia et al., 2017; Farrell et al., 1990, 1991; Gallaugher et al., 2001). However, so far, the potential benefits of aerobic exercise training on tolerance against environmental stressors have been overlooked. Up to now only one study has been carried out to show the potential benefit of aerobic interval training on hypoxia tolerance in goldfish (Fu et al., 2011). Despite promising evidence of the benefits of aerobic exercise training on cardiac physiology (Castro et al., 2013b; Farrell et al., 1990, 1991), the increased robustness of the cardiac system against environmental stressors has not been tested yet, which is the purpose of my thesis.

The introduction of thesis is composed of five main sections. First, I provide an overview of the current knowledge of the 1) thermal tolerance of fish and its link with cardiac performance, and 2) how these can change with plasticity. Then, I examine the 3) principles of exercise physiology and 4) the current knowledge regarding exercise physiology in fish. Finally, I provide a brief introduction to the occurrence of transgenerational effects on offspring phenotypes.

1.2 From fish thermal tolerance to cardiac performance

Global warming exacerbates the severity and the frequency of heatwaves (Breitburg et al., 2018; Oliver et al., 2018; Russo et al., 2014) potentially surpassing fish regulatory capacity to cope with high temperatures. The disruption of fish regulatory capacity has cascading consequences from physiological and behavioural performance (Domenici et al., 2007, 2017; Farrell et al., 2009b; Killen et al., 2012, 2013; McBryan et al., 2013; Schulte, 2015) to population changes, such as altering migratory routes, geographical distribution and, in the worse cases, leading to local extinction (Cavole et al., 2016; Garrabou et al., 2009; Marras et al., 2015; Philippart et al., 2011; Wernberg et al., 2013). Environmental temperature is a major factor affecting the biology of ectothermic animals, such as fish, increasing the rate of biological functions at several levels of biological organisation (e.g. enzyme activity, metabolic rate, heart rate, growth, and swimming performance) (Little et al., 2020; Schulte, 2015). The response of biological functions to temperature can be described by thermal performance curves. Usually, when the temperature rises, the rate of a biological performance rise until reaching a peak at its thermal optimum. After this peak, the rate of biological performance plateaus and starts declining, often with a sharp drop. This trend can typically be described by a thermal performance curve (TPC) (Fig. 1), which defines the temperature range in which a biological function can be sustained (Schulte, 2011). The acute effects of temperature on the organism's performance can also be measured by the temperature point at which the animal loses the capacity to maintain its equilibrium and locomotion became disorganised and erratic. At this point the animal reach its thermal limit and it is considered ecologically death, since it lost the capacity to control its locomotion and avoid deathly situations, such as predator attacks (Desforges et al., 2023). The lower and upper thermal limits are determined by assessing the temperature at which the fish lose their capacity to maintain equilibrium during exposure to acute cooling (known as Critical thermal minimum – CT_{MIN}) or acute warming (known as Critical thermal maximum – CT_{MAX}) (Beitinger and Lutterschmidt, 2011; Lutterschmidt and Hutchison, 1997; McKenzie et al., 2021b). In this thesis, I am referring to these limit points to define the thermal tolerance windows. However, it should be noted that fish cannot ecologically withstand these critical temperatures for extended periods of time.

Although a considerable number of studies have been published on fish thermal tolerance, the mechanisms underlying thermal limits in fish remain unclear. There has been intense debate within the scientific community regarding the existence of a universal mechanism that underpins thermal limits in fishes. Several hypotheses have been proposed, each focusing on different levels of biological organisation, from the molecular to the organism level (see review from Ern et al., 2023). At **molecular level**, the main hypotheses aiming to explain thermal tolerance in fish are: 1) mismatch between the thermal sensitivity of interlinked biochemical reactions; 2) full or partial protein denaturation leading to loss of function, 3) disruption of cellular membranes integrity and loss of function. **At the cellular level**, the main hypotheses are as follows: 1) temperature-dependent deterioration of electrical excitability (TDEE) of cardiac and nervous tissues and 2) failure in mitochondrial function. At **the organismic level**, the main hypotheses are as follows: 1) oxygen- and capacity-limited thermal tolerance (OCLTT), 2) temperature-induced impairments in cardiac performance, and 3) temperature-induced brain damage (Ern et al., 2023). However, it must be kept in mind that these hypotheses are not mutually exclusive. Indeed, the thermal adjustments at lower level of biological organization are part of the response at the higher level. Furthermore, several factors at different biological levels may interact to contribute to thermal tolerance depending on species-specific demands.

Although this topic is still under debate, the focus of the research in thermal tolerance is pointing the failure of heart and/or the brain as possible key organs that fail at high temperature, principally OCLTT but also TDEE hypotheses. Briefly, the OCLTT hypothesis suggests that cardiorespiratory and blood-delivery systems are impaired at high temperatures with cascading hypoxaemia to the tissue. At high temperatures, the cardiorespiratory systems are unable to match the increased oxygen demand from the tissue due to impaired oxygen delivery performance (e.g. temperature-dependent impairment in cardiac electrical excitability, see the TDEE hypothesis). Complementary to OCLTT, Vornanen (2016, 2020) proposed the TDEE hypothesis, suggesting that a mismatch due to differences in thermal sensitivity between the inward Na^+ current (I_{Na}) and outwards K^+ current (I_{K1}) in electrically excitable cells could explain the upper thermal tolerance in fish. This mismatch between I_{Na} and I_{K1} results in temperature-dependent depression of electrical excitability, leading to decreased cardiac performance during warming water. At the organ level, both the OCLTT and TDEE hypotheses identify cardiac performance as a possible weak point that might explain fish thermal tolerance, using a top-down approach for the former and a bottom-up approach for the latter. In salmonid species, data from several studies suggest that the whole organism's thermal tolerance might be related to the tolerance of cardiac function, and therefore follow the predictions of the OCLTT and/or TDEE hypotheses (Eliason and Anttila, 2017; Vornanen, 2020).

1.2.1 Cardiac performance during acute warming

The cardiovascular system is one of the key physiological systems in vertebrates and essential to support vital functions. Its main function is to transport oxygen, carbon dioxide, nutrients and hormones throughout the whole organism. The cardiovascular system of fish consists of a single closed circulation, driven by one heart. Typically, the heart in fish is composed of five sequential chambers: sinus venosus, atrium, atrioventricular segment, ventricle, conus arteriosus or bulbus arteriosus. The atrium and ventricle constitute contractile parts of the heart. The heart of fish pumps the low-oxygenated blood from the tissues to the gills, where it is oxygenated. Oxygenated blood then flows further to the tissues from the gills. Therefore, cardiac performance needs to be modulated according to metabolic demands and organism activities, such as in response to an increase in metabolic rate due, for example, swimming activity, escape from predators, or environmental stressors.

Cardiac performance can be typically simplified as cardiac output (Q), which gives the volume of blood (ml) pumped by the heart in a given period of time (minute) and is normalized by the body mass of the organism. Cardiac output is product of the heart rate (*ƒ*H, heartbeat * minute) and the cardiac stroke volume ($V_{s,}$ volume of blood pumped from the ventricle in one beat):

$$\mathbf{Q = \mathit{f}H * V_s} \qquad \text{(Farrell and Smith, 2017)}$$

The cardiac output is intimately related to the aerobic metabolic rate (MO_2) as shown by Fick equation:

$$\mathbf{MO_2 = Q * (CaO_2 - CvO_2)}$$

where CaO_2 is arterial oxygen content and CvO_2 is venous oxygen content; and (CaO_2-CvO_2) is the tissue oxygen consumption (Farrell and Smith, 2017). Because CaO_2 depends on the amount of blood pumped to the gills, Q plays a key role in MO_2. Therefore, the Fick equation can be modified as:

$$\mathbf{MO_2 = \mathit{f}H * V_s * (CaO_2 - CvO_2).}$$

Because of this tight connection with metabolic rate, cardiac performance is highly influenced by environmental temperature, both at the functional and molecular level. In most fish species, acute exposure to warming temperatures increases heart rate up to a maximum, after which it reaches a plateau when the temperature increases further (Safi et al., 2019). In contrast, stroke volume is less sensitive to temperature changes (Ekström et al., 2017b; Farrell, 2009; Farrell et al., 2009a). Therefore, during acute warming, the cardiac output increases mainly because of the increase in *ƒ*H.

The maximum beating frequency that the heart can achieve at a given temperature is defined as the maximal heart rate (fH_{MAX}). In nature, fishes may reach fH_{MAX} in several energy-demanding situations, such as during maximal swimming performance to escape a predator or to migrate through fast-flowing rapids in rivers (Iversen et al., 2010; Steinhausen et al., 2008). By measuring fH_{MAX} during acute warming it is possible to characterize the thermal performance curve for fH_{MAX} and to determine threshold temperatures that generally correspond to the threshold temperature for aerobic scope (AS, difference between standard and maximum metabolic rate). For example, the Arrhenius breakpoint temperature (T_{ABP}), defined as the temperature at which $f\mathrm{H}_{\mathrm{MAX}}$ first slows down its incremental rate with temperature. In some fishes, $\mathrm{T}_{\mathrm{ABP}}$ appears to closely corresponds to the $\mathrm{T}_{\mathrm{OPT}}$ for AS (Fig. 1) (Casselman et al., 2012; Anttila et al., 2013a; Ferreira et al., 2014). The $\mathrm{T}_{\mathrm{ABP}}$ also marks the temperature above which the fish's cardiovascular system starts to show signs of reduced efficiency. Beyond this temperature, the thermal stress starts to affect the biochemical reactions involved in the fish's metabolism and muscle contraction, and their efficiency begins to decline. This leads to potential negative impacts on the fish's overall performance as the temperature keep rising, such as reduced oxygen transport and impaired cardiovascular function. However, the relationship between $\mathrm{T}_{\mathrm{ABP}}$ for $f\mathrm{H}_{\mathrm{MAX}}$ and $\mathrm{T}_{\mathrm{OPT}}$ for AS has been validated only in few model species and it remains unexplored in most fishes. In addition, sometimes the $\mathrm{T}_{\mathrm{ABP}}$ is not possible to calculate or is overestimated if insufficient measurements are made. The arrhythmia temperature ($\mathrm{T}_{\mathrm{ARR}}$) is also important measure of cardiac thermal limits as it is the temperature at which $f\mathrm{H}_{\mathrm{MAX}}$ starts to become arrhythmic, above which the heart function is compromised and cannot efficiently pump blood to the gills and deliver O_2 to the tissue, compromising the fish's overall performance. $\mathrm{T}_{\mathrm{ARR}}$ occurs usually before the $\mathrm{CT}_{\mathrm{MAX}}$ (Farrell, 2016), therefore it represents a proxy of upper thermal limit. In anesthetised fish with $f\mathrm{H}_{\mathrm{MAX}}$ induced pharmacologically, the capacity of the heart to keep pumping rhythmically may be related to the myocardial oxygen availability and to the anaerobic ATP production.

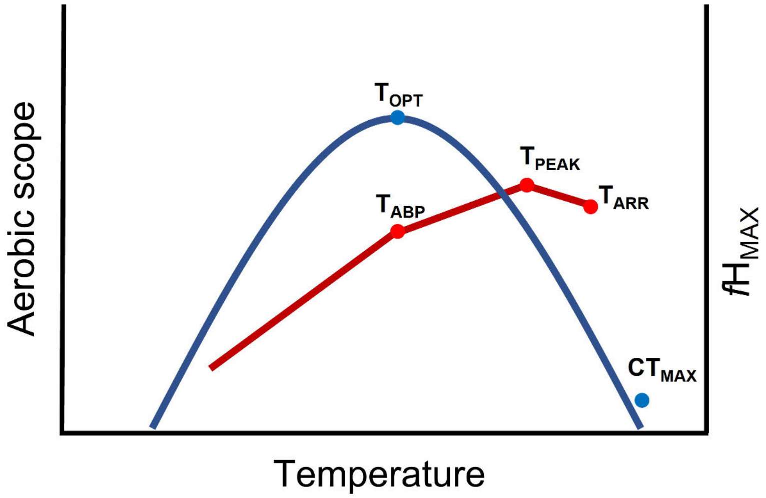

Figure 1. The relationship between the thermal performance curves for Aerobic Scope (AS; blue line) and Maximal Heart Rate ($f\mathrm{H}_{\mathrm{MAX}}$; red line). Figure inspired by studies of Ferreira et al. (2014) and Muñoz et al. (2014). The blue points represent the thermal optimum for the AS, and the Critical Thermal Maximum ($\mathrm{CT}_{\mathrm{MAX}}$). Red points represent the cardiac transition temperature for $f\mathrm{H}_{\mathrm{MAX}}$: The Arrhenius Break Point temperature ($\mathrm{T}_{\mathrm{ABP}}$), temperature at which $f\mathrm{H}_{\mathrm{MAX}}$ first slows down and proxy of the $\mathrm{T}_{\mathrm{OPT}}$ for AS; Temperature at which maximum heart rate ($f\mathrm{H}_{\mathrm{MAX}}$) reached its peak ($\mathrm{T}_{\mathrm{PEAK}}$); the Arrhythmia Temperature ($\mathrm{T}_{\mathrm{ARR}}$), temperature at which $f\mathrm{H}_{\mathrm{MAX}}$ starts to become arrhythmic.

1.2.2 Cardiac acclimation to high temperatures

When fish are exposed to high temperatures for a long period, they undergo physiological changes that lead to an acclimation (or acclimatisation) response to the new thermal environment. Acclimation is a phenotypic response to a consistent change in environmental condition that alters performance and possibly enhances fitness (Seebacher et al., 2014; Van Oppen et al., 2015). The acclimation response of fish to chronic high temperature exposure has been extensively studied, particularly regarding its effects on cardiac performance (Eliason and Anttila, 2017; Farrell and Smith, 2017; Gamperl and Farrell, 2004; Keen et al., 2017; Vornanen, 2016). As more than 30.000 fish species exist from the arctic depth to tropical reefs, species-specific differences in heart function and acclimation capacity are common across fishes, making it difficult to generalise. Nevertheless, the acclimation response in cardiac performance mostly affects cardiac output, through changes in intrinsic heart rate, and only partially through stroke volume (Vornanen, 2016). For example, in most fishes, the heart rate usually decreases after warm acclimation to compensate for the direct effect of temperature. On the other hand, cold acclimation increases the heart rate at a given temperature compared to warm acclimated fish.

The acclimation responses in cardiac performance are mainly due to compensatory changes at the molecular and cellular levels, with the aim of optimising the energy cost/benefit balance at the given temperature and compensating for the temperature-dependent increase in metabolism (Sokolova, 2023). Acclimation to high temperature usually leads to remodelling of the expression of membrane machineries involved with action potential conduction and cardiomyocyte contraction (Keen et al., 2017; Shiels, 2017). It is, therefore, possible that functional changes induced by acclimation may be linked to adjustments in the molecular mechanisms regulating the contractile machinery. The transient currents of calcium ions that occur in cardiomyocytes are fundamental to active the sarcomeres and to ensure the heart contraction. The transient currents of calcium ions from extracellular space and the intracellular storage (Sarcoplasmic reticulum) to cytoplasm are primarily handled by four key calcium cycling channels involved in cardiac excitation contraction coupling (EC-coupling). These proteins are: L-type calcium channel, ryanodine receptor (RyR), sarco(endo)plasmic reticulum Ca^{2+-}ATPase (SERCA), and Na^{+}/Ca^{2+} exchanger (NCX) (Gillis and Johnston, 2017; Hove-Madsen and Tort, 2001; Hove-Madsen et al., 2000; Shiels, 2011, 2017). Since the sarcomere and the associated Ca^{2+} cycling proteins are one of the most ATP-demanding molecular mechanisms in the cardiomyocytes (Schramm et al., 1994), it is therefore possible that acclimation response may remodel these mechanisms to preserve ATP homeostasis.

