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THE PHENOTYPIC PLASTICITY OF THERMAL TOLERANCE AND ITS MODULATION IN A COMPLEX ENVIRONMENTAL SCENARIO

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“The roots of education are bitter,
but the fruit is sweet.”

Aristotle

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GIOVANNA MOTTOLA: The phenotypic plasticity of thermal tolerance and its modulation in a complex environmental scenario

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ABSTRACT

It is well established that climate change constitutes a great challenge for wild animals, with increasing occurrence of heat waves also causing mortality events. However, in nature, environmental stressors are not occurring alone, but several occur at the same time. Among common environmental stressors, presence of trace metals is one of the greatest threats for aquatic animals because of their persistence in the environment and the bioavailability for organisms. Organisms can improve their tolerance to stressors via phenotypic plasticity, i.e., the ability of an organism to change phenotype in response to stimuli from the environment, and this ability might also be transferred to the next generations, through parental effect. In the current doctoral thesis, I investigate how fish respond to extreme environmental events via phenotypic plasticity and via parental effects using laboratory and field approaches.

In Study I, the focus was to elucidate whether the relationship between transcription and translation of the heat shock proteins was shaped by the magnitude of the heat stress in a laboratory-reared zebrafish population. The study showed a coupling of the transcription and translation at high temperature, and an uncoupling at mild temperatures, highlighting the importance of studying the response of fish to heat stress at protein, and not only at gene level.

In the Study II, III and IV, the phenotypic plasticity of thermal tolerance has been individually assessed at both physiological and molecular level, and in two fish species: one temperate species caught from the Baltic Sea (three-spined sticklebacks), and one originally tropical but currently largely laboratory-reared zebrafish. The thermal tolerance of individuals has been measured before and after an acute exposure to heat wave, copper, and combination of the two stressors. In these studies, I observed that the innate thermal tolerance, as well as its phenotypic plasticity, depends on fish species and is shaped by sex, with male stickleback generally showing better plasticity compared to females; but the opposite trend was seen in zebrafish. In sticklebacks, the exposure to heat wave and copper separately, resulted

in an increase of the thermal tolerance, therefore suggesting positive phenotypic plasticity. On the other hand, while the heat wave improved the thermal tolerance of control zebrafish, the same was not observed in the copper exposed group. The response observed in Study I at molecular level, was not replicated in sticklebacks' studies, except for Study IV, suggesting that the molecular response might be also species-specific. In Study IV, the stressor effects were also assessed in the offspring generation. Despite some limitations, Study IV showed that the offspring was strongly affected, as the heat wave and copper negatively influenced the development and thermal tolerance via both developmental and parental exposure.

This research represents an important step forward in the study of the thermal tolerance as it addresses its individual phenotypic plasticity and potential transgenerational occurrence in response to multiple-stressor exposure. The approach used in the current thesis was multilevel, spanning from the biochemical response to the evolutionary potential for adaptation, therefore representing a broad focus on the phenotypic plasticity of thermal tolerance.

KEYWORDS: Phenotypic plasticity, transgenerational plasticity, multiple-stressor exposure, thermal tolerance, copper, heat wave, climate change, sex-specific response, evolutionary adaptation

TURUN YLIOPISTO

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ABSTRAKTI

Ilmastonmuutos on huomattava uhka villeille eläimille ja lisääntyvät lämpöaallot saavat aikaan jopa eläinten massakuolemia. Luonnossa eläimet eivät yleensä kuitenkaan kohtaa vain yhtä ympäristöongelmaa kerrallaan, vaan tavallisesti altistuvat useille ongelmille samanaikaisesti. Metallit ovat vesieläinten suurimpien ympäristöuhkien joukossa biosaatavuutensa ja pysyvyytensä takia. Eläimet pystyvät kuitenkin vastaamaan ympäristöongelmiin esimerkiksi fenotyyppisen plastisuuden kautta. Fenotyyppinen plastisuus on kyky muuttaa ilmiasua ympäristömuutoksen seurauksena. Tämä plastisuus voi myös siirtyä seuraavalle sukupolvelle, jolloin sitä kutsutaan sukupolvien väliseksi plastisuudeksi. Tässä väitöskirjatyössä tutkin laboratorio- ja kenttätöillä, miten kalat pystyvät vastaamaan vakaviin ympäristöongelmiin fenotyyppisen ja sukupolvien välisen plastisuuden kautta.

Osatyössä I keskityttiin tutkimaan laboratoriossa seeprakalojen lämpöshokkiproteiinien lämpötilamuutoksen suuruudesta riippuvaa transkription ja translaation välistä suhdetta. Tutkimukset osoittivat, että transkriptio johtaa translaatioon proteiiniksi vain hyvin korkeissa lämpötiloissa, kun taas vähemmän voimakas lämpötilan nousu ei saanut aikaan kuin transkription. Työ osoitti, että kalojen molekylaarisia vasteita lämpöstressiin on tärkeä tutkia mRNA tason lisäksi myös proteiinitasolla.

Osatöissä II, III ja IV lämpötilatoleranssin fenotyyppistä plastisuutta tutkittiin yksilöllisesti sekä fysiologisella että molekyylitasolla kahdella eri kalalajilla: yhdellä lauhkean ilmastovyöhykkeen lajilla (Itämeren kolmipiikki) ja yhdellä alun perin trooppisen vyöhykkeen, mutta nykyisin suurelta osin laboratorioissa kasvatetulla lajilla (seeprakala). Jokaisen yksilön lämpötilatoleranssi mitattiin tutkimuksissa ennen lämpöaalto-, kupari- ja niiden yhteisaltistusta sekä sen jälkeen. Näissä tutkimuksissa havaitsin, että lämpötilatoleranssi ja sen fenotyyppinen plastisuus olivat lajispesifisiä ja

riippuivat kalan sukupuolesta. Uroskolmipiikit olivat yleisesti ottaen plastisempia kuin naaraat, kun taas seeprakaloilla naaraat olivat enimmäkseen uroksia plastisempia. Kolmipiikeillä lämpöaalto- ja kuparialtistus saivat aikaan lämpötilatoleranssin nousemisen, mikä osoittaa ympäristöstressien aiheuttavan positiivista fenotyyppistä plastisuutta. Toisaalta, vaikka lämpöaalto nosti seeprakalojen lämpötilatoleranssia, kuparialtistus ei saanut aikaan samaa vastetta. Osatyön I molekyylivastetta ei myöskään havaittu kolmipiikeillä ja ainoastaan osatyössä IV seeprakaloilla vasteet olivat samankaltaiset kuin osatyössä I osoittaen molekyylivasteiden lajispesifisyyden. Osatyössä IV tutkittiin myös ympäristömuutoksien vaikutuksia kalojen jälkeläisiin. Jälkeläisten fenotyyppiin vaikuttivat huomattavasti sekä vanhempien altistus että jälkeläisten kasvatusolosuhteet.

Tämä väitöskirjatyö on tärkeä askel eteenpäin lämpötilatoleranssin tutkimisessa, sillä siinä selvitettiin sekä yksilöiden fenotyyppistä plastisuutta että mahdollista sukupolvien välisiä vaikutuksia kahden ympäristöongelman esiintyessä yhtaikaa. Tutkimuksia tehtiin myös usealla eri biologisella organisaatiotasolla lähtien molekyylivasteista adaptaation kautta tapahtuvaan evoluutioon. Tämän takia väitöskirjatyöni antaa hyvin laajan näkökulman lämpötilatoleranssin fenotyyppiseen plastisuuteen.

AVAINSANAT: fenotyyppinen plastisuus, sukupolvien välinen plastisuus, ympäristömuutosten yhteisvaikutukset, lämpötilatoleranssi, kupari, lämpöaalto, ilmastonmuutos, sukupuolispesifiset vasteet, evolutiivinen adaptaatio

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Abbreviations

ANOVA	Analyses of Variance
BCA	Bicinchoninic acid assay
CTmax	Critical thermal maximum
CTmin	Critical thermal minimum
CTRL	Control
Cu	Copper
GLM	Generalized linear model
Hif-1α	Hypoxia-Inducible Factor 1-α
HSE	Heat Shock element
HSF	Heat Shock transcription factor
Hsps	Heat Shock Proteins
HSR	Heat Shock response
HW	Heat wave
KOT, CTRL	Kotka, control
LC50-96h	Median lethal concentration at 96 hours
LM	Linear model
LMM	Linear mixed-effect model
LOE	Loss of equilibrium
LOV, NPP	Loviisa Nuclear power plant
OLK, NPP	Olkiluoto Nuclear power plant
POO, CTRL	Porvoo, control
POR, CTRL	Pori, control
PYH, CTRL	Pyhäranta, control
RAD	Restriction Site Associated DNA Sequencing
Rplp0	Ribosomal protein lateral stalk subunit P0
TGP	Transgenerational plasticity
Topt	Thermal optimum
TPC	Thermal Performance Curve
5dpf	Five-day post fertilization

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 Mottola, G., Nikinmaa, M., Anttila, K. "Hsp70s transcription-translation relationship depends on the heat shock temperature in zebrafish." Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 240 (2020). https://doi.org/10.1016/j.cbpa.2019.110629.