Furthermore, acclimation to high temperatures can lead to improvements in aerobic and anaerobic metabolism and an increase in the activities of metabolic enzymes for efficient ATP production in cardiac mitochondria (Gerber et al., 2020;

Pichaud et al., 2017, 2019, 2020; Voituron et al., 2022). Although not conclusive, some evidence suggests that mitochondrial ATP production may also play a prominent role in the mechanism underpinning cardiac failure at high temperatures (Ekström et al., 2017a; Pichaud et al., 2019). Therefore, improvements in cardiac aerobic and/or anaerobic metabolism may lead to enhanced cardiac performance (Ekström et al., 2017a) and possibly enhanced thermal tolerance (O'Brien et al., 2018; Pichaud et al., 2019). These physiological adjustments in response to warm acclimation contribute to optimize oxygen delivery, ATP production and as well energy utilization in key fundamental cellular process. It is therefore possible that these phenotypic adjustments in response to acclimation to one stressor may provide bidirectional cross-tolerance protection to fish experiencing other stressors that similarly alter the energy balance (Berry and López-Martínez, 2020; Del Rio et al., 2019; McBryan et al., 2013; Rodgers and Gomez Isaza, 2021). This phenomenon is known as cross-tolerance, where exposure to a mild stressor enhances the tolerance to a second stressor through a shared protective mechanism (Rodgers and Gomez Isaza, 2021). Cross-tolerance between high temperature and hypoxia acclimations has long been a question of great interest in the field of fish physiology. However, there is no clear consensus on this, with several studies presenting data that support this hypothesis (Anttila et al., 2013b; Burleson and Silva, 2011; Collins et al., 2021; McBryan et al., 2016), while several other studies failed to provide supporting evidence (Leeuwis et al., 2021; McDonnell et al., 2019; Motyka et al., 2017). Therefore, understanding the mechanism of acclimation is important to assess the potential implications for fish in the current global warming scenario.

Furthermore, the conflicting findings regarding cross-tolerance between high temperature and hypoxia acclimations highlight the need to delve deeper into the mechanisms of acclimation, especially considering the potential implications for fish in the current global warming scenario. In this context, this thesis will investigate the impact of hypoxia (Chapter I) and swimming exercise training (Chapters II, III, and IV) as alternative acclimation stimuli that could potentially enhance thermal tolerance.

1.3 Swimming exercise training

Swimming performance is a fundamental component of the ecology of fishes and is strongly related to their fitness. Predator-prey interaction, mating behaviour, migration and dispersal are, for example, directly related to their locomotor performance (Domenici and Kapoor, 2010). In particular, salmonid species exhibit impressive endurance swimming performance during spawning migration and dispersal behaviours, often reaching up to 90% of the maximal aerobic scope (Eliason et al., 2011; Hayashida et al., 2013).

Typically, fish swimming performance can be represented as a continuum in which the two extremes are burst swimming and sustained swimming, based on the duration, energy metabolism, and muscle fibres on which swimming relies on (Beamish, 1978; Hoar and Randall, 1979; Webb, 1998). Burst swimming primarily relies on white muscle fibres and is fuelled by anaerobic metabolism. In contrast, during sustained swimming fish mainly rely on oxidative red muscle fibres and it is fuelled primarily by aerobic metabolism. Therefore, it could be maintained for a long period. During sustained swimming, the skeletal muscles and cardiorespiratory systems become more active, requiring the organism to increase the aerobic metabolic production of ATP to sustain homeostasis both during and after the physical activity (Farrell, 2009; Farrell et al., 2009a; Gamperl and Farrell, 2004; Hoar and Randall, 1979; Killen et al., 2017). The response to physical exercise is consistent across vertebrates; for example, an acute response to prolonged physical exercise can induce a deficit of O_2 in the organism (hypoxaemia) and a reduction in ATP availability (Bo et al., 2021; Durand and Raberin, 2021).

When fish are reared in captivity, such as in aquaculture conditions, they are deprived from their natural swimming behaviour. Lack of physical activity is particularly evident in salmon and trout aquaculture, where fish are not capable of performing their natural migrations of hundreds to even thousands of kilometres (Hayashida et al., 2013) and are instead confined in sea cages or inland tanks. Current aquaculture conditions, together with domestication, often lead to impairments in physiological functions such as cardiac performance (Twardek et al., 2021), respiratory performance (Zhang et al., 2016), and swimming capacity (Bellinger et al., 2014; Claireaux et al., 2005; Reinbold et al., 2009; Wegner et al., 2018). Farmed fish have also more often cardiac deformities than wild ones (Brijs et al., 2020; Brun et al., 2003; Frisk et al., 2020; Poppe et al., 2003).

To find a solution to these problems, fish scientists have been discussing ideas to stimulate fish swimming behaviour by regulating water speed in farming facilities, and, therefore, applying exercise training programs to aquaculture. In this context, exercise training has been proposed as a useful tool to enhance several physiological functions and mitigate the negative effects of aquaculture rearing conditions (Anttila et al., 2011; Davison, 1997; McKenzie et al., 2021a; Palstra and Planas, 2013).

Swimming exercise training not only improves swimming performance (Gilbert et al., 2014; Lu et al., 2020; Pang et al., 2013) and swimming efficiency (Killen et al., 2016) but also enhances skeletal muscle contraction, Ca^{2+} handling, and oxidative capacity (Anttila et al., 2006; Anttila et al., 2010; Simmonds and Seebacher, 2017). Additionally, an extensive number of studies have reported that exercise training can increase growth rate, muscle anabolism, and enhance feed conversion (Davison, 1989, 1997; Khan et al., 2014; Palstra et al., 2010a, 2015; Perelló-Amorós et al., 2021), although there is no clear consensus of exercise-enhanced growth of the fish

(McKenzie et al., 2021a). Aerobic exercise training can also interact with different diet compositions and enhance nutrient use efficiency (Perelló-Amorós et al., 2021). Contrasting results are also reported regarding the effect of aerobic exercise training on the metabolic rate variables (SMR, MMR and AS) (Brown et al., 2011; Killen et al., 2016; Larsen et al., 2012; Yu et al., 2021, 2022). Similar to mammals, aerobic exercise training also appears to affect fish brain plasticity (Mes et al., 2020), and can induce behavioural changes, e.g., decreasing aggressiveness (Christiansen and Jobling, 1990) as by-product of promoted schooling behaviour. Furthermore, evidence suggests that exercise training can also be used to mitigate stress and promoting recovery (McKenzie et al., 2012; Rodnick and Planas, 2016), for example by reducing cortisol levels. This reduction in stress has also been suggested to improve the resistance of fish to disease (Castro et al., 2011).

Taken together, these studies suggest that exercise training could be beneficial to improve several physiological functions, and therefore improve aquaculture production. However, the effects of exercise training are inconsistent across species and sometimes even within species (McKenzie et al., 2021a). These variations may be due to two important concepts in exercise training physiology: the specificity and optimisation of training. Several factors play a role in determining the type of exercise training performed in fish, similar to that in humans. Different types of exercise training programs can be developed by varying the fundamental elements of exercise training, i.e. intensity, duration, frequency and periodization. The combination of these elements has to be specific according to the desired goal for improvement. For example, endurance training for an Olympic marathon is consistently different from sprint training for a 100-meter dash. In the same way in fish, different combinations of these parameters induce different outcomes and even when using similar combination of parameters, the outcomes can vary, e.g., depending on the natural swimming behaviour of fish species (Anttila et al., 2008a). Therefore, taking the basis of exercise physiology into account is important when planning an exercise training protocol. Thus, in the next paragraph, I review the basis of exercise training physiology, borrowing concepts from human sports science. After that I will review in more detail the effects of training programs on the cardiac physiology and the link with fish thermal tolerance, and whether training can lead to transgenerational benefits.

1.3.1 Basis of exercise physiology and sport science

Exercise refers to a single bout of physical activity that requires energy effort, such as an episodic running, swimming or a long walk in the nature. On the other hand, **exercise training** is defined as repetitive physical activities that are programmed, planned, and structured over a period of time to achieve a specific goal (Siscovick et al., 1985), for example, a planned training program over months to prepare for an

Olympic marathon. In this thesis, I use the term “**exercise training**” referring to planning and structuring a program where physical exercise sessions are repeated on daily basis and last for a long period (weeks). Regular physical activity over a given period of time results in several physiological adjustments that lead to an improved performance in that specific exercise activity (Farrell et al., 2011). Aerobic exercise training causes adjustments of the cardiorespiratory and neuromuscular systems that enhance oxygen uptake from the environment, the oxygen delivery to tissue and enable a tighter regulation and higher efficiency of energy metabolism and contractility of muscles (Jones and Carter, 2000). Each exercise training program is based on a combination of four variables 1) the duration of the training session (how long), 2) the intensity of the training (how hard), 3) the frequency of the training (how often) and consequential resting period. The product of duration and intensity defines the 4) volume of training (Hawley, 2008). The specific combination of these variables determines a specific training response. The specificity of the response is one of the pivotal principles of exercise physiology; the training response is tightly coupled to the volume and duration of the exercise program (Hawley, 2002; McCafferty and Horvath, 2013). Exercise training generates a specific disturbance in the physiological state of the organism that leads to appropriate adaptation (term often used in sports science) or adjustment (synonym term preferentially used in biology), which then leads to improved performance. This is the key concept in the hypothesis of general adaptation syndrome (GAS) (Cunanan et al., 2018; Selye, 1951), also called supercompensation in exercise physiology (Fig. 2). As the main features of muscle physiology and cardiorespiratory systems are common across vertebrate taxa, the basic principle of exercise physiology could potentially be applied to all vertebrates. In sport science, supercompensation is defined as an improvement in the performance elicited after a single session of exercise, and it is the result of the trade-off between exercise volume and recovery period which move the performance to higher level of homeostasis (Bompa and Haff, 2009). If the exercise training program (intensity*duration*frequency) is too mild for the aimed goal/adaptation it may result in no change in performance. On the other hand, if the exercise training is too intense, it may result in an analogous unchanged or even decreased performance as a result of over-training. To achieve consistent adaptation and improvement in performance, the frequency of the training and the resting period have to be considered and planned. This planning is called periodization of the training. The aim of periodization is to achieve a cumulative effect of training that is accomplished by the constant search for optimal homeostatic disturbance. Training adaptation occurs only if the organism is exposed to chain of training stimuli that are constantly challenging the physiological performance of the organism (Mujika and Padilla, 2000) (Fig. 3). However, there is a physiological threshold beyond that the manipulation of the training variables do not produce any further improvement in performance.

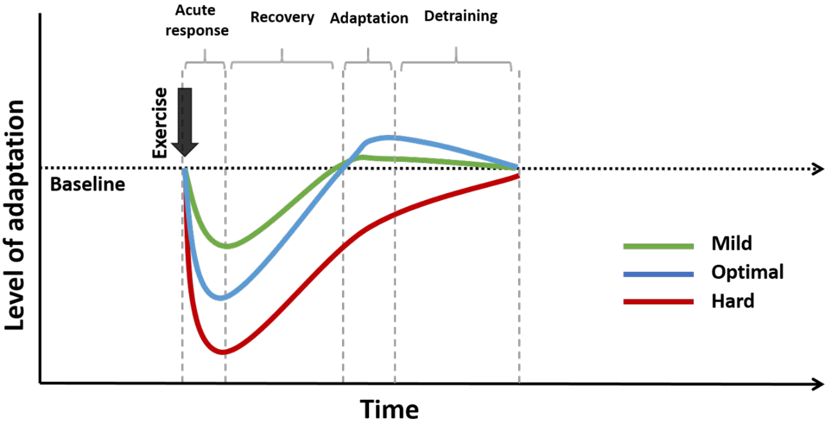

Figure 2. Relationship between exercise intensity and exercise training adaptation. The supercompensation begins with an initial decline in performance, followed by an increase after the recovery period. Different intensities of training lead to different training adaptation/response. Too mild or too hard intensities will not elicit adaptation response and the performance will stay at baseline levels or can even decline. Figure inspired by the review by Cunanan et al. (2018).

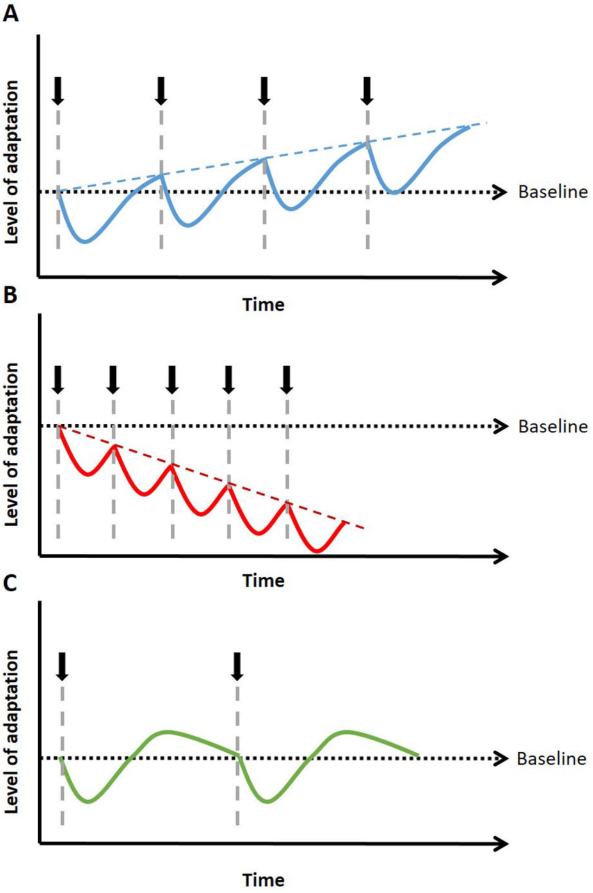

Figure 3. Relationship between recovery time and exercise adaptation. A) Optimal exercise activity results in continual improvements over time; B) exercise activity that is too frequent or too high in magnitude does not permit sufficient recovery, and consequently leading to maladaptation/ overtraining; C) inconstant or insufficient exercise activity results in stagnation or no improvement. Black arrows represent the exercise activity. Figure inspired by the review by Cunanan et al. (2018).

1.3.2 Cardiac acclimation to swimming exercise

The heart is a highly plastic organ that undergoes cellular and metabolic adjustments in response to an increased workload, such as exercise training (Gamperl and Farrell, 2004). Based on the literature on exercise physiology, aerobic exercise training is the most suitable type of training for enhancing cardiorespiratory performance. For example, in mammals, aerobic exercise training has been shown to have several positive effects on cardiac performance (Powers et al., 2014; Serrano-Ostáriz et al., 2011) by improving contractility and increasing the expression of calcium-cycling proteins (Wisløff et al., 2002).

Acute exercise leads to increased workload to cardiovascular system (Chatelier et al., 2005; Claireaux et al., 2005; Driedzic and Hochachka, 1978; Jones and Randall, 1978; Sandblom et al., 2005). For example, in sockeye salmon (*Oncorhynchus nerka*) swimming activity at 1.35 body length * second^{-1} (bl s^{-1}) for 30 min at 15°C increased the oxygen consumption, cardiac output, heart rate, stroke volume, blood cortisol level and decreased the CvO_2 (Steinhausen et al., 2008). Furthermore, the same study showed that resting heart rate increased linearly with temperature, without collapsing at high temperatures. On the other hand, heart rate during swimming activity increases linearly with increasing temperature but reaches a plateau and then collapses at high temperatures (Steinhausen et al., 2008).