II Mottola, G., López, M. E., Vasemägi, A., Nikinmaa, M., & Anttila, K. Are you ready for the heat? Phenotypic plasticity versus adaptation of heat tolerance in three-spined stickleback. Ecosphere, 13(4) (2022). https://doi.org/10.1002/ecs2.4015.

III Mottola, G., Nikinmaa, M., & Anttila, K. (2022). Copper exposure improves the upper thermal tolerance in a sex-specific manner, irrespective of fish thermal history. Aquatic Toxicology, 246 (2022). https://doi.org/10.1016/j.aquatox.2022.106145.

IV Mottola, G., Keituri, E., Pape, A., Anttila, K. Phenotypic plasticity of thermal tolerance and its developmental and parental effects in response to anthropogenic environmental stressors (manuscript).

1 Introduction

Organisms are living in habitats that are increasingly impacted by anthropogenic activities that alter, for example, the global climate, shift ecological interactions by invasive species, and increase pollution (Todgham and Stillman 2013). Climate change is currently also resulting in increasing occurrence of heat waves. Heat waves are sudden thermal events and in the marine and freshwater environments, which are defined as at least 5-day-long events when the temperature exceeds the 90th percentile of a 30-year historical period (Woolway et al. 2021). Heat waves constitute a great challenge for wild animals, especially for ectotherms, whose body temperature depends on the surrounding environment. Moreover, when the magnitude of heat wave exceeds the organism's thermal tolerance, i.e., the maximum temperature they can handle, mass mortality events in both terrestrial and aquatic environments can occur (Till et al. 2019). It is, therefore, becoming crucial to understand how wild organisms will react to sudden temperature increase and which strategies they will adopt to survive in a changing climate. Nevertheless, in nature, environmental stressors are not always occurring one at the time. Rather, the occurrence of one stressor can be accompanied by the modification of another environmental feature, which might agonize or antagonize the responses to natural condition, as well as resulting in no effect (Todgham and Stillman 2013). Among the additional environmental stressors, trace metals are one of the greatest threats for aquatic animals because of their persistence in the environment and the bioavailability for organisms. Copper, for instance, is biologically important at low concentrations (Sappal et al. 2015b), but if it bio-accumulates further, it leads to impairments at different biological levels also causing mortality (Forouhar Vajargah et al. 2020). The toxicity of copper in aquatic environment has been extensively studied and it has been seen to depend on temperature, with high temperature generally increasing the metabolic rate of some organisms, which in turn allows for increased uptake. However, such interaction is complex as increased metabolic rate could also enhance the detoxification processes (Peruzza et al. 2021). Therefore, so far there are no definite conclusions how organisms react to co-exposure to heat wave and copper. This is extremely important to understand as copper run-off from mines and industrial activity constitutes still an ongoing phenomenon in several part

of the world (Perlatti et al. 2021, Punia 2021). Residues from copper industrial process could lead to serious environmental problems (Latorre et al. 2018), such as biodiversity loss and ecosystem modification. Moreover, similarly as for heat waves, copper exposures might occur suddenly in nature, and we do not know whether individuals already facing climate change would be able to handle another extreme event.

It is therefore important to assess the strategies that organisms adopt to counteract environmental perturbations and evaluate the organism responses to a multiple-stressor scenario.

1.1 How organisms respond to environmental perturbations?

1.1.1 Evolutionary thermal adaptation

Adaptive capacity in its broadest sense includes both evolutionary changes and plastic responses, i.e. acclimation (Williams et al. 2008). The major part of literature refers to "acclimation" to climate change as the phenomenon occurring when animals can remodel their physiology to compensate for the effect of temperature variation by phenotypic plasticity (Hoffmann and Sgró 2011, Seebacher et al. 2015). Therefore, in the current thesis, the word "adaptation" or "adaptive capacity" always refer to the evolutionary adaptation, i.e. via genetic means; while the word "acclimation" refers to all the physiological coping mechanisms involving the phenotypic plasticity.

The biochemical and physiological perturbations caused by the environment can be defined as stress. However, although the word "stress" generally has a negative connotation, this is not always the case in biology. Indeed, one of the most interesting aspects of life is that it is able to shape itself to such an extraordinary diversity of environments (Somero et al. 2017). This is possible due to the ability to adapt to different environmental factors, in other words, to cope with "stress". Among the environmental factors, temperature has an important effect on biological functions from molecules to ecosystems (Hochachka and Somero 2002), thus resulting as one of the most critical abiotic factors shaping the distribution and abundance of organisms (Schulte et al. 2011). Organisms have been adapted to different thermal niches, spanning from cold and stable temperatures in Antarctica (Hofmann et al. 2005), to more variable thermal environments in temperate areas, and further to tropical temperatures. While there has been several pieces of evidence suggesting

local adaptation in response to variation in the environmental features (Guo et al. 2015), we also know that evolutionary adaptation is a mechanism that generally requires many generations to occur. Therefore, despite organisms possessing the capacity to adapt to different thermal niches, the current pace of the climate warming and especially high frequency of extreme thermal events, might be too fast for evolution to occur. Moreover, despite examples of rapid evolution of thermal tolerance in laboratory (Geerts et al. 2015), and in nature (Barrett et al. 2011), some species, especially the ones with a long reproduction cycle, might not be able to adopt the evolutionary adaptation as strategy for survival. In other words, evolutionary adaptation might not keep the same pace as climate change.

1.1.2 Phenotypic plasticity of thermal tolerance and its transgenerational occurrence

A crucial mechanism that organisms utilize to respond to changes in environment is the phenotypic plasticity. Phenotypic plasticity is the ability of an individual genotype to change phenotype (e.g., physiological, morphological and behaviour adjustments) in response to stimuli from the environment. In thermal biology, numerous studies have been performed to assess the extent to which the phenotypic plasticity of thermal tolerance is improving the survival capacity of individuals in warming environments (as reviewed by Somero 2005, 2010). The effects of temperature on performance traits within the zone of tolerance can be visualized using a thermal performance curve (TPC) (Schulte et al. 2011) (Fig. 1). TPC is an empirical representation of how a certain performance (e.g., growth, heart rate, biochemical mechanisms) of an individual is shaped by temperature. Virtually, the TPC shows similar shape across individuals, generally starting from a critical thermal minimum (CTmin), which is the lowest temperature at which a certain performance can be achieved; then the performance starts to increase with increasing temperature until a temperature which is optimal for the individual performance (Topt); after which, with temperature exceeding the optimum, the performance starts to decrease until it reaches a critical thermal maximum (CTmax).

It has been shown in literature that, via mechanisms of adaptation or phenotypic plasticity (also called acclimation), the TPC may sHift towards more thermal tolerant phenotypes or increase the optimum temperature, or increase the width of tolerated temperatures (i.e., the thermal breadth) (Fig. 1).

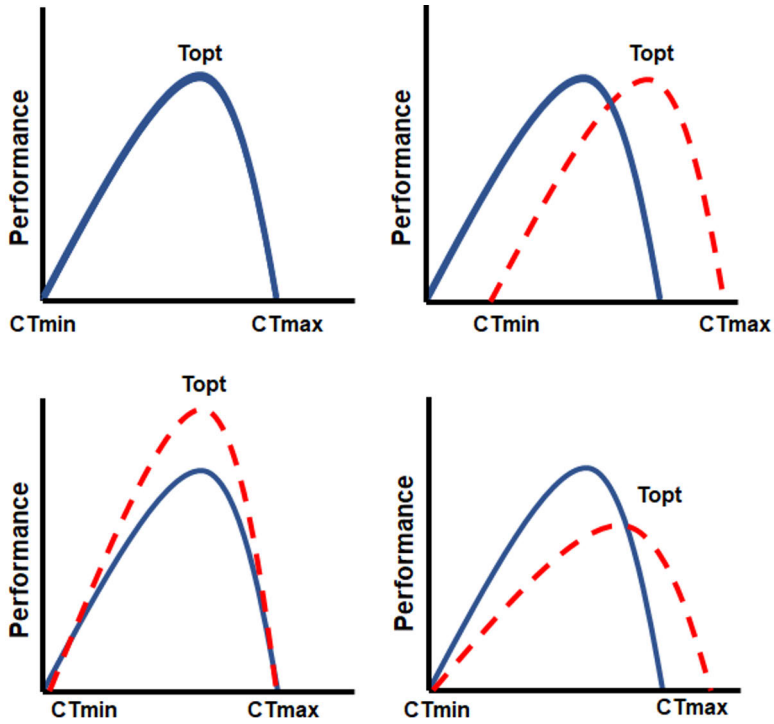

Figure 1. Representation of an empiric thermal performance curve (TPC) and some of its possible variations due to acclimation or adaptation to high temperatures (red dashed lines). The y-axis represents a hypothetical individual performance (growth rate, heart rate, reproduction), while the x-axis represents the temperature. Themal optimum (T_{opt}), CT_{min} (Critical thermal minimum), CT_{max} (Critical thermal maximum).