Chronic response to exercise training results in various cardiovascular adjustments, e.g. it increases cardiac growth (Hochachka, 1961), maximum cardiac output, activity of cardiac enzymes, arterial O_2 content, haematocrit, skeletal muscle capillarity and tissue O_2 extraction (Castro et al., 2011; Chen et al., 2021; Davison, 1997; Farrell et al., 1990, 1991; Gallaugher et al., 2001; Palstra et al., 2014; Solstorm et al., 2015). Moreover, aerobic exercise leads to several changes at cellular and at molecular level (Chen et al., 2021; Takle and Castro, 2013). For example, aerobic exercise training program remodelled the cardiac proteome already during early phases of the training program (Dindia et al., 2017). Additionally, exercise training increases the expression of cardiac genes related to Ca^{2+}-handling proteins (Castro et al., 2013b; Rissoli et al., 2017); improves twitch tension, cardiac pumping capacity, and contraction rate (Rissoli et al., 2017); stimulates cardiomyocyte proliferation (Castro et al., 2013b; Rovira et al., 2018); and promotes the expression of angiogenesis markers (Castro et al., 2013b; Takle and Castro, 2013). Nevertheless, the cardiac adjustments induced by exercise are variable across types of training program and fish species (Davison, 1989; Gamperl and Farrell, 2004). Taken together, these studies provide evidence that exercise training can influence several aspects of cardiorespiratory performance.

1.3.3 Swimming exercise training and thermal tolerance

As said earlier, the capacity of fish to cope with warming water can be affected by their cardiorespiratory performance. Since thermal tolerance seems to be related to cardiorespiratory performance, any improvements induced by aerobic exercise training in cardiac performance at functional or molecular level might improve the thermal tolerance of fish (Gomez Isaza and Rodgers, 2022). In this context, critical swimming speed (U_{CRIT}) has been associated with both cardiorespiratory performance and thermal tolerance in Atlantic salmon (*Salmo salar*) populations screened by good and poor swimmers (Anttila et al., 2014).

Adjustments induced by exercise training could also be beneficial to cope with acute warming, such as increase in cardiac output, heart rate scope, increased activity of cardiac aerobic and anaerobic enzymes, mitochondrial efficiency, haematocrit levels, and the affinity and concentration of haemoglobin and myoglobin. Even though swimming exercise primarily results in an increase in stroke volume rather than heart rate, aerobic exercise training program may lead to a slight improvement in maximum heart rate capacity over the long term which could be beneficial in improving thermal tolerance. This is because, during acute warming, the increase in cardiac output is mainly supported by an increase in heart rate. The molecular and functional changes induced by training may allow fish to maintain oxygen delivery to tissues or to improve metabolic efficiency under energy-demanding situations, such as at elevated temperatures, potentially providing improved tolerance to acute warming. Furthermore, exercise training has been found to improve tolerance to hypoxia in goldfish (*Carassius auratus*) (Fu et al., 2011) and survival in zebrafish (*Danio rerio*) larvae exposed to extreme hypoxia (Bagatto et al., 2001). However, so far the potential benefits of aerobic exercise training on the capacity of fish to cope environmental stressors, such as heatwaves, have been largely overlooked.

1.4 Transgenerational plasticity

In the previous sections, I provided examples of phenotypic plasticity in response to temperature, hypoxia or exercise within one generation. However, phenotypic plasticity can also occur between generations as a response to parental experiences. The phenomenon is known as transgenerational plasticity (TPG) (Earhart et al., 2022; Shama et al., 2014). When fish of the parental generation experience a mild stressor, TGP can provide an adaptative advantage to the next generation (Costantini, 2022). However, TGP is not always adaptative; indeed, when parents are exposed to a severe stressful condition that overcomes their physiological limits, it can be disadvantageous to offspring (Donelson et al., 2018; Sopinka et al., 2016). When TGP is adaptive, it represents a big advantage to the population giving them capacity to cope with the anthropogenic environmental stressors and it can buy time for genetic evolution to occur (Donelson et al., 2018). While phenotypic plasticity allows individual organisms to adjust to immediate changes in their environment, TGP allows populations to adapt to longer-term changes and potentially pass these adaptations to future generations. Environmental conditions could induce changes in gene expression and epigenetic modifications, which can be transmitted to the next generation through epigenetic inheritance mechanisms but also via other non-genetic inheritance mechanisms, such as hormones, maternal investment, egg provisioning and changes in expression of miRNAs. Overall, both phenotypic plasticity and TGP play important roles in allowing organisms to adapt to changing environments. There are several examples how a change in the parental environmental condition can affect next generation. For example, when females of Atlantic salmon experienced warmer water temperature during the last two months of egg maturation, the mass of eggs produced by next generation was higher compared to those that experienced unheated river water (Jonsson and Jonsson, 2016). Parental temperature acclimation can affect also offspring growth rate (Salinas and Munch, 2012; Shama and Wegner, 2014; Shama et al., 2014) and the metabolic capacities of offspring heart mitochondria (Shama et al., 2014). However, the TGP effects on fish thermal tolerance are largely overlooked. Recent studies suggest that in salmonid species the TGP on thermal tolerance following parental thermal exposure is highly variable (Butzge et al., 2021; Penney et al., 2021, 2022). Moreover, exposing captive-reared Atlantic salmon to natural river environments, (e.g. variable water flow) during early life can enhance the survival of the next generation after release in the wild (Evans et al., 2014). It has been reported that environments with different water flows can have a transgenerational effect also in anemonefish (*Amphiprion chrysopterus*), as breeding pairs located at high water flow sites produced offspring with greater caudal fin shape, but lower growth rate and survival, than offspring from breeding pairs from low water flow sites (Cortese et al., 2022). Furthermore, studies in mammals show that exercise can, in some instances, have transgenerational effect on the next generation (McGreevy et al., 2019; Short et al., 2017; Yin et al., 2013). Hence, it could conceivably be hypothesised that maternal swimming exercise, induced by increasing water flow in the tank, might affect the phenotypes of offspring in fish, such as survival, growth, or thermal tolerance.

In addition to TPG, the offspring phenotype is also determined by genetic inheritance. Therefore, in order to cope with climate change, populations can rely not only on transgenerational plasticity but also on genetic inheritance of advantageous traits, such as higher thermal tolerance, that can help to adapt to the new thermal environment (Gienapp et al., 2008). Populations can evolve and adapt to the new thermal environment via genetic inheritance only if their phenotypic traits show a certain degree of variation in the population, possess heritability and are under a selective pressure selection (Charmantier et al., 2014; Falconer and Mackay, 1996). Therefore, assessing the magnitude of the heritability of a trait it is important to determine the evolutionary rate and direction of a population. For example, low level of heritability for a trait will reduce its potential of evolution (Charmantier and Garant, 2005). Heritability (h^2) is a measure of the proportion of variation in a trait that can be attributed to genetic differences between individuals within a population (Charmantier and Garant, 2005). The environmental conditions can also affect the genetic inheritance not only via natural selection, but also changing the heritability of a trait, this is called Genotype-by-Environment interactions (GxE) (Charmantier and Garant, 2005). For example, the set of genes that govern the occurrence of a specific trait may change in a different environment, leading to difference in heritability. For instance, unfavourable environment can directly shape evolution rate by decreasing the heritably of the traits (Charmantier and Garant, 2005; Hoffmann and Merilä, 1999). Phenotypic plasticity to acclimation to environmental temperature has also been shown to affect the heritability of related traits. For example, higher acclimation temperatures diminished the heritability of CT_{MAX} in Atlantic salmon (Debes et al., 2021). Similarly, different thermal environments can alter the heritability of body mass in brook char (Crespel et al., 2013). Therefore, it is also possible that environmental conditions experienced by the parental generation, such as maternal exercise training, could also affect the heritability of specific physiological traits by changing the set of genes regulating these traits. However, it is still not known whether parental environment, such as exercise training, can affect the heritability of traits in the next generation.

1.5 Aims

The main goal of this thesis was to investigate the phenotypic plasticity of cardiac performance and cardiac thermal tolerance in fish in response to exercise training. This doctoral thesis covered three main topics: cardiac phenotypic plasticity to high temperature and hypoxia, and harnessing phenotypic and transgenerational plasticity via exercise training.

1. Warming waters and hypoxia events are serious problems for both wild and farmed fish. The phenotypic plasticity in cardiac performance seems to be related to the capacity of fish to cope with these stressors. A specific example of phenotypic plasticity is cross-tolerance protection, defined as the ability of an organism to respond to a mild environmental stressor conferring a survival advantage in the face of different subsequent stressors. The functional and molecular changes underlying the cross-protection are not fully understood. Therefore, this thesis aims to:

    - Investigate the cross-protection mechanism in cardiac thermal performance after acclimation to high temperature and acclimation to hypoxia.

- Explore the existence of shared molecular mechanisms underpinning the changes in the cardiac cycle related to cross-protection of those stressors.

2. Phenotypic plasticity can be harnessed for aquaculture and conservation purposes. In this context, exercise training in aquaculture has been shown to improve the health and growth of farmed fish. It also improves different physiological traits of fish including swimming performance and cardiovascular performance. Cardiac performance appears to be related to thermal tolerance and robustness. However, very little is known about the cardiac phenotypic plasticity in fish following acclimation to aerobic exercise training and the possibility to improve cardiac thermal tolerance. Therefore, the aim of the present thesis was to:

   - Find the optimal exercise training program to improve the cardiac performance and the cardiac thermal tolerance in different farmed salmonids, both in juvenile and in adult stage.
   - Investigate the physiological mechanisms underlying the cross-tolerance protection between exercise training and thermal tolerance.

3. Adult fish in the reproductive phase are particularly sensitive to thermal stresses. This issue is particularly relevant for brood-stock fish kept in captivity for commercial aquaculture as well as for restorative aquaculture. Therefore, new practices are urgently needed to enhance their thermal tolerance. Optimal exercise training may be crucial in the management of brood stocks in captivity. It is known that parental experience can impact offspring physiology through transgenerational plasticity (TGP). However, little is known about the transgenerational effects of maternal exercise training on the next generation's physiology and survival. This thesis aims to:

   - Investigate the transgenerational impact of maternal exercise training on key physiological traits in hatchery-reared fish.
   - Evaluate the applicability of exercise training programs in real aquaculture settings and their implications for both commercial and restoration aquaculture.

The objectives of each chapter are explained in detailed here below:

In **chapter I**, the objective was to assess the extent of phenotypic plasticity in fish cardiac function. The focus was on studying physiological and molecular changes that occur during cardiac acclimation to high temperature and hypoxia, which contribute to cross-protection in thermal tolerance. Cardiac thermal tolerance and ECG traits were measured during acute warming, and then linked the molecular changes in calcium cycling proteins.

In **chapter II**, the aim was to investigate the effect of exercise training (1 bl s^{-1}) and functional feeds on cardiac performance in juvenile rainbow trout (*Oncorhynchus mykiss)*. Specifically in this thesis I will mainly focus on the effects of exercise training on the cardiac performance rather than the effect of functional feeds.

In **chapter III**, the goal was to optimize the training program by studying the effects of three different training intensities on cardiac thermal performance and associated molecular and haematological adjustments in juvenile rainbow trout. Control speed, medium-intensity training (2 bl s^{-1}), and high-intensity training (3 bl s^{-1}) were tested to determine the optimal intensity.

In **chapter IV**, the objectives were to assess the potential implications of exercise training for restorative aquaculture and conservation programs. I addressed this issue studying the applicability of the exercise training program in brown trout (*Salmo trutta*) and investigating the transgenerational effect of maternal exercise training on cardiac thermal performance, CT_{MAX}, life history traits, and heritability of these traits.

2 Materials and Methods

2.1 Animals

In this thesis I investigated the plasticity of cardiac thermal performance in three fish species:

2.1.1 European sea bass *(Dicentrarchus labrax)*

European sea bass is a temperate teleost from the family of Moronidae, widely spread in Eastern coast of Atlantic Ocean and in Mediterranean Sea. The European sea bass is an important species for commercial fisheries and for aquaculture, it is considered a delicacy in many countries. It has high economic value since it is one of the most cultured species in Europe as well as one of the most exploited fish species in fishery (Bjørndal and Guillen, 2018). European sea bass habits coastal areas, lagoons, and estuaries (Sánchez Vázquez et al., 2014). In its natural habitat, European sea bass are often exposed to heatwaves and low oxygen saturation. For these reasons, it represents a good animal to investigate the molecular and functional cardiovascular plasticity of fish to cope with high temperature and hypoxia.

2.1.2 Rainbow trout (*Oncorhynchus mykiss*)

Rainbow trout is a salmonid native in the North Pacific Ocean. It is a euryhaline species having migratory anadromous populations but also resident freshwater populations. Due to its migratory nature, rainbow trout are strong and active swimmers. The anadromous form has a complex life cycle, it spends most of its life at sea and migrates to the river to spawn. Usually rainbow trout reach sexual maturation after 2–5 years, when mature fish start to migrate to the river where they were born. Depending on the population, migration can occur during late summer (river maturing) or during winter (ocean maturing). Spawning period is in spring for both strains. After hatching, the juveniles live for 2–3 year in the rivers before starting their down-stream migration to the sea. Rainbow trout is widely adaptable fish species that can withstand broad range of temperatures (0–27°C). However, its thermal optimum window for cardiorespiratory functions, for growth and preferred temperature range from 16.5°C to 20.5°C (Anttila et al., 2013a). Nevertheless, recent data showed that some Californian population can have high aerobic scope until almost 25°C (Verhille et al., 2016). Due to its remarkable capacity to adapt to different environments, its high fecundity and ease for domestication, rainbow trout is now one of the most widely cultured salmonids in the world. For all these reasons, rainbow trout has rapidly become an ideal model species in fish biology, especially in the field of physiology and ecology.

2.1.3 Brown trout (*Salmo trutta*)

Brown trout is a salmonid species native in European waters. Due to its variety of subspecies, it is often considered as species complex. As with many other salmonid species, brown trout can have a resident freshwater form and an anadromous form that migrate to the sea at smolt stage. The freshwater form can spend the whole life cycle in rivers and lakes. The anadromous form has a life cycle that is similar to the Atlantic salmon and rainbow trout, although the spawning migrations have usually shorter duration and smaller spatial scale. Contrary to rainbow trout, the reproductive season of brown trout is from fall to early winter. Mature fish migrate from the sea to the rivers and look for a spawning ground rich in gravel sediment. They dig a nest in the gravel, called redd, the female deposits there the eggs and then the male fertilizes them. Then the adults return to the sea after covering the redd with more gravel. The alevin stay under the gravel until the yolk sac is absorbed. Then they become fry and begin their exogenous feeding phase, establishing their territory in the river. The juveniles (fry and parr stages) stay in the river for 2–3 years. Then at smolt stage, they start their migration to sea (anadromous form) or lake (potamodromous form) in which they reach sexual maturation after 2–3 years and they migrate back to the natal rivers for spawning. In conservation categories brown trout is classified as a species of Least Concern, although in several areas in Europe there are populations of endemic subspecies of *Salmo trutta* that are in danger of extinction due to habitat loss due to climate change, overfishing and introgressive hybridization with other subspecies artificially introduced. Brown trout has gained in popularity as a model organism in different fields of biology due to its ecological relevance as bioindicator and its complex lifestyle. It is studied in conservation biology, ecotoxicology but also in genetics and physiology due to its peculiar adaptability and genetic variety of subspecies (Dvorak et al., 2020; Frank et al., 2011; Jacobs et al., 2018).