Therefore, individuals genetically adapted (by evolutionary adaptation) or acclimated (i.e. via phenotypic plasticity) to a warmer environment, generally shape their performance curve accordingly. Some studies have focused on the variation of the thermal performance curve after acute (Eliason et al. 2011) or chronic exposures to increased temperature (Robinson and Davison 2008, Bilyk et al. 2012). These studies represent milestones for the study of the thermal tolerance. However, while describing the acclimation process under different thermal regimes, they do not take into consideration the complexity of the natural environment, where multiple other features might change (due to temperature changes or in an independent way). It is therefore important to introduce a more realistic environmental scenario, to

understand whether phenotypic plasticity, in this case in response to thermal events, might be shaped by other factors.

Besides phenotypic plasticity, which occurs within a generation, it has been observed that the environmental condition experienced by the parents might influence the offspring generation. Parents experiencing a warming condition might transfer this information to the offspring, by non-genetic routes. These parental experiences may sometimes also have influence at developmental level leading to positive or negative effects (Uller 2008). These parental effects are considered as an important aspect to study when addressing the phenotypic strategies that individuals adopt to survive in changing climate. Parental effect has recently got a vast focus by the scientific community, especially studying the mechanisms promoting the occurrence, after one generation of multigenerational plasticity events (Donelson et al. 2018). However, there is a gap of knowledge on the occurrence of parental effects in a multiple-stressor scenario, and whether the promotion of such mechanism might be influenced because of the complex interactions among the different environmental stressors. However, this is extremely important to understand, as the parental effects, as well as the multigenerational ones, might potentially be responsible for transgenerational effects (via methylation and histone modification) that could lead to the acquisition of traits that can become heritable and therefore have a role in adaptive evolution (Greenspoon and Spencer 2018).

1.1.3 Physiological responses to copper exposure

Copper is one of the most important metals for humans (Gravenmier et al. 2005) as it is an essential micronutrient for many biological processes since it interacts with many proteins and it drives several biochemical mechanisms (e.g. in transcriptional regulators, cell receptors and transporters, etc.) (Festa and Thiele 2011). Moreover, copper has been extensively used for human purposes like electronics and plumbing, as well as to control the growth of algae, bacteria, and fungi (Newman 2009). Its importance in many fields led to intensive extraction, which in turns have led to its presence in many surface waters around the world.

Waterborne copper may interact with wild aquatic organisms and lead to a variety of physiological effect (Johnson et al. 2007). It has previously observed that copper impairs some behavioural functions and muscle activity (Haverroth et al. 2015), as well as reduce the ability to orientate in a current (Johnson et al. 2007, Da Silva Acosta et al. 2016) in zebrafish. Additionally, copper accumulation induces oxidative stress in tissues, leading to cellular damage (Sanchez et al. 2005), as well

as reduction in oxygen consumption and therefore metabolism (De Boeck et al. 2006).

Despite its toxicity when accumulating in higher than physiological concentrations, organism might be able to respond to copper exposure via different ways, resulting in potential copper acclimation. For instance, Buckley et al. 1982 found that after several copper chronic sub-lethal exposures, coho salmon was able to acclimate to more elevated copper concentration and increase its LC50 (median lethal concentration), suggesting phenotypic physiological adjustments. Moreover, copper exposure during zebrafish embryogenesis induced reprogramming by significantly upregulating genes involved in gene metylation processes (Dorts et al. 2016). It has been also observed that many organisms were able to genetically adapt to the presence of copper in nature (Hoare et al. 1995, Fisker et al. 2011, Gerstein et al. 2014).

These studies suggest that organisms are able to cope with the presence of copper in several ways. However, the occurrence of copper in natural environments may be accompained by several other stressors that can occur indipendently, like temperature increase and hypoxia events. When copper has been associated with high temperature in killifish (Poecilia vivipara), the rate of bioaccumulation into tissues increased at higher temperatures, suggesting a temperature effect on metabolism and consequent accumulation capacity (Dornelles Zebral et al. 2019). Moreover, in a study conducted by Lapointe et al. 2011 co-exposure of heat and copper caused the most sinificant changes in gene transcription level and enzyme activity in fathead minnow (Pimephales promelas), resulting in a lower capacity to handle the heat stress, when copper was present. Although the previous studies led to a negative synergistic effect of copper in combination with high temperature, this does not seem to be as straightforward when copper was coupled with other environmental stressors, like hypoxia, where the latter seemed to play an antagonist role, at least during the early development (Fitzgerald et al. 2016a). It is crucial to understand how individuals respond to copper exposure, under unpredictable environmental variability, such as under climate change driven events, and whether the mechanisms that organisms adopt could be adaptive.

1.1.4 Inter and intra-individual variation of phenotypic plasticity

While one could think that the mean phenotypic response of a population to a certain environmental stressor is informative enough to infer on the future population evolutionary trajectories, this concept is not completely correct. Organisms use

different traits to respond to environmental perturbations, and when a population experiences the same perturbation, one would expect that all individuals respond in the same way, thereby having low phenotypic variation. However, individuals might differ not only from each other (Nikinmaa and Anttila 2019), but also from their former selves (O'Dea et al. 2021).

The population phenotypic variability may be the result of evolutionary, and/or developmental mechanisms. Depending on the environmental stressor, the population might either increase or decrease the phenotypic variation while increasing or decreasing the mean plasticity (or there is no change either in variation/and or plasticity) (Fig. 2). Such variation may not only shape the individual inner tolerance to environmental stressor, but hypothetically may influence the capacity to respond again to the same (or another) stressor, in other words, the scope of plasticity, resulting in an increase of the population tolerance. In a climate change scenario, where the climate variability and unpredictability are extremely high, it is important to assess the phenotypic response of a population at individual level, and within the same individual. In the current thesis, the individual phenotypic variation

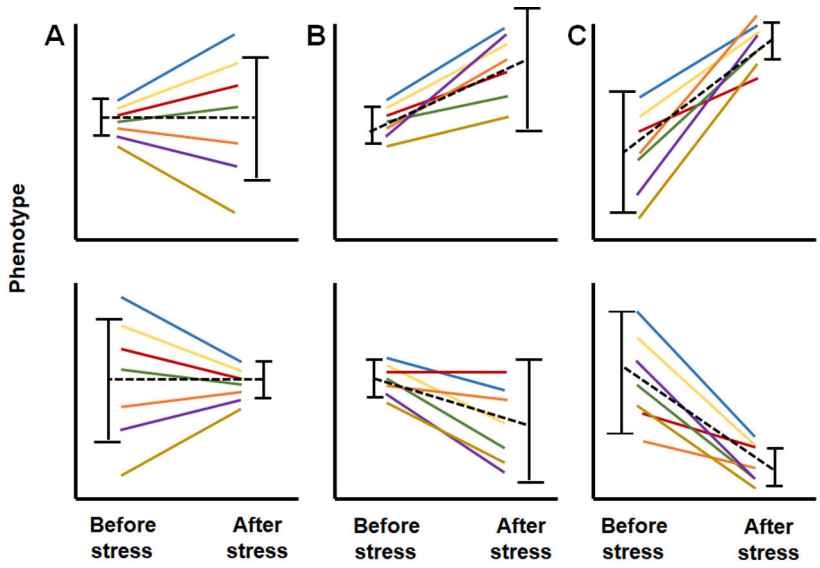

Figure 2. Representation of the potential trajectories of the population phenotypic variance after the exposure to a hypothetical stressor. A, increase or decrease of the phenotypic variance without change in the mean phenotypic response. B, Increase of the phenotypic variance with a increase or decrease of the mean phenotypic response. C, Decrease of the phenotypic variance with a increase or decrease of the mean phenotypic response.

and phenotypic plasticity has been assessed at individual level in three studies, through individual fish tagging and by using statistical models which address the phenotypic variation of thermal traits among and within individuals.

1.1.5 Biochemical responses to a warm and polluted environment

The phenotypic and short- and long-term phenotypic capacity of ectotherms to withstand environmental fluctuations are based, in part, on abilities to modulate gene expression (Logan and Somero 2011). These cellular responses depend on several features, such as the timing of exposure and the magnitude of the stress. Moreover, the cellular responses have considerable phenotypic plasticity, such that the prior state of acclimation (or acclimatization) of an individual has measurable effects on the timing and intensity of expression of many components of the cellular stress response (Somero 2020a). Therefore, the cellular stress response represents a key tool to understand the molecular basis of the physiological response of the organisms, especially in the context of climate variation. I, therefore, studied the expression pattern of the Heat-Shock Proteins, both at mRNA (hsp) and protein level (Hsp) and the Hypoxia-Inducible Factor (Hif), as molecular response to the exposure to temperature increase and copper exposure.

1.1.5.1 Heat Shock Proteins

It is now well understood that one of the heat stress responses in cells is the activation of the heat-shock proteins (Hsps). Hsps are chaperone proteins and their role is to adjust the native structures of thermally unfolded proteins, as well as to avoid the occurrence of damages to the proteome by, for example, reducing propensities for formation of insoluble aggregates of unfolded proteins, which result toxic to the cell (Somero 2020b). The heat shock protein induction results from binding a transcription factor, called heat shock transcription factor (Hsf), to a heat shock element (Hse) upstream of heat shock protein genes (Morimoto et al. 1992). Most of the inducible heat shock protein genes do not contain introns, therefore, the mRNA is rapidly translated into nascent protein within minutes following exposure to a stressor (Somero et al. 2017). This assures a rapid response to environmental stress, which contributes to enhancing the survival and health of the stressed organism.

The Hsps are divided in several protein families defined on their molecular weight, such as Hsp90, Hsp70, Hsp40 (Airaksinen et al. 1998). Each of these families shows different function in the cell. The HSP90, support various components of the cytoskeleton and steroid hormone receptors (Basu et al. 2002). On the other hand, Hsp70 assists the folding of new polypeptide chains, act as a molecular chaperone, and repairs and discard the altered or denatured proteins (Basu et al. 2002).

The heat shock response (HSR) has been characterized for a wide range of species and was found to exhibit a high degree of conservation in its basic properties from bacteria to animals (Tomanek 2010). In fish, the vast majority of studies have focused on effects of temperature stress on HSR, but several studies have shown that Hsp levels increased in fish tissues also in response to a variety of environmental and biological stressors (Iwama et al. 1999). HSR occurs in the majority of fish species, except in Antarctic notothenioids fish, which are lack the heat shock response as a result of adaptation to stable cold environment (Hofmann et al. 2005). The role of the Hsps in the acclimation response to increasing temperature alone, and in combination with other environmental stressors, has been previously studied in fish (Fangue et al. 2006, Logan and Somero 2011, Metzger et al. 2016) and invertebrates (Tomanek and Somero 1999). Indeed, besides being upregulated during a stress induced by warm temperatures, expression of Hsps is also modulated by the presence of other stressors, such as pollutants, including copper (Jing et al. 2013, Dorts et al. 2016). These studies highlight a mechanism of plasticity of the heat shock response, which can be shaped by thermal acclimation to higher, or lower, temperatures, as well as by other stressors. However, the phenotypic plasticity of the heat shock response seems to be species-specific and, therefore, reflecting the separate evolutionary histories of each species, which in turns can reflect the thermal limits and the biogeographical distributions. Moreover, it is still not clear whether the HSR occurs in short-term environmental temperature perturbations, like during an environmental heat wave. Therefore, it is important to understand whether a short temperature event may trigger the HSR, resulting in a cellular damage repair.

1.1.5.2 Hypoxia-Inducible Factor

The hypoxia-inducible factor (Hif) pathway is a key regulator of cellular O2 homeostasis and an important orchestrator of the physiological responses to hypoxia (low O2) in vertebrates (Semenza 2012, Mandic et al. 2021). Hif is a heterodimeric transcription factor that is composed of two basic helix–loop–helix proteins — Hifα and Hifβ (Schofield and Ratcliffe 2004). The Hifα/β dimer binds to a core DNA motif (G/ACGTG) in hypoxia-response elements (HREs) that are associated with a

broad range of transcriptional targets (Schofield and Ratcliffe 2004, Semenza 2012). These target genes are highly involved in systemic responses to hypoxia, such as angiogenesis and erythropoiesis, but also in cellular responses, such as alterations in glucose/energy metabolism (Semenza et al. 1994). Hif has been primarily characterized in mammals. Nevertheless, the numerous fluctuations of oxygen content in aquatic environments offered a unique opportunity to a group of researchers to investigate the role of Hif-1α in regulating the physiological responses to hypoxia in fish (Soitamo et al. 2001).

Besides being implied in the oxygen regulation, Hif-1α is also activated in response to other environmental stressors, such as copper. Copper regulates Hif activity by promoting the formation of the Hif-1 transcriptional complex (Feng et al. 2009). Moreover, it has been showed that copper sulphate (CuSO4) induces the expression of Hif-1α in breast and hepatic cancer cells promoting cell migration (Rigiracciolo et al. 2015) and, it seems to regulate the activity of prolyl-4-hydroxylase domain (PHD) enzymes, which senses the cellular oxygen partial pressure and activates Hif-1α (Martin et al. 2005).

There is increasing evidence that Hif is also involved in the response to other environmental stressors, such as thermal stress. Temperature and hypoxia have an interactive and synergistic effect in the environment (O'Brien et al. 2020). At the organismal level, temperature increase could cause higher metabolic rate and oxygen demand in fishes, potentially leading to hypoxic conditions in some tissues. For instance, it has been observed that the critical thermal maximum (CTmax) of Atlantic salmon was positively correlated with hypoxia tolerance (Anttila et al. 2013). Moreover, CTmax was lower under hypoxic conditions in Fundulus heteroclitus (Healy and Schulte 2012). In mammalian cells, Hsp90–Hsp70 complex binds Hif-1α protein protecting Hif-1α against oxygen-independent proteosomal degradation in both normoxia and hypoxia (Isaacs et al. 2002, Katschinski et al. 2004, Zhou et al. 2004). Thus increased amounts of Hif-1α as a result of increased temperatures might be a consequence of induction of heat shock proteins of the 90 and 70 family (Katschinski et al. 2002). This suggests that the oxygen and the ability to deliver it within different body compartments might be a shaping factor of thermal tolerance in fish (Rissanen et al. 2006), however recent work found contrasting results (Joyce and Perry 2020).

The upregulation of Hif-1α might also be induced as a secondary hypoxia-induced effect by copper exposure. It has been shown that copper induced Hif-1α activation in response to gills oxygen-transport impairment (Fitzgerald et al. 2019) and that increased the gill epithelium thickness impeding oxygen to flow through gills properly (van Heerden et al. 2004, Blewett et al. 2017). This caused a hypoxic condition at the tissue level, which in turns activated the Hif pathway to restore the normoxic condition. However, the compensatory response might not be enough

when fish are also exposed to environmental hypoxia, resulting in a decrease of hypoxia tolerance because of copper exposure. For these reasons, studying the expression pattern of Hif-1α becomes valuable when assessing the effect of heat waves, copper exposure and the combination of both on the physiological response of fish to stress and the phenotypic plasticity of thermal tolerance.

2 Aims

The aim of this thesis is to investigate the phenotypic response of fish to acute environmental stressors, e.g., heat wave and copper exposure through the mechanism of phenotypic plasticity using laboratory and field approaches. Moreover, to understand the basis of the phenotypic variation, the developmental plasticity and the occurrence of parental effect will be studied in fish in their early life-stage. The environmental effect will be assessed at ecological (life history traits), physiological (upper thermal tolerance) and molecular (Hsp and Hif) level, and in laboratory reared and wild fish populations living in thermally polluted and pristine environments. Moreover, the potential sex-specific response to stress exposure will be assessed. The thesis will thus address these main questions:

I. What are the molecular responses of fish after a sudden increase of temperature in controlled laboratory conditions? Is the response pattern dependent on magnitude of the thermal stress and how long does the molecular response last?
II. Does the upper thermal tolerance of fish have an adaptation potential for fish caught nearby thermally polluted areas, compared to fish from pristine areas? Is the phenotypic plasticity of the upper thermal tolerance after a heat wave exposure dependent on their thermal history?
III. Can environmental pollution (i.e. copper exposure) influence the phenotypic plasticity of thermal tolerance in populations potentially adapted to warm temperature from thermally polluted areas? What are the molecular mechanisms involved in the response?
IV. What are the combined effects of the exposure to two abiotic stressors; heat wave and copper exposure, on the upper thermal tolerance of adult fish and larvae development? What is the potential for parental effects in the offspring generation?