2.2 Experimental design

2.2.1 Study I) Cardiac plasticity in response to high temperature and hypoxia

In the study I, to study the physiological and molecular changes in cardiac phenotype in response to high temperature and hypoxia, European sea bass (mean body length

18.3 cm, mean body weight 76.4 g) were acclimated for 6 weeks to three acclimation conditions (Fig. 4): 1) control condition: seasonal average temperature of 16°C with normoxia; 2) high temperature condition: temperature of 25°C with normoxia; 3) hypoxic condition: temperature of 16°C with moderate hypoxia of 50% air saturation.

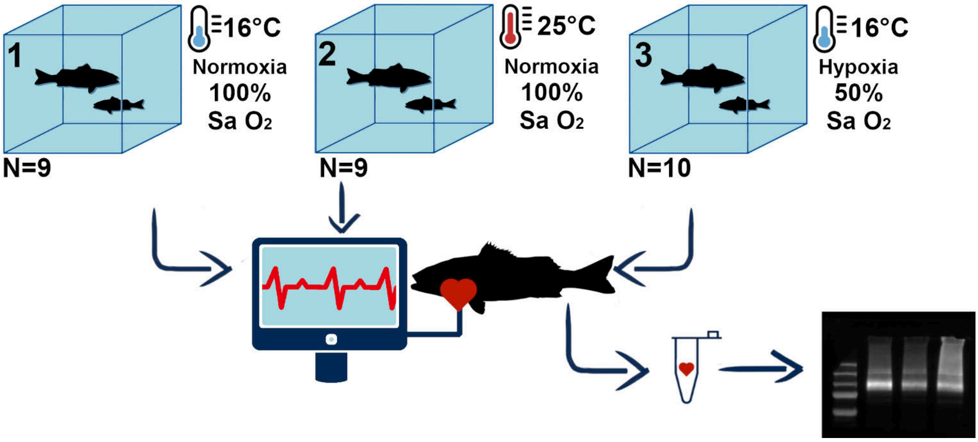

Figure 4. Experimental design study I: European sea bass were randomly assigned to three experimental conditions: 1) Control condition 16N (16°C and 98% sat. O2); 2) High temperature condition 25N (25°C 98% sat. O2) and 3) Hypoxia condition 16H (16°C; 50% sat. O2). The fH_{MAX} was measured after 6 weeks of acclimation. After the fH_{MAX} measurements, the ventricle from each fish was sampled for molecular analysis of NCX and SERCA protein levels.

2.2.2 Study II and study III) Cardiac responses to swimming exercise training

In the study II and III, I studied the effect of aerobic exercise training programs on the cardiac thermal performance of the juvenile rainbow trout. To do so trout were exposed to aerobic exercise training using different training intensities by regulating the waterflow in the tank (Fig. 5).

Specifically, in study II the rainbow trout (mean body length 17.2 cm, mean body mass 66.3 g) were randomly divided into one of two experimental groups: **1) Control group** (no training group), swimming constantly at an average water flow velocity of 0.3 bl s^{-1} (0.05 m s^{-1}). **2) Trained group**, exposed to average water flow velocity of 1.0 bl s^{-1} (0.17 m s^{-1}) for 6 h per day and at 0.3 bl s^{-1} for the rest of the day (18h). The exercise training was carried out for 6 h per day (8:00–14:00), 5 days per week for a total of 6 weeks. The control velocity for this, and all the training studies, was always the water flow velocity that was used in normal fish rearing for that age-group and species of the fish in fish hatchery.

Based on the results of study II, I planned a follow-up experiment to investigate if the cardiac thermal tolerance can be enhanced by higher intensity of the training.

Therefore in study III the rainbow trout (mean body length 10.6 cm, mean body mass 14.9 g) were randomly designated to one of three experimental group: **1) Control group**, exposed to constant water flow velocity of 0.9 bl s^{-1} (0.06 m s^{-1}); 2) **Medium intensity** of training with a water flow velocity of 1.7 bl s^{-1} (0.11 m s^{-1}) and 3) **High intensity** of training with a water flow velocity of 2.9 b1 s^{-1} (0.17 m s^{-1}). The training program of study III consisted of a training session of 6 h per day, 5 days per week for a total of 5 weeks. During the resting period (18 h per day) the water flow velocity was reduced to the same speed as control group. The fish were fed on daily basis 1 h after the exercise training session.

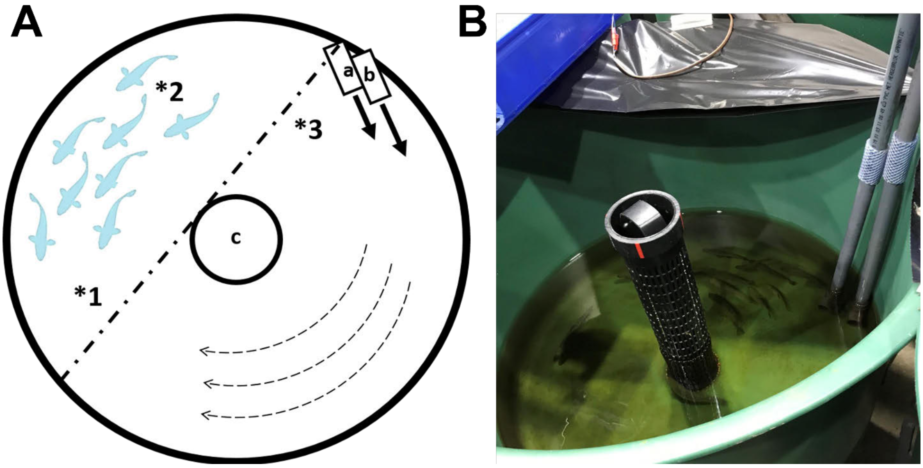

Figure 5. Top view of the tanks Chapter II and III. A) Dashed line represents the shelter area covered by a black plastic blind so that the fish preferentially swim in that area. Asterisks * represent the three points where the speed of water were measured. Letter a correspond to the inflow that provided the control/resting water flow (1 bl s^{-1}), letter b represents the inflow for the training water flow. Letter c represent the central column with the outflow. B) Picture of a fish tank.

2.2.3 Study IV) Cardiac response to swimming exercise training and maternal effects of training

In the study IV, I investigated how maternal exercise training can affect the cardiac thermal tolerance and life history traits in the offspring of brown trout via transgenerational plasticity. This experiment was divided into four phases (Fig. 6): Phase 1) the assessment of the effect of exercise training on adult phenotype. Phase 2) the assessment of the effect of training on the reproductive success. Phase 3) the assessment of the transgenerational effect of maternal exercise training on the cardiac phenotype, thermal tolerance, survival, and heritability. Phase 4) Long term effect of maternal exercise on morphology of the offspring.

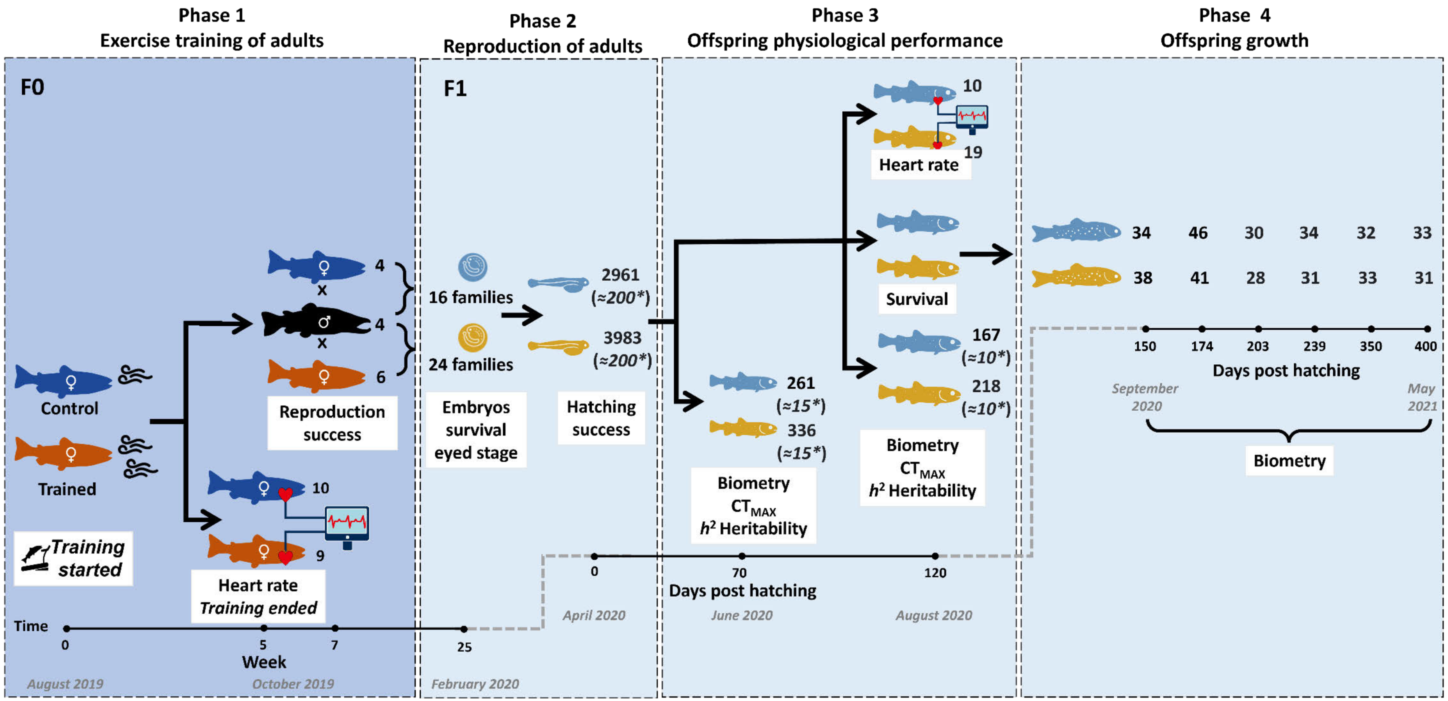

Figure 6. **Experimental design of study IV**: The experiment was divided into four phases. **Phase 1**: Training the adult brown trout and evaluating the training effect on the TPC for the maximal heart rate (F0: dark blue = control; dark orange = training). **Phase 2**: the effect of training on the reproductive success of the adults. **Phase 3**: the effect of maternal exercise training on the survival, CT$_{MAX}$ and the thermal performance curve for the maximal heart rate of the offspring, and the heritability of the offspring body mass, fork length, and CT$_{MAX}$ (F1: light blue = control mother; light orange = trained mother). **Phase 4**: the long-term effect of maternal exercise training on the biometry from 150 until 400 dph. The sample sizes of each group are indicated for each measurement. Italic numbers represent the sample size per each family. In Phase 1, the statistical unit is the mother. In Phase 2, the statistical unit is the family. In Phase 3, the statical units are the offspring individuals. In Phase 4, the statical units are the offspring individuals without family information.

2.2.3.1 *Adult rearing conditions*

The adult brown trout (mean body length 56.5 cm, mean body mass 2666.2 g), which were part of a population of brood stock stocked for conservation purposes, were randomly assigned to one of two experimental group: **1) Control group** (no training group), swimming constantly at an average water flow velocity of **0.2 bl s^{-1}** (0.12 m s^{-1}). **2) Trained group**, exposed to average water flow velocity of **0.7 bl s^{-1}** (0.41 m s^{-1}) for 6 h per day and at 0.2 bl s^{-1} for the rest of the day (18h). The training program of study IV were conducted in 5 days per week for a total of 5 weeks. In study IV, the water velocity of the training program was chosen as the maximum possible speed achieved by input of the rearing tank in the aquaculture facility. The training occurred between September – October to simulate the spawning migration timing of brown trout. The heart rate was measured directly after the training program ended. After the heart rate measurements, the brood-stock fish were allowed to rest for two weeks. Then mature eggs and milt were collected for artificial reproduction from anesthetized fish. Families were produced by a full factorial mating design crossing four untrained mothers with four trained fathers and six trained mothers with the same four trained fathers (14 individuals in total). By doing so, forty families were obtained (16 untrained families and 24 trained families).

After the artificial reproduction, the adult fish were euthanised and morphological and reproductive investment variables were measured. These included body mass, fork length, condition factor [(K)=(body mass/fork length)3*100)], ventricle mass, relative ventricle mass [(RVM)=(Ventricle mass/body mass)*100], liver mass, hepatosomatic index [(HSI) =(Liver mass/body mass)*100], clutch mass (defined as the g of ovulated eggs that was possible to strip out manually), relative fecundity [(clutch mass/body mass)*100] and egg diameter. Additionally, to estimate the effect of exercise training on the myocardial oxygen supply, the dry mass of the spongy and of the compact myocardium, spongy/compact myocardium ratio, and the compact myocardium percentage ((dry mass of compact myocardium/dry mass of ventricle) *100) was measured by dissecting the ventricles along the sagittal plane and fixing in 70% ethanol. Then the compact and spongy myocardium were divided, and the dry masses were measured (Farrell et al., 2007b).

2.2.3.2 *Offspring rearing conditions*

To avoid bias of developmental plasticity, all fertilized eggs and offspring were reared under the same conditions: i.e. temperature, oxygen saturation and photoperiod followed seasonal fluctuations. The unfertilized eggs were carefully removed, and the fertilized eggs were incubated in duplicate racks, with each family was placed in its own slots in both racks. The dead eggs were counted and removed daily. At 124 days post fertilization, the survival to eyed stage was assessed for each family ((number of eyed eggs/total number of fertilized eggs)*100). After the survival to eyed stage assessment, a subsample of 200 eyed eggs were randomly selected from each family and each family was placed into a rearing tank.

All the families hatched at around 180 days post fertilization (dpf), and hatching success was assessed for each family as ((number of hatched eggs/number of eggs moved to rearing tanks)*100). Families were kept separated in the rearing tanks until 120 days post hatching, at this stage the survival was calculated for each family as: ((number of fish survived at 120 days post hatching/total number of hatched fish) * 100). At this moment, all the families from the trained and all the families untrained mothers were grouped together and transferred into bigger tanks (2.06 m, one tank for each training group). Growth performance was measured until 400 days post hatching (dph).

2.3 Maximum heart rate ($f\text{H}_{\text{MAX}}$) measurement

Maximum heart rate ($f\text{H}_{\text{MAX}}$) is defined here as the absolute maximal heartbeat frequency recorded at a given temperature during an acute warming. To achieve the $f\text{H}_{\text{MAX}}$, the fish was pharmacologically treated with atropine to prevent vagal cardiac inhibition and with isoproterenol to provide maximum beta-adrenergic cardiac stimulation, according to Casselman et al. (2012).