3 Materials and Methods

3.1 Study species

The phenotypic response to thermal and pollution stress has been assessed in two fish species, both representing valid model organisms suitable for eco-physiological and ecotoxicological studies.

3.1.1 Laboratory fish: Zebrafish (*Danio rerio*) (I, IV)

Zebrafish (Danio rerio) is a small freshwater cyprinid fish indigenous to South Asia, and in the wild broadly distributed across parts of India, Bangladesh, Nepal, Myanmar, and Pakistan (Lawrence 2007). Laboratory-reared zebrafish is undoubtedly the most widely used model species of fish in studies spanning from medicine to ecotoxicology. However, despite its extensive medical use, this species has been also studied from an ecological point of view, with a focus on thermal background (López-Olmeda and Sánchez-Vázquez 2011a, Sidhu et al. 2014, Morgan et al. 2019). Previous studies on zebrafish thermal biology have shown that laboratory zebrafish reared at 20 ºC have a Critical thermal maximum (CTmax) of 39.2 ºC ± 0.3 and a Critical thermal minimum (CTmin) of 6.2 ºC ± 0.3 (López-Olmeda and Sánchez-Vázquez 2011a). However, zebrafish acclimated to 30 ºC showed an increase of CTmax until reaching 41.7 ºC ± 0.4 and a CTmin of 10.6 ºC ± 0.5, highlighting the eurythermal nature of zebrafish (López-Olmeda and Sánchez-Vázquez 2011a). Zebrafish thermal window, like that of other fish species, is therefore shaped by acclimation as well as genetic mechanisms. However, also developmental, and multigenerational plasticity might play an important role in shaping the thermal tolerance of this species. Being an easy-to-handle and breed organism, compared to most other fish species, and having a relatively short lifespan, zebrafish is highly valuable when studying multigenerational, or developmental effect.

3.1.2 Wild fish: Three-spined sticklebacks (*Gasterosteus aculeatus*) (II, III)

The three-spined stickleback (Gasterosteus aculeatus L.) is a small mesopredatory teleost fish from the family of Gasterosteidae, with habitats that range from full marine to freshwater bodies across the Northern hemisphere (Katsiadaki et al. 2007). Many aspects of stickleback biology (ecology, evolution, behavior, physiology, ecotoxicology) have been well documented and, with a large scientific database at our disposal, three-spined stickleback has become a model system for studying many evolutionary processes, such as speciation and adaptive radiation (Ostlund-Nilsson et al. 2006). In the Baltic Sea, marine three-spined sticklebacks utilize the shallow coastal zones for reproduction, but are believed to spend a large part of their life in pelagic open sea areas (Bergström et al. 2015). There is a strong evidence for genomic divergence driven by local adaptation of stickleback populations along a salinity and temperature gradient (Guo et al. 2015) in the Baltic Sea. This suggests that the environmental heterogeneity of the Baltic Sea shaped the population structure leading to a local adaptation of this species. For these reasons, three-spined stickleback represents a very useful model species for studying the mechanisms of local adaptation and phenotypic plasticity to different thermal regimes.

3.2 Experimental design for Study I

The thermal regime of zebrafish generally spans from 18 to 34 ºC (López-Olmeda and Sánchez-Vázquez 2011b), therefore, in order to trigger a thermal stress and study its effects at molecular level thirty fish were exposed to acute heat shock (30 minutes) at 26→26°C (handling control), +5°C increase 26→31°C (mild heat shock) and +7°C increase 26→33°C (strong heat shock). After 30 minutes exposure, fish were transferred back to their original aquarium at normal rearing temperature. At this point, ten fish from each aquarium were sacrificed with cranial percussion at the following time-points: (1) immediately after the exposure, (2) two hours and (3) twenty-four hours later. Six fish that were not exposed to any of the conditions were sacrificed and used as naïve untreated control. All procedures were approved by the Finnish Animal Experiment Board (ESAVI/10413/04.10.07/2016).

3.3 Experimental design for Study II and III

3.3.1 Study area

3.3.1.1 The Baltic Sea

The Baltic Sea is a semi-enclosed postglacial sea surrounded by nine developed and industrialized countries (Reusch et al. 2018). It is a relatively young sea (8000 years old) with an average water depth of only 58 m. Moreover, being open to the North Sea, it is characterized by a strong salinity gradient from marine to almost freshwater (Reusch et al. 2018). Due to these geographical features, the Baltic Sea is currently experiencing many environmental stressors such as eutrophication, warming, oxygen depletion and acidification (Johannesson et al. 2011). However, it is one of the most intensely studied marine system with high data density and many long-term data series (Johannesson et al. 2011, Bergström et al. 2015, Reusch et al. 2018). For these reasons, the Baltic Sea constitutes a highly important coastal and marine system and an important open laboratory of coastal and marine system for conducting research on ecological as well as evolutionary aspects of the marine fauna.

3.3.1.2 Nuclear power plant as evolutionary laboratories

For probing questions around thermal adaptation on evolutionary timescales, we must rely on natural or semi-natural systems (Jutfelt 2020). Nuclear power plant areas can offer interesting opportunities since organisms living nearby these areas might have experienced increased temperatures already for generations. Moreover, since nuclear power plants are spatially replicated over relatively small areas, they represent a statistically powerful tool to make predictive inferences. In the Study II and III, six locations situated along the Finnish coastline of the Baltic Sea were chosen to study the phenotypic plasticity of thermal tolerance in three-spined sticklebacks. Two locations were situated near cooling water discharge areas of two nuclear power plants (NPP), while the others were in more pristine areas. The water in these areas is generally warmer compared to the water in pristine areas. This is because the controlled fission reaction taking place into the reactor core generates heat in the fuel bundles, which needs to be cooled by water taken from the sea. When the water passes through the reactors its temperature increases up to 10 ºC. This water must be discharged in the open sea where it finally mixes with sea water. However, although this effluent is mixing and cooling while reaching the

environment, the water of the whole discharge area remains warmer compared to pristine areas. The discharge water from the Olkiluoto and Loviisa nuclear power

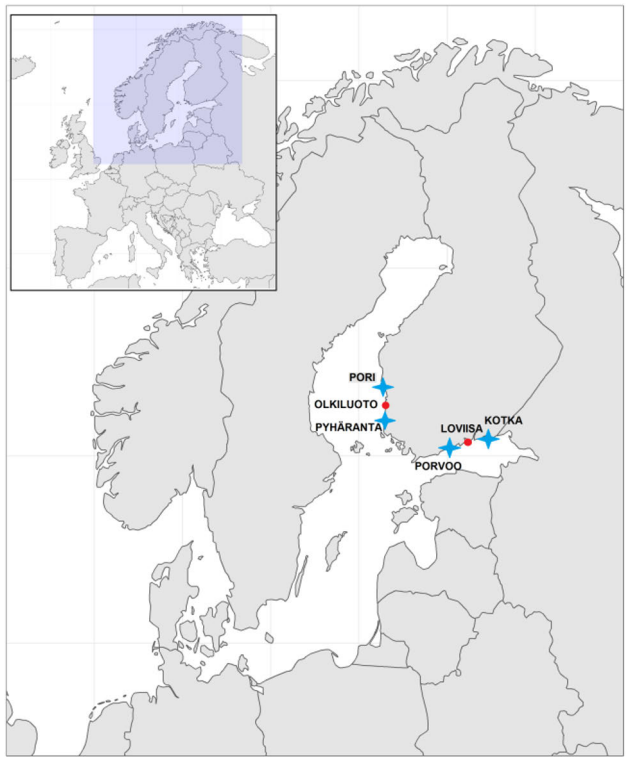

Figure 3. Map of the study area and sampling locations. Red dots indicate the nuclear power plant areas (NPP), while blue crosses indicate the control locations (CTRL).

plant has been reported to warm the temperature of the water in these areas by 2-5ºC compared to the inlet water areas (Ilus 2009). Both the plants were built during 1971-1980 and they both use around 40 to 70 m3s-1 of seawater to cool down the nuclear units. Depending on the weather conditions, a temperature increase can be observed at an approximate distance of as far as 3-5 km from the discharge point. This causes changes also in the ice conditions, as the cooling water discharge area remains unfrozen throughout the winter. The size of the unfrozen and weak ice area varies depending on the winter in Finland, being maximally around 7 km2 (Ilus 2009).

Adult individuals of three-spined sticklebacks were sampled during May 2018 from Olkiluoto nuclear power plant (OLK, NPP), Pori (POR, CTRL) and Pyhäranta (PYH, CTRL) in the Gulf of Bothnia and from the Loviisa nuclear power plant (LOV, NPP), Porvoo (POO, CTRL) and Kotka (KOT, CTRL) in the Gulf of Finland.

Fish were transferred to the fish facilities of the University of Turku, where they were reared. In order to evaluate the individual phenotypic plasticity, the fish were tagged and allow to recover for 2 weeks before experiments. The upper thermal tolerance of each fish was measured using the Critical thermal maximum (CTmax) method (Sidhu et al. 2014). After two weeks of recovery from the first CTmax measurement (CTmax1), fish were exposed to three different conditions for one week: handling control (CTRL), heat wave exposed (HW, 16→26°C) and copper exposed (Cu, 100 µg/L). During the one-week exposure, a group of fish (N = 25) from each condition was sampled and sacrificed at two different time points for molecular analyses: (1) 4 days of exposure and (2) 7 days of exposure. The remaining fish from each condition continued the exposure until the 7th day, when the second CTmax (CTmax2) was performed to assess the effect of each exposure on the individual phenotypic plasticity of the thermal tolerance. Therefore, the phenotypic plasticity of thermal tolerance was calculated at the individual level by subtracting the CTmax1 from the CTmax2 relative to each individual (Phenotypic plasticity = CTmax2 – CTmax1). All fish procedures were performed according to the Finnish Animal Care permission (ESAVI/2867/2018).

3.4 Experimental design for Study IV

In the previous studies of the current thesis, the phenotypic plasticity of thermal tolerance has been assessed at the individual level under exposure to a single source of stress, artificial heat wave or copper exposure. In Study IV, phenotypic plasticity was assessed under the combination of the two stressors together. Adult, individually tagged, zebrafish were reared in the fish facilities of the University of Turku. The first CTmax was measured for each fish two weeks after tagging. Thereafter, fish were divided into four groups and exposed to the following conditions for one week: (1) 27 ºC (control), (2) 27 ºC + 25 µg/L of copper, (3) 33 ºC (heat wave condition), (4) 33 ºC + 25 µg/L of copper in filtered tap water…At the sixth day of exposure, fish were moved to reproduction tanks and kept there overnight for the breeding under the same exposure conditions. CTmax of adults was measured again after reproduction and thereafter fish were sacrificed for the molecular analyses.

The embryo produced by each group were split into two water conditions: (1) same water condition as the parents and (2) control water condition. Another group of adult fish that were not tested for CTmax and/or exposed to any of the conditions were also allow to reproduce, and the offspring generation was exposed to heat wave and/or copper exposure for 5 days to unveil the developmental effect in zebrafish larvae. During the exposure, life-history traits (mortality, hatching success, embryo size and growth) were recorded. Moreover, the occurrence of malformations

(oedema, spinal malformation, and swimming bladder inflation) were checked. CTmax and molecular analyses were also performed on hatched larvae at 5 days post-fertilization (5dpf). The experimental design was repeated 6 times in total. All fish procedures were performed according to Finnish Animal Care permission (ESAVI/32228/2020).

3.5 Quantification of the thermal tolerance

Thermal tolerance is usually quantified with two different experimental methods, labelled 'static' and 'dynamic' (Rezende et al. 2011). In the current thesis, the thermal tolerance was assessed using the dynamic method (introduced and defined by Cowles and Bogert 1944), which defines the Critical thermal maximum (CTmax). During the CTmax measurement the organisms are exposed to gradual increase of water temperature until "the locomotory activity become disorganized and the animal loses its ability to escape from conditions that will promptly lead to its death" (Cowles and Bogert 1944). Therefore, this method allows to measure the maximum temperature that an organism might potentially tolerate (short-term tolerance) given its physiological condition in the absence of any other hazard (Santos et al. 2011). CTmax test is a well-known and extensively used test in animal physiology (Lutterschmidt and Hutchison 1997, Morgan et al. 2018). The endpoint at which the organism locomotory activity becomes disorganized is also called Loss of Equilibrium (LOE).

In the current studies, the CTmax measurements of three-spined sticklebacks and zebrafish were performed as described in Sidhu et al. (2014). Briefly, fish were placed into an experimental aquarium/set up and allow to familiarize with the new environment for one hour at 16 °C (Study II and III) or at 27 °C (Study IV). Thereafter, water temperature was increased by 0.3 °C/min until 27 °C (Study II and III) or 37 °C (Study IV), after which the thermal ramp speed was decreased to 0.1 °C/min until loss of equilibrium (LOE). LOE was defined differently for stickleback and zebrafish. For sticklebacks it was defined as the absence of active movement after a gentle poke, while for adult zebrafish LOE was indicated by the fish turning upside down for the first time. For the zebrafish larvae LOE was defined as the loss of response to stimulus applied to the tail of the larvae (three pokes with three seconds between them) as described by Andreassen et al. 2020. When an individual lost equilibrium, the temperature was quickly recorded, and the fish was removed from the setup and placed in a recovery tank. CTmax was usually measured every day at the same time in order to minimize potential diel fluctuations (Lydy and Wissing 1988). Post-trial mortality was followed for the two weeks of recovery.

3.6 Molecular measurements

3.6.1 Sample processing

The molecular response to heat stress was assessed by studying the expression pattern of the Heat Shock Proteins (Hsp70, Hsp90) at mRNA (Study I) and protein level (Study I-IV) and the Hypoxia-Inducible factor (Hif-1α) (Study III and IV). In the Study I and IV, zebrafish were flash frozen in liquid nitrogen immediately after the sampling and white muscle tissues were used for molecular analyses. In the Study II and III, the liver tissue of three-spined sticklebacks was collected and immediately frozen in liquid nitrogen. Sample processing was performed at the University of Turku.

3.6.1.1 mRNA (Study I) and protein extraction (Study I-IV)

Isolation of RNA from the white muscle pieces was performed by homogenizing the samples in TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and 1-bromo-3-chloropropane (BCP, Sigma Aldrich, St. Louis, MO, USA). The samples were also treated with DNase I (Promega, Madison, WI, USA). The amount and purity of RNA was measured with Nanodrop 2000 (Thermo Scientific, Wilgminton, DE, USA) and only samples with an A260/280 ratio of $\geq$ 1.8 were used in downstream applications. RNA integrity was confirmed by agarose gel electrophoresis (Aranda et al. 2012). Only samples with conspicuous 2:1 28S to 18S rRNA ratio were used for the cDNA synthesis.

Protein characterization was performed according to Mottola et al. (2020). Tissues were homogenized in 6 volumes of lysis buffer (62.5 mM Tris-HCl, 1 µg m-1 leupeptin, 1 µg m-1 pepstatin, 1 mM PMSF, pH 6.8) using TissueLyser (Qiagen, Hilden, Germany). Lysates were centrifuged at 10,000 g for 10 min at 4°C. Supernatants were denatured in Laemmli buffer (Laemmli 1970) for 7 min at 95°C. Protein concentrations were determined using BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA) and the protein concentrations were read at 570 nm using a Wallac EnVision 2103 Multilabel Reader (Perkin Elmer, Turku, Finland).

3.6.1.2 RT-qPCR for mRNA expression (Study I)

RNA was reverse transcribed to cDNA. The primers for hsp70 and reference genes β-actin and Rplp0 mRNA were obtained from Tiedke et al. 2013. Quantitative PCR was conducted in QuantStudio 12K Flex Real Time PCR System (Life Technology, Carlsbad, CA, USA) located in Turku Centre of Biotechnology. Each plate contained also non-template controls to detect potential contamination in

reaction mixes. QuantStudio 12 K Flex software version 1.2 (Life Technology) was used for analyzing the data. All primer pairs gave a single peak in the dissociation curve and PCR efficiency was within the range of 104% to 110%. Target (hsp70) and reference gene (rplp0 and β-actin) reaction quantities were determined from a standard curve generated from a 1:1 to 1:64 serial dilutions of randomly chosen and pooled samples. The relative expression level was calculated with 2ΔΔCT method (Livak and Schmittgen 2001) against the average expression level of genes in fish from untreated control group using Rplp0 expression in normalization.

3.6.1.3 Protein quantification (I-IV)

The quantification of proteins was performed using Western Blot. The samples were loaded in acrylamide gels, 12% (BioRad, Cat#1610185) and the proteins were separated by size. From the gels the proteins were transferred to a Whatman nitrocellulose membrane, pore size 0.45 µm (Perkin Elmer, Boston, MA, USA) incubated in Tris-buffered saline (TBS) blocking solution containing 5% non-fat powdered milk. After that, membranes were incubated overnight with primary antibody in TBS-0.1% Tween-5% milk at +4°C. Next morning, the membranes were incubated in TBS-0.1% Tween-5% milk with secondary antibody for the detection of bands, respectively. After TBS-0.1% Tween membrane washing, the bands were visualized at ChemiDoc MP Imaging System (Biorad, Hercules, CA, USA). Densitometry was performed using ImageLab. Each gel contained gel loading control sample to take gel-to-gel variation into account in calculations. For estimating the relative protein levels, band intensities were divided with total protein gel/control protein band intensities. More details about the Western Blot protocol are given in Table 1.