The advantages of the $f\text{H}_{\text{MAX}}$ method are: 1) $f\text{H}_{\text{MAX}}$ TPC can be performed easily and faster compared to the TPC based on AS, 2) the pharmacologically stimulation of the $f\text{H}_{\text{MAX}}$ prevents the confounding factors of behaviour and autonomic control on the $f\text{H}_{\text{MAX}}$, 3) the pharmacological stimulation of the $f\text{H}_{\text{max}}$ provides the maximum limit of heart rate beyond which the heart is not capable to pump efficiently the blood to the gills, 4) provides ecologically relevant threshold temperatures (T_{ABP}, T_{PEAK}, T_{ARR}) and $f\text{H}_{\text{MAX}}$ values, that are similar to the $f\text{H}$ values of un-anesthetised fish that were chased to exhaustion during acute warming, 5) T_{ARR} can be used to estimate the thermal limit of the fish as a sub-lethal endpoint rather than the more extreme Critical thermal maximum (CT_{MAX}) (Adams et al., 2022; Casselman et al., 2012; Ekström et al., 2016; Eliason and Anttila, 2017; Eliason et al., 2013; Farrell, 2016; Gilbert et al., 2022; Mauduit et al., 2016; Penney et al., 2014).

The limitations of the protocol are: 1) Inaccurate measurement of resting heart rate, leading to unreliable heart rate performance calculations. Endogenous stimulation of catecholamines during fish capture and placement in measuring setup causes elevated heart rate, even after a resting period. 2) In natural setting, fish may not be able to maintain $f\text{H}_{\text{MAX}}$ due to accumulation of plasma lactate and potassium. Pharmacological method only provides physiologically maximal capacity, and not how long the $f\text{H}_{\text{MAX}}$ can be maintained in natural condition.

Fish were initially anesthetised with first dose of 100 ppm tricaine (MS-222), to measure body mass and fork length. Then, each fish was immersed in the heart rate chambers with a low dose of MS-222 (60ppm). The anaesthesia during measurements was needed to stop skeletal muscle movements in order to prevent electrical artefacts in the ECG signal.

To avoid hypoxemia, the gills were continuosly irrigated with 100% air saturated water via customized mouthpieces. Two electrodes were placed in proximity of the heart to detect the ECG signal. After the resting period, the maximal heart rate was induced pharmacologically with two sequential injections (see more details in Table 1). The first injection of atropine blocked the vagal afference that down regulates the *f*H. After fifteen minutes from the atropine injection, the injection of isoproterenol was given to maximally stimulate beta-adrenergic receptors.

Fifteen minutes after the isoproterenol injection, the water temperature was increased by 1°C stepwise increments every 6 min (for adults the acute warming was 1°C/15 min). The acute warming ramp started at the rearing temperature until the onset of cardiac arrhythmia. Cardiac arrhythmia was defined as clear and abrupt skipping of the ventricle contraction (missing QRS complex) (Casselman et al., 2012; Pettinau et al., 2022) (Fig. 7). The fish was considered to have reached the experimental end point and therefore removed from the experimental setup after cardiac arrhythmia was first observed. The temperature at this point was recorded as the arrhythmia temperature (T_{ARR}). T_{ARR} was considered as the upper critical thermal limit for cardiac function, due to intrinsic impairments of the heart contraction or action potential conduction, likely caused by thermal sensitivity of the ion-channels involved with cardiac contraction (Vornanen, 2016, 2020).

Figure 7. A representation of continuous recording of electrocardiogram (ECG) of European sea bass showing normal maximal heart rate (*f*H$_{MAX}$) in the left side and, immediately upon in the right side, the abrupt onset of cardiac arrhythmias (arrow) at critical high temperature during acute warming.

After the data collection, the *f*H$_{MAX}$ at each temperature was calculated from the ECG recordings using 15 continuous heartbeats, sampled at the end of each warming step (Casselman et al., 2012). In study I, beside the *f*H$_{MAX}$, also the electrical intervals associated with a cardiac cycle (i.e., PQ, PR, RR, QT, PQ, and QRS) were calculated by processing the ECG signals using ‘ECG Averaging View’ software in the PowerLab library.

For each fish, the thermal performance curve (TPC) for *f*H$_{MAX}$ was also drawn by plotting the natural logarithm of *f*H$_{MAX}$ against each measuring temperature.

Based on the TPC for $f\mathrm{H}_{\mathrm{MAX}}$, the thermal transition temperatures were calculated such as $\mathrm{T}_{\mathrm{ABP}}$, i.e. proxy for the thermal optimum for aerobic scope ($\mathrm{T}_{\mathrm{OPT}}$) of an organism (Casselman et al., 2012; Ferreira et al., 2014). This was done according to Yeager and Ultsch (1989). From the TPC also the highest $f\mathrm{H}_{\mathrm{MAX}}$ (peak $f\mathrm{H}_{\mathrm{MAX}}$) and the temperature where it was achieved ($\mathrm{T}_{\mathrm{PEAK}}$) were obtain.

Table 1. Differences in the maximal heart rate stimulation across the four studies. The correct doses were determined as instructed in Casselman et al. (2012).

	STUDY	SPECIES	BODY MASS (g)	INJECTION TYPE	CONCENTRATION
Atropine	I	*Dicentarchus labrax*	76.4	intraperitoneal	3 mg/kg
Isoproterenol	I	*Dicentarchus labrax*	76.4	intraperitoneal	3.2 µg/kg
Atropine	II	*Oncorhynchus mykiss*	105.5	intraperitoneal	2.5 µg/g
Isoproterenol	II	*Oncorhynchus mykiss*	105.5	intraperitoneal	8 ng/g
Atropine	III	*Oncorhynchus mykiss*	14.9	intraperitoneal	2.5 µg/g
Isoproterenol	III	*Oncorhynchus mykiss*	14.9	intraperitoneal	8 ng/g
Atropine	IV	*Salmo trutta*	2666.2	intravenous	1.25 µg/g
Isoproterenol	IV	*Salmo trutta*	2666.2	intravenous	4 ng/g
Atropine	IV	*Salmo trutta*	3.1	intraperitoneal	2.5 µg/g
Isoproterenol	IV	*Salmo trutta*	3.1	intraperitoneal	8 ng/g

2.4 $\mathrm{CT}_{\mathrm{MAX}}$ of offspring

In study IV, the Critical thermal maximum ($\mathrm{CT}_{\mathrm{MAX}}$) of the offspring was measured at two time points from each family, in June (70 dph) and in August (120 dph). The $\mathrm{CT}_{\mathrm{MAX}}$ was measured in a thermal-insulated tank (78 x 56 x 43 cm, 60-liter) (Fig 8). The three metal coils connected to a chiller-heater (RE 1050 GN, Lauda) were placed at the bottom of the tank. To maintain homogenous water conditions of temperature and oxygen saturation, the water was mixed with air-pumps and water-pumps, carefully avoiding any direct water flow to the fish. During the measurement, five fish from each family were kept separated in mesh boxes to keep track of the family information (11 x 6 x 9 cm for 1st $\mathrm{CT}_{\mathrm{MAX}}$, and 18.5 x 11.5 x 10.5 cm for 2nd $\mathrm{CT}_{\mathrm{MAX}}$ measurement). The boxes were suspended in a frame with their edges above the water surface but mostly below it. The setup was able to carry eight boxes, therefore 40 fish were tested simultaneously. We ensured the repeatability of the CT_{MAX} protocol by programming the automatic heating ramp in the chiller/heater and by recording the water temperature with Fibox 3 Set (PreSens Precission Sensing GmbH, Regensburg, Germany). The fish were acclimated in the boxes for one hour at 16±1°C. Thereafter, the water temperature was increased 0.3°C min^{-1} for the first 30 minutes and then the speed of temperature increase was decreased to 0.1°C min^{-1} (Sidhu et al., 2014), to permit a precise evaluation of the temperature for the loss of equilibrium (LOE), corresponding to the thermal limit (CT_{MAX}). The LOE was defined as the temperature where the fish displayed a loss of equilibrium and inability for locomotor activity for over 3 seconds (Morgan et al., 2018). When the LOE was observed, the temperature was recorded, and the fish was removed from the setup and placed in an individual recovery box. Then, the fish were euthanized with overdose of MS-222 (200ppm) and the body mass and length were measured.

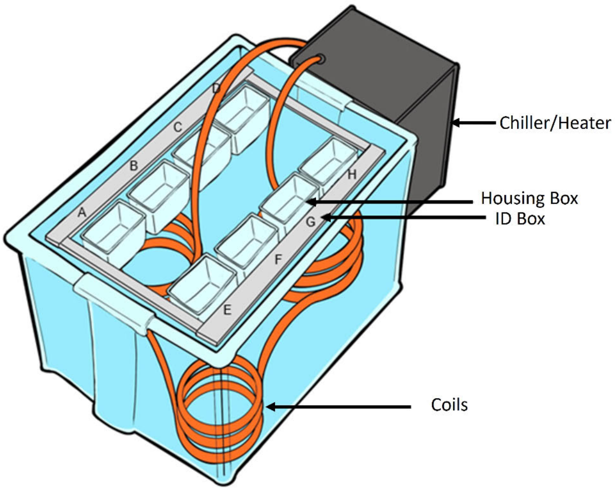

Figure 8. CT_{MAX} experimental setup consisted in a thermal-insulated tank equipped with three metal coils connected to a programmable chiller-heater. Eight mesh boxes were hanged in the water surface. Five fish per family were placed in each box to keep track of the family origin. The figure used here is based on a figure from Saarinen's Master thesis (Saarinen, 2022).

2.5 Molecular and haematological analysis

In studies I and III, the cardiac acclimation to high temperature and hypoxia as well as the acclimation to exercise training were also investigated at molecular level, by quantifying the level of key proteins involved in cardiac E-C coupling and oxygen delivery to tissues, but also measuring the activity of key aerobic and anaerobic enzymes. Fish intended for molecular analysis were euthanized using an overdose of MS-222 (200ppm). The ventricle was then quickly sampled, weighed, frozen in liquid nitrogen and stored at -80°C until molecular analysis.

2.5.1 Western blotting

In studies I and III, the proteins in the ventricles were quantified using Western blot analysis. The ventricles were homogenised in six volumes of ice-cold homogenization buffer using Tissuelyser and were then centrifuged at +4°C and 5000g. The supernatants were denatured in 1:1 v/v of Laemmli buffer. The denatured samples were pipetted into TGX Stain-Free gels (Bio-Rad, Hercules, CA, USA) and the proteins were separated by molecular weight using gel electrophoresis (see more details in Table 2). Pictures of the gels were taken with ChemicDoc MP Imaging system (Bio-Rad) to calculate the relative amount of total protein per sample.

The proteins were then transferred from the gel onto a Whatman nitrocellulose membrane via Western blotting at 4°C. To block the non-specific antibody binding to membranes, the membranes were soaked in 5% skim milk + Tris-buffered-saline solution (TBS). Membranes were incubated overnight in primary antibody solutions (in TBS + 0.1% Tween +5% skim milk) at 4°C (Table 2). After the overnight incubation in the primary antibody, the membranes were incubated in a solution TBS-0.1% Tween-5% milk with secondary antibody (Table 2).

Pictures of the membranes were acquired again as mentioned above for the gels, and the intensities of the detected bands were measured (Image Lab 6, Bio-Rad). The intensities of the bands were related to the total protein intensities of the bands in each sample giving the relative protein level. A control sample (mix of samples of three individuals) was pipetted in each gel to control and to normalize the gel-to-gel variation.

Table 2. Details of western blot analyses in studies I and III.

Tissue sampled	Study	Protein concentration loaded to each well	Target protein	Primary antibody and its dilution	Secondary antibody and its dilution
ventricle	I	30µg protein	SERCA	Anti-SERCA2 ATPase (1:2000, ab 91032; Abcam, Cambridge, UK)	Goat anti-rabbit IgG StartBright Blue 700 (1:5000; Bio-Rad, Hercules, CA, USA)
ventricle	I	30µg protein	NCX	Anti-NCX1 (1:1000; ab2869, Abcam)	Goat anti-mouse IRDye 800cw (1:5000; Lot No. C80306–03, Licor, Lincoln, NE, USA)
ventricle	III	30µg protein	SERCA	SERCA (1:2000; ab 91032, Abcam)	Goat anti-rabbit IgG StartBright Blue 700 (1:5000; Bio-Rad)
ventricle	III	30µg protein	HIF	HIF (1:2000; ab2185, Abcam)	Goat anti-rabbit IgG StarBright Blue 800 (1:5000, Bio-Rad)
ventricle	III	30µg protein	VEGF	VEGF (1:2000; ab209835, Abcam)	Goat anti-rabbit IgG StarBright Blue 800 (1:5000, Bio-Rad)

2.5.2 Enzyme assays

In study III, the activities of key aerobic (citrate synthase, CS, EC 2.3.3.1) and anaerobic (lactate dehydrogenase, LDH, EC 1.1.1.27) enzymes of energy metabolism were also measured. The ventricle was homogenised with a TissueLyser. Then, the homogenate was diluted for CS and LDH samples with a 50mM Tris solution (pH 8.0 and 7.4 for CS and LDH, respectively). The CS and LDH measurements followed the method described by Dalziel et al. (2012). The activity of both enzymes was measured at room temperature with an EnSpire 2300 Multilabel Reader spectrophotometer (Perkin Elmer, Turku, Finland). The protein concentration was spectrophotometrically measured with a BCA protein assay kit (ThermoFisher, Waltham, MA, USA).

2.5.3 Haematocrit and haemoglobin concentration

In studies I, II and III, haematological parameters were measured to investigate the oxygen carrying capacity to the tissues. To quantify the haematological traits, the fish was anesthetized (100ppm MS 222) and the blood sample was immediately taken from caudal vein into heparinized syringe. A part of the blood was collected in haematocrit capillaries and the haematocrit value was read after centrifugation with the capillary centrifuge for 10 min. Additionally, 10 µl of blood was diluted with 1 ml of Drabkin's solution for haemoglobin concentration analyses (0.6 mM $K_3[Fe(CN)_6]$, 0.77 mM KCN, 1.2 mM KH_2PO_4). The haemoglobin concentration was measured using a EnSpire 2300 Multilabel Reader (Perkin Elmer) according to Clark et al., 2008.

2.6 Statistical analysis

Throughout the whole thesis, the values are given as means and s.e.m and $p < 0.05$ was chose to indicate statistical significance. Data management and statistical analysis were performed using SigmaPlot 14 (for calculation the T_{ABP} for the chapters I, II, III and IV) (SyStat Software, San Jose, CA, USA); SPSS ver. 26 (Studies I and III) and SPSS ver. 27 (Chapter IV) (IBM Corp. Released 2019, IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY, USA) and RStudio version 3.6.1 (Study IV) (R Core development team 2022).

In **Study I**, two-way repeated measure ANOVA was used to test the effects of acclimation to high temperature and to hypoxia on the fH_{MAX} and ECG traits, with experimental conditions and temperatures during acute warming as main factors. One way ANOVA was used to test the effect of acclimation to high temperature and to hypoxia on T_{PEAK}, T_{ARR} and also on the protein level of SERCA and NCX. Spearman correlation was used to assess the relationship between the protein level of either SERCA or NCX on the fH_{MAX} and ECG traits. Multiple linear regression was used to evaluate the contribution of SERCA and NCX on the T_{ARR}.

In **Study II**, a two-way ANOVA was used to test the effect of exercise training and diet on the Hb, Hct, RVM, HIS, fH_{PEAK}, T_{ABP}, T_{PEAK} and T_{ARR}. The effect of exercise training and diet on fH_{MAX} was tested using a three-way ANOVA with training, diet and temperatures during acute warming as main factors.

In **Study III**, a linear mixed model with repeated measures was used to test the effect of training on the fH_{MAX} , with training and temperatures during acute warming as main factors, and day of the measurement as random factor. A linear mixed model, with exercise training as fixed effect and day of sampling as random factor, tested the effects of exercise training on the cardiac thermal performance (T_{ARR}, T_{ABP}, T_{PEAK}, TS, fH_{PEAK}) and molecular variables (enzyme activity of CS and LDH, HTC and Hb, proteins levels of SERCA, HIF and VEGF).