Table 1. Tissue type and mass, loaded protein concentration into each well and the antibodies utilized to measure the protein concentrations.

	Study I	**Study II and III**	**Study IV**
Tissue	White muscle	Liver	white muscle (adult) whole body (larvae)
Tissue mass	25 mg	25 mg	
Protein concentration	20 ug	20 ug	20 ug (adult) 4 ug (larvae)
Primary Antibody			

Hsp70	rabbit polyclonal anti-salmonid inducible Hsp70 (1:5,000) (AS05061A) (Agrisera, Vännas, Sweden)	mouse monoclonal Anti-Hsp70 (1:10,000) (SAB4200714) (Sigma-Aldrich, St. Louis, MO, USA)	mouse monoclonal Anti-Hsp70 (1:10,000) (SAB4200714) (Sigma-Aldrich, St. Louis, MO, USA)
Hsp90		mouse monoclonal Hsp90 beta (1:10,000) (ab53497) (Abcam, Cambridge, UK)	
Hif-1		rabbit polyclonal Hif-1 alpha (1:2,000) (ab2185) (Abcam, Cambridge, UK)	rabbit polyclonal Hif-1 alpha (1:2,000) (ab2185) (Abcam, Cambridge, UK)
ß-actin	rabbit polyclonal anti-β-actin (1:5,000) (ab8227) (Abcam, Cambridge, UK)		
Secondary Antibody			
Hsp70	HRP-conjugated Goat Ab to Rb IgG (1:10,000) (AB6721) (Abcam, Cambridge, UK)	IRDye 800CW Goat anti-Mouse IgG (1: 10,000) (Licor, Lincoln, NE, USA)	StarBright Blue 700 Goat Anti-Mouse IgG (1:10,000) (Bio-Rad, Hercules, CA, USA)
Hsp90		IRDye 800CW Goat anti-Mouse IgG (1: 10,000) (Licor, Lincoln, NE, USA)	
Hif-1		Goat Anti-Rabbit IgG (1:10,000) StarBright Blue 700 (Bio-Rad)	StarBright Blue 800 Goat Anti-Rabbit IgG (1:5,000) (Bio-Rad, Hercules, CA, USA)
ß-actin	HRP-conjugated Goat Ab to Rb IgG (1:10,000) (AB6721) (Abcam, Cambridge, UK)		

3.6.2 RAD sequencing and genetic library construction (Study II)

In order to quantify the genetic divergence among the three-spined sticklebacks from different locations, the genomic DNA was extracted from caudal fin using a salt extraction protocol. Library preparation, sequencing and demultiplexing was conducted at an external service provider (MGX – Montpellier GenomiX, Montpellier, France). RAD library was prepared according to the protocol described by Baird et al. 2008. Sequencing was performed on an Illumina NovaSeq 6000 using a NovaSeq Reagent Kit. Altogether, 745,126,832 reads were retained after quality filtering and 30 individuals were sequenced per location. The obtained RAD-data were analysed using Stacks (version 2.41) (Catchen et al. 2011, 2013). Thereafter, reads were realigned to the latest three-spined stickleback reference genome (Nath et al. 2021). SNP calling was carried out with Stacks v2.0 (Catchen et al. 2013) using gstacks module. In order to exclude markers associated with sex determination in three-spined stickleback as the analysed samples contained varying proportion of males and females (despite the attempt to equalize sex ratio), we performed an association testing for sex determination using the egscore function implemented in GenABEL (Aulchenko et al. 2007). To perform population structure analyses, we further filtered our dataset, removing SNPs deviating from Hardy-Weinberg equilibrium using PLINK (Purcell et al. 2007). We estimated the level of pairwise population genetic differentiation using the unbiased FST estimator (Weir and Cockerham 1984) in the StAMPP R package (Pembleton et al. 2013, Pembleton and Pembleton 2020). Significance of FST values and 95% confidence intervals were computed using bootstrapping as implemented in the package. To visualise population structure, a discriminant analysis of the principal components (DAPC) was performed with R/adegenet (Jombart and Ahmed 2011). The optim.a.score function was used to choose the optimum number of PCs to retain.

3.6.3 Statistical analyses and Softwares

The statistical analyses were performed using SigmaPlot14 (Study I) (SyStat Software, San Jose, CA, USA) and RStudio version 3.6.1 (Study II, III, IV) (R Core development team 2019). The residual distribution of morphological, physiological, and molecular variables was visually assessed using DHARMa (Hartig and Hartig 2017) or performance (Lüdecke et al. 2021) in R environment, while a Shapito-Wilk

and Brown-Forsythe test were used to check for normality and equal variance, respectively in SigmaPlot. When needed, data were transformed using log-transformation to fulfil the assumptions of normality. Linear model (LM), general linear model (ANOVA), linear mixed effect models (LMM), generalized linear models (GLM) and generalized linear mixed effect model (GLMM) were fitted using the lm, glm and aov function in stats or lmer function in the lmerTest package (Kuznetsova et al. 2017) and with the function glmer in the lme4 package (Bates 2010). Minimum adequate models were derived by model simplification using F or chi-squared tests based on analyses of deviance (Crawley 2007). Multiple comparisons for all the models were performed using the function glht into the "multcomp" package (Bretz et al. 2021) and considering the covariates and the interactions (Hothorn et al. 2008), when needed (Study II and III). The pairwise contrast was estimated with Tukey test using the package emmeans (Russell 2019) (Study IV). Data visualization has been performed using ggplot2 (Wickham 2016). Model structures of the different studies are showed in Table 2.

Briefly, in Study I, to assess the differences in the expression pattern of the hsp70 mRNA and Hsp70 protein by exposure temperature and at different timepoints, a 2-way ANOVA was performed.

In Study II, a linear mixed effect model was utilized in order to assess whether the CTmax change was significant among populations, sex, and exposure to heat wave or control. In the model, fish individual has been run as a random factor in order to account for repeated measures of the same individual. Mass has been used as a covariate in the model. Moreover, the differences in the individual plasticity (CTmax2 – CTmax1) were assessed by running a linear model and using population and sex as fixed factor. Mass has been used as a covariate in the model. A linear model has been run to understand the relationship between the CTmax1 (innate thermal tolerance) and the phenotypic plasticity of each individual (CTmax2 – CTmax1). This has been assessed by using population as a fixed factor to see whether this relationship was depending on the thermal history of each population. In order to study the differences in the expression pattern of the protein among population, exposure (control and heat wave) and sampling time point, a 1-way ANOVA has been run.

In Study III, a linear mixed effect model was utilized in order to assess whether the CTmax change was significant among populations, sex, and exposure to copper or control. In the model, fish individual was run as a random factor to account for repeated measures of the same individual. In order to study the differences in the expression pattern of the protein among populations, exposure (control and heat wave), a linear mixed effect model was run using the sampling day as random factor in order to account for variability given by different sampling time.

In Study IV, the life-history traits (mortality, hatching success) and the malformation occurrence in dependence of the exposure (developmental plasticity) and parental exposure (parental effect) to heat wave, copper, and their combination in larvae of zebrafish was assessed by running a generalized linear model or a generalized linear mixed effect model (using replicate experiment as a random factor when this was having a significant effect on the dependent variable) with a binomial error structure. A linear mixed effect model was utilized in order to assess whether the CTmax change was significant among different treatments (heat wave, copper and combination) in adult zebrafish. Sex was included as fixed factor, while fish identity was included as random factor for accounting the repeated measurement of the same individual. The protein expression pattern was assessed by using a linear mixed effect model, using heat wave temperature and copper exposure, and interaction, as fixed factors. The number of replications of the experiment was considered as random factor in one analysis as this was having a significant effect on the protein expression when previously tested.

More information about the statistical analyses is available in the publications.

Table 2. Statistical models and structures utilized in each Study.