In **Study IV**, the effect of exercise training on fH_{MAX} of the adults was tested using a linear mixed model with repeated measures, with training and temperatures during acute warming as main factors, while day of the measurement and chamber of heart rate setup were taken as random factors. The transgenerational effect of exercise training on fH_{MAX} of the offspring was tested using a linear mixed model with repeated measures, with training and temperatures during acute warming as main factors, while mother ID, day of the measurement and chamber of heart rate setup served as random factors. The effects of exercise training on the cardiac transition temperatures for cardiac performance (T_{ABP}, $T_{PEAK,}$ T_{ARR}) were tested using a linear model in the adults (direct effect) and also in the offspring (transgenerational effect). To test the influence of exercise training the on morphological variables of the adults (body mass, fork length, condition factor, ventricle mass, RVM, compact myocardium dry mass, spongy myocardium dry mass, spongy/compact myocardium ratio, compact myocardium percentage, liver mass, and HSI) as well as on the reproductive variables (clutch mass, relative fecundity and egg diameter) were tested using independent-samples T-test with training group as factor. The transgenerational effect of maternal exercise training on the survival to eyed stage, hatching success and parr stage survival (120 days after hatching) was tested using Mann–Whitney U test. A linear mixed model was used to the transgenerational effect of maternal exercise on the $CT_{MAX,}$ body mass, body length and condition factor of the offspring. Maternal training and time of the CT_{MAX} measurement (June – 70 dph and August – 120 dph) were used as main factor, while ID of CT_{MAX} box, day of the measurement, and mother and father IDs as random factors. The factorial breeding design allowed to assess the narrow sense of heritability (h^2) of CT_{MAX}, body mass, body length in the offspring, using an animal model (Lynch & Walsh, 1998) with the ASReml software (Version 4.2; www.vsni.co.uk; Gilmour et al., 2015). For each trait, the narrow-sense heritability was then estimated as the proportion of the additive genetic variance (VA) to the total phenotypic variance (VP): h^2 = VA/VP. The transgenerational effect of maternal training on the long-term changes in morphology (body mass, body length and condition factor) was tested using linear models with maternal exercise and time of the measurements as fixed factors. Detailed information about the statistical analysis performed in each chapter are available in the attached publications.

3 Main Results & Discussion

The aim of this thesis was to investigate the phenotypic plasticity of cardiac thermal performance in fish following acclimation to energy demanding/limiting stimuli. In **Study I**, I studied this issue by acclimating European sea bass to high temperature and hypoxia. By comparing the two acclimation responses, I found that European sea bass were able to acclimate to the two stressors. However, the stressors do not possess shared cardiac mechanisms at functional or molecular level that could support cross-tolerance protection between high temperature and hypoxia.

In **Studies II, III** and **IV**, I studied the phenotypic plasticity of cardiac thermal performance following acclimation to aerobic exercise training. I found that the acclimation response to aerobic exercise training was species-specific and it was highly dependent on the intensity of the training.

In these studies, optimal aerobic exercise training, on the other hand, provided evidence of cross-tolerance protection by increasing the cardiac thermal limits, measured as arrhythmia temperature (**Studies III** and **IV**).

Moreover, in **Study IV**, I investigated the occurrence of transgenerational plasticity in response to maternal exercise training. In this last Study, I found that maternal exercise training was able to increase the reproductive success of the mothers and the growth rate of the offspring. Although there was direct evidence of phenotypic improvement of the cardiac thermal performance in the mothers following 5 weeks of aerobic exercise training, this did not translate to TGP improvements in either the cardiac thermal performance or CT_{MAX} of the offspring.

3.1 Plasticity to high temperature and hypoxia

Six weeks of acclimation to high temperature significantly affected the cardiac phenotype of the European sea bass both at functional and molecular level. Warm acclimation to 25°C (25N) reset the $f\mathrm{H}_{MAX}$ to a lower rate across the entire warming ramp compared to the control (16°C, normoxia – 16N) and the hypoxia (50% oxygen saturation at 16°C – 16H) acclimated fish (Fig. 9). This reduction in $f\mathrm{H}_{MAX}$ was associated with a prolonged diastolic phase of the ventricular contraction. This phenotypic plasticity of heart rate, however, was only a partial compensation since the $f\mathrm{H}_{MAX}$ of warm acclimated group measured at 25°C remained higher than that of cold acclimated groups measured at 16°C (16N and 16H). Additionally, warm acclimated fish showed significantly higher T_{ARR} compared to the other groups (+2.7°C as compared to normoxia and +3.4°C as compared to hypoxia). Therefore, European sea bass showed relatively high cardiac phenotypic plasticity when exposed to high temperature. These changes in heart rate and thermal limits are consistent with previous studies that have investigated the cardiac effects of warm acclimation in fish (Aho and Vornanen, 2001; Badr et al., 2016; Ekström et al., 2016; Farrell et al., 2007a; Safi et al., 2019; Sutcliffe et al., 2020). The increase in cardiac thermal limits following warm acclimation may allow the heart to keep beating rhythmically, and therefore enable the efficient transport of gases, nutrients, and chemical wastes even at high temperatures. On the other hand, hypoxia acclimation at 50% O_2 saturation did not alter the TPC of fH_{MAX} and the T_{ARR} compared to the normoxia acclimated group. This result suggested that 6 weeks of hypoxia acclimation at 50% O_2 saturation, did not induce a cross-tolerance protection to acute warming. Previous studies have shown mixed evidence about cross-tolerance between hypoxia acclimation and thermal tolerance. While some studies supported this relationship (Anttila et al., 2013b; Burleson and Silva, 2011; Collins et al., 2021; McBryan et al., 2016) others showed contrasting findings (Leeuwis et al., 2021; McDonnell et al., 2019; Motyka et al., 2017). This discrepancy could be attributed to the different levels of hypoxia used for the acclimation, as well as the species-specific differences in hypoxia tolerance. Further studies, which consider comparison between species at several levels of hypoxia acclimation, will need to be undertaken to better understand the relationship between hypoxia acclimation and thermal tolerance and the physiological reasons why there seems to be such relationship is some species but not in others.

Warm acclimation decreased the protein levels of both NCX and SERCA compared to control group (16N). On the other hand, hypoxia acclimation significantly reduced the protein level of SERCA but not the one of NCX compared to control group (16N). SERCA contributes to relaxation of cardiac contraction by actively consuming ATP to re-sequestrate Ca^{2+} from the intracellular space back into the sarco(endo)plasmic reticulum (SR). NCX also contributes to the relaxation phase. However, it uses the electrochemical gradient of Na^{+} to re-sequestrate Ca^{2+} into the extracellular space (Shiels et al., 2002; Vornanen, 2017). Previous studies have also shown that warm acclimation can reduce the rate of SR Ca^{2+} release/uptake and the SERCA transcript expression in rainbow trout (Aho and Vornanen, 1999; Keen et al., 1994; Keen et al., 2017). It is possible that in sea bass, the reduction of ATP-consuming SERCA, in both high temperature and hypoxia, could be attributed to the need to optimize the ATP balance, as a compensation mechanism to restore functional and energetic homeostasis (Tikkanen et al., 2016). On the other hand, the maintained level of NCX in hypoxia and cold acclimated group and reduced level in warm acclimated group might suggest that NCX is used to modulate the heart rate without affecting the ATP energy balance. Indeed, the NCX level was associated with higher heart rate, shorter diastole duration and higher T_{ARR}. However, SERCA did not seem to have connection to the function. Therefore, NCX seems to have much higher functional importance in sea bass than SERCA.

In conclusion, European sea bass showed great capacity to acclimate to both high temperature and hypoxia, which reflects its coastal and estuarine habitat preference. Indeed, these environments are characterized by great fluctuation in temperature and oxygen level. High temperature had a more significant influence on reshaping the cardiac phenotype than hypoxia, as the latter merely affected the SERCA levels. The current data in European sea bass do not provide clear evidence of cross-tolerance between hypoxia acclimation and cardiac thermal tolerance, as the functional and molecular mechanisms investigated here do not provide a common ground supporting the cross-tolerance. More mechanistic studies, possibly involving within-individuals correlation analysis, are needed to unravel the cross-tolerance relationship between high temperature and hypoxia.

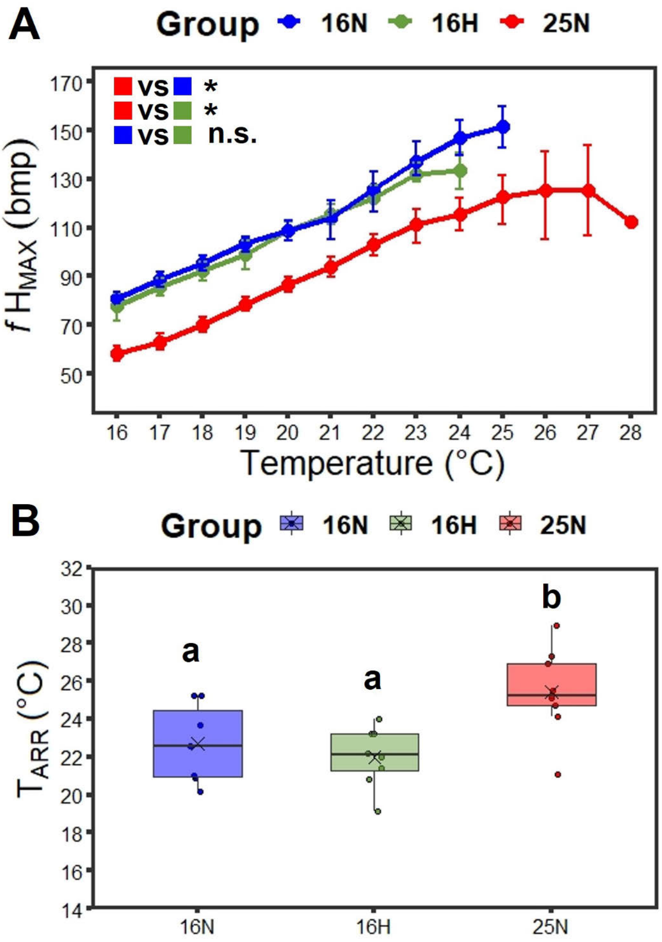

Figure 9. Effect of acclimation to 16 °C and normoxia (16 N); 16°C and hypoxia (16 H) or 25°C and normoxia (25 N) in European sea bass on A) fH_{MAX} (average ±s.e.m.) and B) arrhythmia temperature. * Represents significant difference between groups. Different letters represent significant differences between the groups.

3.2 Plasticity to aerobic exercise training

In Study II, III and in the first part of Study IV I have investigated the effects of aerobic exercise training programs at different training intensities in two salmonid species, rainbow trout and brown trout, and three different life stages. The training session was of 6 h per day for 6 weeks in the Study II and 5 weeks in the studies III and IV, which allowed an easy comparison between the experiments. I will first go through the main results of each chapter and then concentrate on the comparison of the findings and general conclusions.

3.2.1 Study II

In Study II, I studied the interactive effects of functional feeds and exercise training. However, in this thesis I will focus only on the effects of exercise training in order to provide a fluent comparison with the other training experiments (Studies III and IV). In Study II, the most important results were that aerobic exercise training at 1 bl s^{-1} for 6 weeks increased the $f\mathrm{H}_{\mathrm{MAX}}$ at high temperatures (Fig. 10) and also the temperature at which heart rate reaches its peak (T_{PEAK}) compared to the control group (constantly swimming at 0.3 bl s^{-1}). Furthermore, the trained fish had a significantly higher haematocrit (Hct), but not haemoglobin concentration. The increased Hct and $f\mathrm{H}_{\mathrm{MAX}}$ may suggest that exercise training program at 1 bl s^{-1} started to affect the aerobic capacity of the trained fish by improving the potential oxygen delivery to tissues, although it did not translate into higher cardiac arrhythmia temperature. However, increased Hct alone, without changes in Hb, did not necessary indicate increased oxygen delivery. A previous study in rainbow trout has shown that aerobic training at training intensity higher than 1 bl s^{-1} for 4-7 weeks increased stroke volume, cardiac output, and maximum power output but not heart rate measured *in situ* (Farrell et al., 1991). Moreover, exercise training, with Ucrit swimming test on alternate days for 4 months, of Chinook salmon (*Oncorhynchus tshawytscha*) did not induce differences in heart rate, cardiac output or stroke volume, although trained fish had significantly higher MO_2 during U_{crit} swimming challenge (Gallaugher et al., 2001). However, in my study the effects of exercise training on the thermal performance curve of heart rate and the potential benefits on cardiac thermal tolerance were evaluated for the first time. Only one previous study before mine had investigated the benefits of aerobic exercise training on hypoxia tolerance providing evidence of cross-tolerance between exercise training and tolerance against low oxygen levels (Fu et al., 2011).

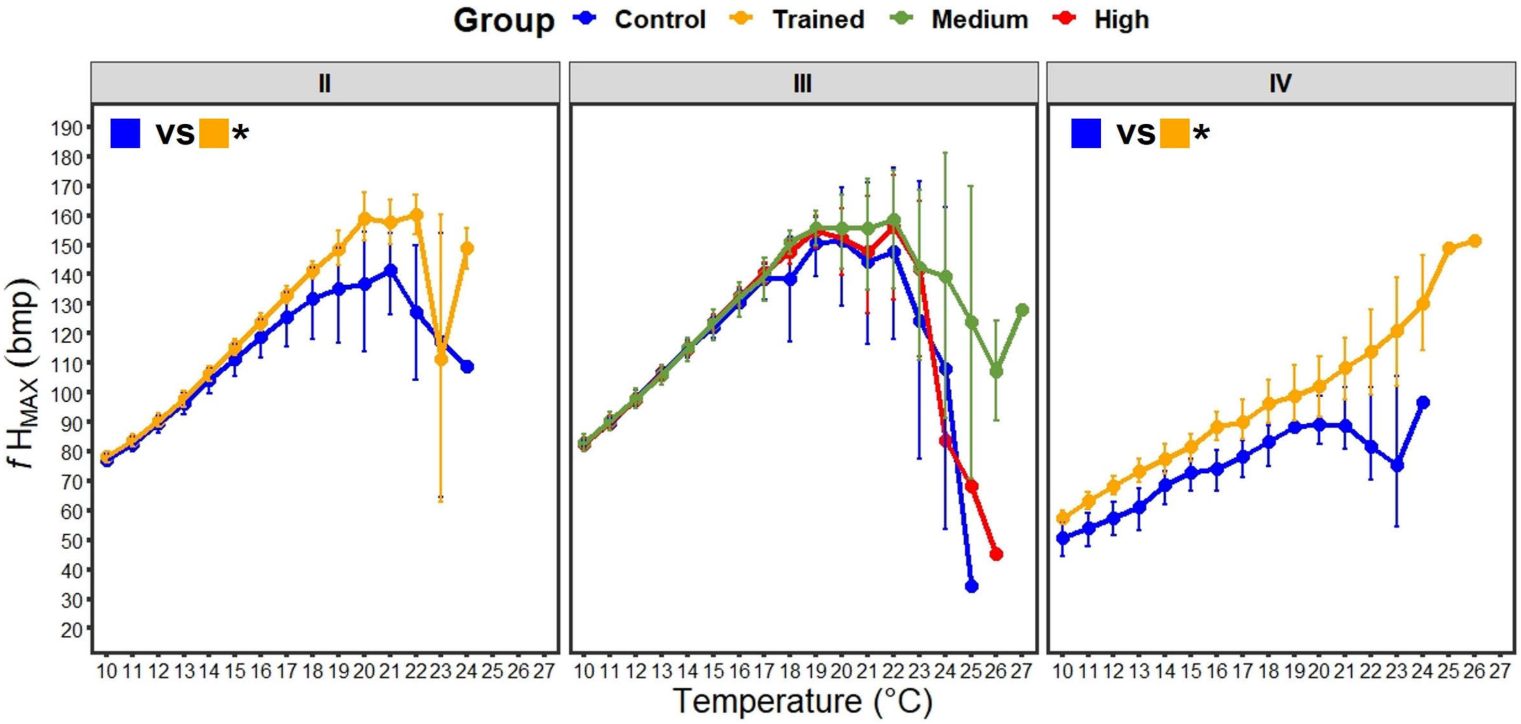

Figure 10. The effects of training programs thermal performance curves for fH_{MAX} (average ± s.e.m) during acute warming in study II, III and IV. In study **II**, fH_{MAX} was measured in rainbow trout (≈100 g – 1 year old) exposed to control speed (0.3 bl*s^{-1}) and training speed (1 bl*s^{-1}). In study **III**, fH_{MAX} was measured in rainbow trout (≈15 g– 6 months old) exposed to control speed (1 bl*s^{-1}), medium training speed (2 bl*s^{-1}) and high training speed (3 bl*s^{-1}). In study **IV**, fH_{MAX} was measured in adult brown trout (≈2.6 kg) exposed to control speed (0.3 bl*s^{-1}) and training speed (0.7 bl*s^{-1}). * represent significant main effect of training.

In my study despite the improvements in TCP for the *f*H$_{MAX}$, the aerobic exercise training did not increase T$_{ARR}$ (Fig. 11), the temperature at which the *f*H$_{MAX}$ starts to be arrhythmic. It is possible that higher intensity was needed to enhance the thermal limits of the fish. Furthermore, the current study did not show changes in relative ventricle mass, the hepatosomatic index (HSI), or in condition factor. No changes in HSI and condition factor suggested that exercise training at 1 bl s^{-1} did not evoke the energy deficit to trigger a sufficient training response. Despite the promising results, this study indicated that a higher training intensity may be needed to challenge the heart function and to reveal if training could enhance the cardiac thermal limits.

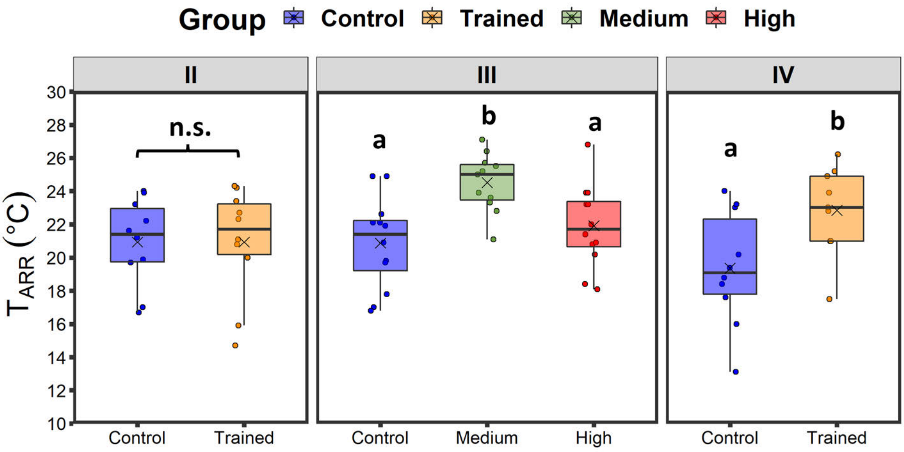

Figure 11. The effects of training programs arrhythmia temperature (T$_{ARR}$) measured during *f*H$_{MAX}$ measurements in study II, III and IV. In study **II**, T$_{ARR}$ was measured in rainbow trout (≈100 g – 1 year old) exposed to control speed (0.3 bl s^{-1}) and training speed (1 bl s^{-1}). In study **III**, T$_{ARR}$ was measured in rainbow trout (≈15 g – 5 months old) exposed to control speed (1 bl s^{-1}), medium training speed (2 bl s^{-1}) and high training speed (3 bl s^{-1}). In study **IV**, T$_{ARR}$ was measured in adult brown trout (≈2.6 kg) exposed to control speed (0.3 bl s^{-1}) and training speed (0.7 bl s^{-1}). Different letters represent significant difference between the training groups in each experiment.

3.2.2 Study III

Based on the results of the previous experiment (Study II), I hypothesised that in order to increase the cardiac thermal tolerance the fish should be trained at higher intensities. Therefore, in this study I investigated the effect of three different training intensities, control water flow at 1 bl s^{-1}, medium intensity at 2 bl s^{-1} and high intensity at 3 bl s^{-1}. This study demonstrated, for the first time, that optimal aerobic exercise training can be used to enhance the cardiac upper thermal tolerance in fish. The most important result was that only medium intensity was able to enhance the cardiac upper thermal limit (T$_{ARR}$) by 3.6 °C as compared to the control group (Fig. 11). High intensity did not, however, significantly increase the T$_{ARR}$ of trained fish.

Moreover, medium intensity training increased also the T_{PEAK} and the Thermal Scope (TS $=T_{ARR}-T_{ABP}$) that informs about the upper thermal window for the heart rate.

Higher tolerance of the cardiovascular performance might translate into a higher tolerance to acute warming in the whole organism (Ferreira et al., 2014; Gilbert et al., 2020). Previous studies showed that aerobic exercise swimming can improve cardiac performance in fish. For example, exercise training can enhance twitch tension, contraction rate and cardiac pumping capacity (Rissoli et al., 2017). Additionally exercise training can increase stroke volume and energy metabolism of cardiomyocytes (Farrell et al., 1990, 1991). Therefore, it is possible that exercise training can improve the efficiency to respond to energy demanding stressors, such as high temperature, because of these systemic improvements in cardiovascular performance. This idea is supported by the results I obtained from the enzyme activities of the aerobic and anaerobic metabolism, citrate synthase (CS) and lactate dehydrogenase (LHD), respectively. Aerobic exercise training significantly increased the CS activity as compared to control meaning that the higher capacity to produce ATP aerobically was associated with the wider and higher cardiac thermal tolerance. On the other hand, only fish trained at high intensity showed a significant reduction in LDH activity. These results are consistent with previous studies that showed an increment in CS activity and a reduction in LDH activity in the ventricles of trained rainbow trout (Farrell et al., 1990) as well as in the red muscle of trained Atlantic salmon (Anttila et al., 2006, 2008b; Zhang et al., 2016).

Contrary to what was expected, the fH_{MAX} did not significantly differ across the groups (Fig. 10). However, this result is consistent with what was reported by Gallaugher et al. (2001) in trained Chinook salmon. They found that the heart rate during critical swimming speed (U_{CRIT}) did not differ between the trained and untrained fish (Gallaugher et al., 2001). These results seem to be consistent with human athlete's physiology, where aerobic exercise training mainly affects the resting or the routine heart rate and not the maximal rate (D'Souza et al., 2014), a phenomenon called Athletic bradycardia.

It is interesting to note that high intensity training at 3 bl s^{-1} did not improve the T_{ARR}, T_{PEAK} or TS. These results could be explained by overtraining syndrome, probably the intensity and/or recovery time within the daily sessions (18h) were not optimal. Indeed, fish trained at high insistently displayed significantly lower condition factor, as well lower LDH enzyme activity in the ventricle as compared to control group. High intensity training may have induced an overtraining syndrome, that compromised the benefits induced by exercise training and did not improve the cardiac thermal tolerance. Interestingly, also a recent study by Gomez Isaza & Rodgers reported that Chinook salmon trained at 4bl s^{-1} for 3 weeks had marginally higher, but not significant, CT_{MAX} compared to control fish (CT_{MAX}: trained =28.6°C; control =28.2°C) (Gomez Isaza and Rodgers, 2022). These data are consistent with our results at high intensity training, suggesting that exercise-induced increment in thermal tolerance might be intensity dependent. In human, overtraining usually induce a stress response with elevation of stress biomarkers, such as cortisol level (Grandou et al., 2020). Similar results have been found also in fish, and usually an optimal exercise training decrease the cortisol levels and promote faster recovery after handling stressors (Boesgaard et al., 1993; Herbert et al., 2011; McKenzie et al., 2012; Palstra et al., 2020; Woodward and Smith, 1985), while the exercise training at high water flow (2.5 bl s^{-1}) increased the cortisol level in Atlantic salmon (Timmerhaus et al., 2021). A recent study by Bard et al. also reported that high cortisol levels were associated with lower T_{ARR} and lower CT_{MAX} in subordinate rainbow trout as compared to dominant fish, although no significant differences in heart rate were reported (Bard et al., 2021). Although in this study, I did not measure the cortisol levels, the lower T_{ARR} in the fish trained at high intensity as compared to medium intensity might indicate a high cortisol level induced by excessive high intensity training. These raise intriguing questions that could be address in future research.

To elucidate the molecular mechanisms induced by exercise training that may increase the cardiac thermal tolerance, I also measured the effect of exercise training on Hct and Hb concentrations, as proxy of oxygen carrying capacity. Furthermore, I also measured from the ventricle the protein levels of SERCA, hypoxia-inducible factor (HIF) and vascular endothelial growth factor (VEGF), as proxy of angiogenesis of new coronary vessels. However, exercise training did not significantly affect the haematological parameters. According to previous studies, the response of Hct following acclimation to exercise training is highly variable as some studies reported increase in Hct (Davison, 1997; Gallaugher et al., 2001; Thorarensen et al., 1993) and others no changes (Gomez Isaza and Rodgers, 2022; Palstra et al., 2020; Skov et al., 2011). One reason of these contrasting results is probably that Hct can rapidly change, via fish spleen contraction and/ or changes in adrenergic tone, when the fish are under stress, e.g. during swimming activity (Gallaugher et al., 1992; Joyce and Axelsson, 2021). Furthermore, exercise training did not affect the ventricular levels of SERCA, VEGF and HIF. These results suggest that the increase in cardiac thermal tolerance occurred preferentially through increasing the ventricular aerobic metabolism, rather than oxygen carrying capacity or EC coupling efficiency. Despite these interesting results, additional research is needed to better understand the molecular mechanism underpinning the increase in thermal tolerance after exercise training.

3.2.3 Study IV

In Study IV, the main goal was to assess the usefulness of aerobic exercise training in the context of conservation and restocking programs in order to improve the resilience of hatchery-reared salmonid to global warming (within and across generations). Therefore, I studied this issue at the parental level (effect of training in F0) and at the transgenerational level (transgenerational effect of maternal exercise in F1), in a brown trout population kept in captivity for restocking purposes.

3.2.3.1 Cardiac thermal tolerance

In this study, aerobic exercise training not only increased the TPC for $f\text{H}_{\text{MAX}}$ but also the cardiac transition temperatures (T_{ABP}, T_{PEAK} and T_{ARR}) for $f\text{H}_{\text{MAX}}$, shifting the cardiac transition temperature towards higher temperatures (Figures 10 and 11). In particular, exercise training increased the cardiac thermal limit (T_{ARR}) by 3.5°C (Fig. 11). This result is highly relevant since especially adult female salmon in the reproductive stage have low tolerance to high temperatures, making them particularly vulnerable to global warming (Eliason et al., 2013; Hinch et al., 2021). Trained fish had consistently higher $f\text{H}_{\text{MAX},}$ along the acute warming as compared to the control group (Fig. 10). The heart of trained fish was able to keep beating rhythmically at higher rate, even at high temperatures. This result suggests that the heart of trained fish is thermally more resilient. By been able to keep beating regularly at high temperatures, it is able to ensure the oxygen delivery to the tissues, but also it receives enough oxygen for its function even at high temperatures (Farrell, 2002). Exercise training did not, however, affect the proportion of the compact and spongy myocardium or relative ventricle mass. This suggests that the increased cardiac thermal tolerance in this species was not associated with a higher myocardial oxygen supply or increased ventricle mass as proxy of stroke volume.

3.2.3.2 Reproductive success

In this experiment, the exercise training program was aimed to simulate the spawning migration, and to assess the effect of excessive training on reproductive success. In the wild, just before spawning, many salmonid species, such as salmon and trout, undertake a strenuous upstream migration (Jonsson and Jonsson, 2011; Hayashida et al., 2013). In this study I have found that trained adult brown trout produced more eggs and had 16% higher embryo survival to eyed stage as compared to the control group. No significant differences were found between the groups in egg diameter, hatching success or offspring survival at parr stage (120 days post hatching). Since no significant changes in egg diameter were observed in trained females, we can speculate that the higher survival rate of embryo until eyed stage may be due to differences in vitellogenesis and nutrition investments. However, to date there are no studies that could provide a clear mechanistic explanation for these changes in the offspring survival. Remarkably the increment on reproductive success was achieved even if exercise trained female had lower HSI, indicating lower body energy, yet training did not affect the condition factor of the fish. These results may be the direct consequence of the energy cost of exercise training during reproductive stage. A previous study found similar results in trained adult sockeye salmon (Patterson et al., 2004). Patterson et al found that exercise training increased the proportion of mature females in the population and also increased the embryo survival to eyed stage. Although Patterson et al. did not found any increment in stress biomarkers, previous studies reported that confinement stress due to captivity reduced the egg survival and the number of offspring in several wild salmonids (Campbell et al., 1994). A sub-optimal training program, e.g. a too long training duration with no resting periods, could impair the physiological performance possibly due to stress and allocation of energy to swimming, leaving less energy for reproduction. A previous study in pubertal rainbow trout reported that exercise training at 0.75 bl s^{-1} without resting periods can suppress ovarian and oocyte development and downregulate ovarian transcriptomic response (Palstra et al., 2010b). These discrepancies might be explained by differences in training load volume (i.e. intensity * training duration * sets of training) (Hawley et al., 2014; MacInnis and Gibala, 2017) that have to meet the needs of the species and life stage. It is possible that the training in Palstra et al. (2010) was too intense for pubertal rainbow trout.

3.2.3.3 Transgenerational effect of maternal exercise

Interestingly, offspring from trained mothers had significantly higher body mass and body length as compared to offspring from untrained mothers during the studied growth period (from 150 dph to 400 dph) (Fig. 12). Mammalian studies have shown that parental exercise can have an effect on offspring health and metabolism (Gorski, 1985; Harris et al., 2018; Kusuyama et al., 2020). However, my result is incredibly novel since it was the first example in all vertebrates showing growth improvement induced by maternal exercise. My study did not clarify the mechanism that could explain this effect. However, offspring growth rate could be enhanced by non-genetic inheritance mechanisms, e.g., by egg nutritional provisioning, maternal hormones, or epigenetics changes (Donelson et al., 2018; Munday, 2014). These results are similar to what has been found in previous studies that have shown the occurrence of transgenerational plasticity (TGP) in growth following parental acclimation to high temperature (Salinas and Munch, 2012; Shama et al., 2014).

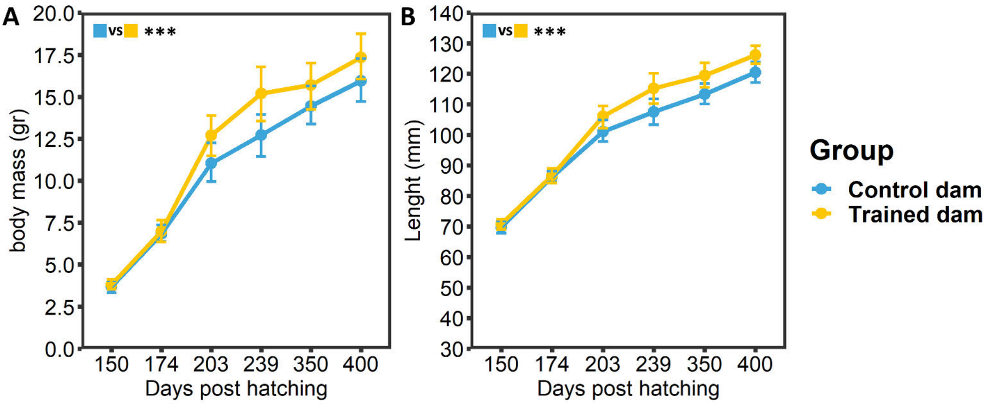

Figure 12. Trangenerational effect of maternal exercise training on A) body mass B) fork length of the offspring from 150 to 400 dph. * Represents significant main effect of the maternal training.

The increased cardiac performance and thermal tolerance of the adults in response to training did not, however, lead to similar improvements in the offspring. Offspring from trained mother had the same TPC for *f*H_{MAX} and the same cardiac transition temperatures as the offspring from untrained mothers. Consistent with this results, maternal exercise did not affect the CT_{MAX} (Fig. 13), which was affected only by the time period when it was measured (June = 70 dph vs August = 120 dph).

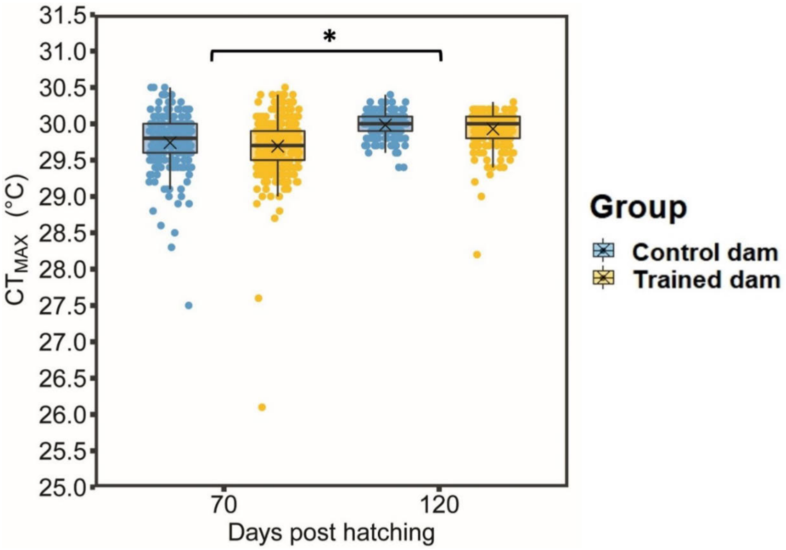

Figure 13. Trangenerational effect of maternal exercise training on the CT_{MAX} of the offspring at two time points (70 dph – June;, 120 dph -August). No significant transgenerational effect of maternal training group was detected. * Represents significant difference between the time points.

Transgenerational plasticity following difference in temperature or water flow of the parental environment has been documented in different fish species (Cortese et al., 2022; Donelson et al., 2018; Evans et al., 2014; Lee et al., 2020; Shama and Wegner, 2014; Shama et al., 2014). However, only three previous studies have assessed the occurrence of transgenerational plasticity in thermal tolerance. Consistent with the data of my Study, these studies similarly showed that the thermal tolerance in offspring from parents acclimated to high temperature (Penney et al., 2021, 2022) or to hypoxia (Ho and Burggren, 2012) was not affected. This finding may suggest there is very little potential for TGP to affect thermal tolerance. Thermal tolerance seems to possess high plasticity within generation, but might lack mechanisms for trans-generational effect.

Besides the transgenerational effect of maternal exercise training, the results of this study showed that maternal experience can affect the heritability of some traits in the offspring. Indeed, maternal exercise training increased the heritability of CT_{MAX} and body length in the offspring, but not body mass (Fig 14). This finding suggests that maternal exercise training has the potential to accelerate the evolutionary response of these traits in the trained population, especially if these traits will be under the selection in the new warmer environment, as predicted by global warming. Despite the interesting results, the question on the possible mechanisms underpinning this increase in heritability remains, as this study does not allow any conclusion to be made. However, the present study raises the possibility that transgenerational plasticity may influence the heritability in the same way as plasticity affects the heritability. Indeed, previous studies showed that environmental conditions, such as temperature acclimation, can interact with the genotype (genotype-by-environment interactions) and can affect the heritability of growth traits (Crespel et al., 2013; Shama et al., 2014) and thermal tolerance (Debes et al., 2021). It is, therefore, likely that parental environment can interact with the genotype in a similar way when it comes to exercise training.

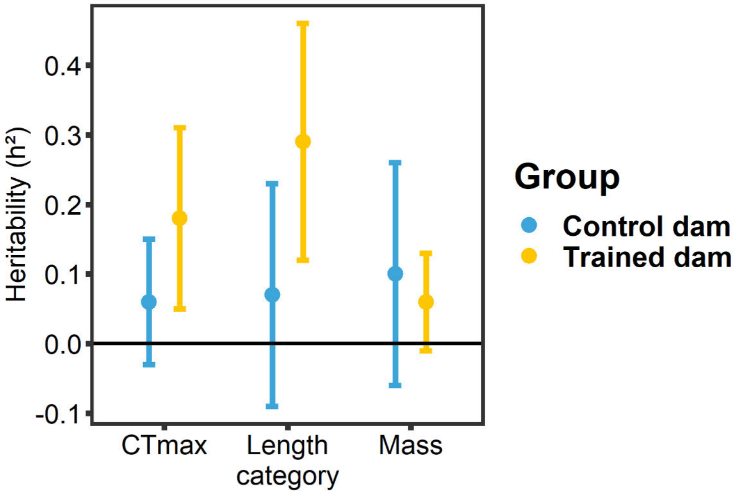

Figure 14. Comparison of the Heritability [*h2* (SE)] of CT_{MAX}, body length and body mass in the offspring from control and trained mothers.

3.3 Comparison between Study II, III and IV

An interesting finding to emerge from the data from this thesis is that the effects of exercise training on cardiac performance and cardiac thermal tolerance appears to be dependent on the intensity of the training.

By comparing the results from Studies II and III, it is possible to see that within the same species, the intensity of the training can significantly affect the cardiac thermal tolerance (Fig. 10 and Fig. 11). A possible explanation for this might be related to the energy cost of swimming, also defined as the cost of transportation (COT) that increases exponentially with the swimming speed (McKenzie, 2011; McKenzie et al., 2021a; Tudorache et al., 2008). Therefore as COT increase during exercise, more workload is required by the cardiorespiratory system to sustain swimming. However, if exercise is too mild, it will not reach the threshold to elicit a cardiac response. Too high intensity of training might, on the other hand, lead to overtraining as discussed in part Study III. Indeed, the physiological response to exercise training follow an hormetic curve, where only appropriate levels of stress elicit beneficial physiological adjustments (Peake et al., 2015). Therefore, it is possible that to increase cardiac thermal performance in fish cardio training should be planned with intensity close to the maximum aerobic threshold, i.e. the maximal effort is possible to be sustained with only aerobic metabolism. Besides training intensity, there are other significant aspects of exercise training that have to be taken in consideration, such as the duration and the frequency of the training sessions (initially studied by altering training sessions and resting periods). For example, in trained fish, daily resting period reduces the stress induced by swimming as compared to continuous training (Castro et al., 2011).

However, fishes exhibit significant variation in swimming performance, that is directly linked to the specific ecological niches they occupy (Blake, 2004). Each fish species possesses unique morphological and physiological adaptation that matches their environment and lifestyle, and that defines their swimming performance. A clue of this species-specific effect of training can be seen comparing the trained rainbow trout (II and III) and the trained brown trout (IV). Trained brown trout showed an increase in T_{ARR} despite being trained at lower relative speed (0.7 bl s^{-1}) as compared to the rainbow trout (2 bl s^{-1}). This discrepancy in training response could be explained by the different swimming habits between brown trout and rainbow trout. Indeed, brown trout occupy low-stream rivers and are known to perform shorter migrations (Heggenes, 1996; Heggenes et al., 1999; Jonsson and Jonsson, 2011). Similarly, a previous study reported that brown trout responded better to training at lower intensity (Anttila et al., 2008a).

Interestingly, not only brown trout responded better to lower intensity of exercise training showing higher T_{ARR}, but also they showed a consistently higher fH_{MAX} at all temperatures (Fig. 11). In contrast, in Study III, rainbow trout (15 g – 6 months old) displayed an increment in T_{ARR} without any increment in fH_{MAX}, while in Study II an increase in fH_{MAX}, but not in T_{ARR}, was observed with larger juveniles (105.5 g- 1 year old) (Fig. 11). These differences can be explained in part by different strategies being used to maintain a high cardiac output in fish of different age or size. It is possible that adult brown trout (2.6 kg) might increase the cardiac output by increasing the fH_{MAX}., while juvenile rainbow trout might rely more in the increase of stroke volume to increase the cardiac output. It is difficult to explain these results, but it might be related to aging effect. Aging has been shown to affect the trainability and decrease the U_{CRIT} performance in zebrafish (Gilbert et al., 2014). Therefore, it is possible that older brown trout might have higher collagen deposit in the myocardium (Murphy et al., 2021) increasing the stiffness of the ventricle and compromising the modulation of the volume stroke (Keen et al., 2016, 2017).

Taken together, these findings suggests that for exercise training programs to be effective they should be meticulously planned considering the optimal training volume (intensity, session duration, resting periods) associated with the goal of the training (e.g. increase growth, or increase robustness to stressors etc..) and the physiological and behavioural characteristics of the species, likewise as in human sport science.

3.4 Applications for aquaculture and conservation

Global warming is predicted to have a significant negative impact on the aquaculture of cold-water fish species, such as salmon and trout. Indeed, massive death events are often associated with heatwaves. Therefore, there is an urgent need to increase the resilience of hatchery-reared fish against climate change. In this context, the results of this thesis showed that optimal aerobic exercise training programs can became an important tool to improve the cardiac thermal performance and help hatchery-read fish to cope with heatwaves. Interestingly, the data of this thesis showed that aerobic exercise training can improve cardiac thermal performance in two different salmonid species, rainbow trout (II and III) and brown trout (IV), but also at two different life stages, as parr (II and III) and adults (IV). As these experiments were directly carried out in regular aquaculture tanks, these data provided insights about the direct applicability of exercise program in aquaculture facilities. Exercise training provided benefits beyond the cardiac performance. Indeed, training brood-stock increased their reproductive success, the embryo survival and enhanced the growth rate of their offspring. These results mean that by just increasing the water flow in the brood-stock and simulating the spawning migration it could be possible to obtain more and bigger offspring. These findings are highly relevant also in the context of management and conservation of endangered salmonid populations. Previous studies showed that body size is considered as one of the most important factors influencing the survival of early life stages with also positive effects in adulthood, life history traits and later fitness (Ahti et al., 2020; Miller et al., 1988). Therefore, restocking bigger fish could potentially increase their survival in nature. Conservation managers could be encouraged to incorporate exercise training program into their breeding and restocking programs, to precondition the juvenile hatchery-reared fish before they are released in the nature or the adult fish before the spawning period. Exercise training could be scheduled to correspond to the time of their natural spawning migration, aiming to improve the reproduction as well as the survival and growth rate of the next generation. Taken together these results showed that aerobic exercise training programs could be beneficial for every sector involved with rearing fish, from commercial aquaculture to conservation aquaculture and stock enhancement.

4 Conclusions

Global warming is increasing the magnitude and frequency of heatwaves challenging the physiological limits of fish to cope with high temperatures. For this reason, global warming is predicted to negatively impact fish farming of all kinds, be it for food purposes or for conservation and restocking purposes. This thesis aimed to increase the understanding in the capacity of fish to acclimate to global warming stressors, such as high temperature and hypoxia. This thesis focused especially on the importance of phenotypic plasticity of the cardiac function to cope with these stressors. Furthermore, this thesis proposed aerobic exercise training as a useful tool to increase the thermal tolerance of the cardiac performance in hatchery-reared fish, and therefore to improve the resilience and sustainability of aquaculture to global warming.

The results from study I provide a better understanding of cardiac plasticity following high temperatures and hypoxia. European sea bass possess high cardiac phenotypic plasticity that allowed them to acclimate to high temperature and hypoxia at both functional and molecular levels. However, the acclimation to these stressors did not provide evidence for shared acclimation mechanisms at cardiac level that could support cross-tolerance between hypoxia and thermal tolerance.

The findings from the study II showed that rainbow trout acclimated to aerobic exercise training displayed higher $f\mathrm{H}_{\mathrm{MAX}}$ at high temperatures and possibly increase the oxygen delivery to tissues. However, the higher $f\mathrm{H}_{\mathrm{MAX}}$ did not lead to an increase increment in cardiac thermal tolerance of trained fish. Study III demonstrated for the first time that aerobic exercise training can provide cross-tolerance protection and increase thermal tolerance. However, the response to exercise was dependent on intensity of the training. Indeed, only rainbow trout trained at 2bl s^{-1}(medium intensity) showed a 3.6 °C higher T_{ARR} as compared to the controls that was associated with an enhanced CS activity in the ventricle. High intensity training did not significantly enhance the cardiac thermal tolerance, probably due to overtraining syndrome.

Data from study IV, not only confirmed that aerobic exercise training can provide a cross-tolerance protection in another fish species, but also that it can have transgenerational effects on the next generation. Maternal exercise training increased the embryo survival and the growth rate of the offspring. Despite these benefits maternal exercise training did not improve the cardiac thermal performance nor the CT_{MAX} of the offspring. To develop a full picture of transgenerational effects of exercise training, further studies should focus also on the transgenerational effect of paternal exercise training. Notwithstanding, these results shed new light on transgenerational effect of exercise and demonstrated that phenotypic plasticity and transgenerational plasticity can be harnessed via acclimation to exercise training to improve the phenotype of the hatchery reared fish.

Taken together, the findings of this thesis provide important implications for the salmonid aquaculture sector, with benefits not only for commercial aquaculture but also for farming fish for restocking and conservation programs. Despite these encouraging findings, many questions remain. Building upon this research, the next step could be to examine if exercise training can affect the whole organism thermal tolerance and survival rate during real heatwave. More broadly, future studies are also needed to determine if and what kind of exercise training is optimal to increase the thermal tolerance of non-salmonid species. Although not conclusive about the molecular mechanisms underlying the response to exercise training, this thesis brings important advances in the field of fish physiology, trying to build a bridge between exercise physiology in fish and human sport science. Indeed, fish represent a good animal model to study exercise physiology due to their innate instinct to swimming against water currents (rheotaxis) that makes it easy to apply and manipulate exercise training protocol.

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And now the last pages of my PhD thesis... only now I am starting to realize that I am reaching a significant milestone in my academic career. My PhD journey was metaphorically similar to a freediving dive, initially filled with excitement as I delved into a new world, followed by the awareness of the tiring ascent from the deep water that demanded persistent effort, culminating in the gratifying moment of breaking through to the surface. This amazing adventure was possible thanks to the support of many people. So, as I come to the end of my PhD journey, I want to express my immense gratitude to these amazing people I have met and who have supported me along this way.

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