Study	Structure	Model
I	**Molecular response ~ Timepoint + Temperature*	2-way ANOVA
II	*Temperature ~ Trial + Population + Mass + Sex + Mass:Sex + Trial:Population + (1 | fish identity).*	LMM
	Plasticity ~ Population + Sex + Mass.	LM
	Plasticity ~ CT_{max1} + Population.	
	**Molecular response ~ Population/Temperature/Timepoint*	1-way ANOVA
III	*Temperature ~ Trial + Sex + Trial:Sex + Population + (1 | fish identity).* *Molecular response ~ Exposure group + Population + Sex + (1|Day of sampling).*	LMM
IV	***Life history traits/Malformations ~ Temperature * Treatment*	GLM, GLMM
	*Temperature ~ Time * Temperature * Treatment * Sex + (1 | fish identity)*	LMM
	**Molecular response ~ Temperature * Treatment + (1|Replicate)*	
	**Molecular response ~ Temperature * Treatment*	2-way ANOVA
	*Size/Temperature ~ Temperature * Treatment*	

*Molecular response: mRNA and/or protein expression
**Mortality (both in adults and larvae), Hatching, Spinal malformation, Oedema, Swim bladder inflation (only in larvae)

4 Main results and discussion

The future projections of climate change are predicting increasing occurrence of extreme thermal episodes, like heat waves. Therefore, it is necessary to study the phenotypic responses of wild organism under thermal extremes and the potential implications for evolution in thermally unpredictable climate scenario. Moreover, the heat waves are not the only stressor that may affect the thermal plasticity of fish, and multiple stressors, which occur at the same time, generally affect the phenotypic strategies that wild populations adopt. Therefore, besides understanding the impacts of climate warming on fish, it is fundamental to understand the evolutionary trajectories of the thermal tolerance and its plasticity in a complex environmental scenario. Therefore, the aim of the current thesis is to evaluate the thermal response of laboratory and wild fish populations belonging to different thermal niche (temperate and tropical), at different life stages and at two main levels of biological organization, under acute thermal episodes and in presence or absence of another environmental perturbation (copper). Moreover, I assess whether a long-time exposure to thermally polluted areas of nuclear power plant has resulted in evolutionary thermal adaptation to elevated temperature, what are the potential limits for thermal adaptation in the wild and how this reflect on the capacity of fish to respond to sudden extreme events.

4.1 Molecular mechanisms behind the thermal response

4.1.1 Cellular response to changing temperatures (Study I, II, IV)

In the current thesis, I studied the patterns of expression of the Hsp70, Hsp90 and Hif-1α as molecular responses for assessing the biochemical mechanisms

underlining the thermal tolerance and its phenotypic plasticity at physiological level. The magnitude of the heat stress chosen was species-specific, making comparison between studies difficult. However, while comparisons between species may be speculative, the expression pattern in response to different exposure timing is important to assess.

In Study I, the aim was to assess the molecular response of fish to a sudden temperature increase, how long the response lasts and whether the response was triggered only at mRNA level or also at protein level in dependence of the magnitude of the heat stress. The Hsp70 transcription-translation relationship was function of the magnitude of the heat shock, with zebrafish exposed to the highest temperature (33 ºC; 7 ºC increase from control) showing a coupling of the transcription and translation, compared to the fish exposed to milder temperature, in which the mRNA for the hsp70 was expressed, while the protein was not translated. This result is extremely important as it shows that studying the expression pattern of the proteins, and not only the gene transcription, is crucial to understand the molecular mechanisms that underpins the physiological response. This is because the protein has the key role in regulating the cellular response to heat stress, and not its relative mRNA only. However, the coupling of transcription-translation as a response to the heat shock was observed only immediately after the 30-minutes exposure, and the protein translation decreased already after 2 hours of recovery until it returned to control level after 24 hours, suggesting that the cellular heat shock response was immediate and decreases in relative short recovery time. These result are, however, species-specific, with stenothermic species with low upper thermal tolerance showing a coupling of the transcription and translation at lower temperature than eurythermal species having higher upper thermal tolerance (Lewis et al. 2016). This suggests that the coupling not only depends on exposure temperature, but also on the thermal niche of the species.

Contrary to Study I, during which the expression of the Hsp70 was studied after a sudden acute exposure to heat stress, in Study IV the molecular changes in the Hsp70 expression were evaluated after 1-week exposure to heat wave, copper exposure and the combination of both in adults and larvae of zebrafish. Furthermore, in Study IV the relationship between the critical thermal maximum of fish and the protein expression pattern was investigated. There were some technical issues during the experiment associated with copper contamination in the water supply, which add some uncertainty to the results, but the findings, nevertheless, add important insights to our understanding of the response of fish to heat waves. The expression of the Hsp70 increased after one week of exposure in zebrafish from Study IV, compared to the control group, suggesting that the translation of the Hsp70 was kept active during the whole acclimation. It should be noted that in Study IV, the fish handling occurred several times because of the repeated measurements; therefore, the

expression of the Hsp70 might have been impacted by the fish handling. However, all the fish were handled equally during the experiment, suggesting that the differences between exposure groups in this Study can likely be attributed to the temperature conditions. Nevertheless, addition of a naive group (which was not exposed to handling), would have represented a more reliable handling control. Furthermore, it is important to point out that the expression of the Hsp70 in Study IV was measured immediately after a CTmax challenge in fish exposed to 1-week long heat wave. CTmax represents a thermal shock for fish, implying the activation of the cellular stress response. However, the evidence that only the heat wave exposed group increased the expression of Hsp70 after the CTmax measurement suggests that the mechanism of translation was upregulated in this group, probably due to the greater accumulation of mRNA before the CTmax determination, compared to the control. This suggests that either zebrafish may be especially vulnerable to thermal increase, or that they possess a susceptible molecular thermal response, which is able to restore the thermal capacity.

On the other hand, in Study II, sticklebacks that were exposed to 1-week long heat wave (26 ºC; 10 ºC increase from control) were not having higher expression pattern of either of the two Hsp families studied (Hsp70 and Hsp90), compared to control fish. Also in this Study, the aim was to assess the molecular response to the heat wave using the expression profile of the Hsps. The lack of response in sticklebacks was observed during both the 4th and 7th day of exposure, suggesting that the molecular heat stress response depends on the species and how severe the heat stress is for the species. The lack of Hsp expression in sticklebacks from Study II, after an heat wave acclimation, was unexpected and in contrast with previous findings (Logan and Somero 2011, Oksala et al. 2014). Indeed, some studies have shown an upregulation of the Hsps and/or mRNA in fish acclimated to different temperatures for long and short periods (Logan and Somero 2011, Madeira et al. 2014, Oksala et al. 2014). There are several explanations why such response was not observed in stickleback.

First, the molecular heat stress response has been observed to be immediate in the cells (Study I, Schulte et al. 2011). However, this might not be the case when more gradual exposure and longer acclimation period are performed. The heat shock response is upregulated when cells are exposed to a stress that impairs the proper structure and function of the proteins. If this stress is absent, no upregulation of the chaperones is observed in the cell (Study I, no protein upregulation in mild stress). The sampling timepoint for sticklebacks of the Study II were at the 4th and the 7th day of acclimation. It could be that these timepoints were not optimal for observing a molecular heat stress response, as sticklebacks were already acclimated to the heat wave temperature by the 4th day and the Hsps, that most probably were upregulated

during the initial phase of the heat wave exposure, went back to control levels before 4 days of exposure and stayed at control level thereafter.

Second, the sticklebacks were extremely plastic when adjusting their thermal tolerance in response to heat wave, suggesting that the heat wave acclimation was not representing a stress temperature for sticklebacks and did not induce any molecular heat stress response, especially because the mode of increase was gradual. Both sticklebacks in Study II and zebrafish in Study IV increased their upper thermal tolerance (CTmax) after the exposure to heat wave, suggesting that an acclimation response was triggered. However, the molecular response was found to be different between the two species, with only zebrafish expressing high level of Hsp70 after the heat wave. This evidence suggests that the two species have different plasticity levels, with wild sticklebacks generally showing a higher plasticity compared to the laboratory reared zebrafish. Sticklebacks from the Baltic Sea were experiencing high temperature daily fluctuation during spring-summer time, most probably inducing a higher plasticity compared to the zebrafish population that, despite being eurythermal in the wild, were acclimated to stable temperatures in laboratory conditions for generations. The heat wave temperatures chosen in the current studies were mimicking heat waves occurring in nature in both the Baltic Sea (recorded by temperature data loggers) and in zebrafish wild habitat. The different molecular response between the two species underpins the different thermal plasticity, with zebrafish being under higher cellular stress and potential damage, which did not occur in sticklebacks. Therefore, the latter might have had carried a molecular response at mRNA level, which has not been translated into proteins as no induction was triggered after 1-week acclimation.

4.1.2 Cellular response to copper exposure (Study III, IV)

The Hsp expression was also studied in response to copper exposure, to understand whether population with different thermal history were showing different Hsp expression patterns in response to copper exposure, compared to control population living in pristine areas. Moreover, differences between copper exposed and control exposed fish were studied to assess whether copper was triggering the heat shock proteins expression. The expression pattern of the Hsps did not change after the exposure to a sub-lethal copper concentration in sticklebacks from Study III and zebrafish in Study IV. Previously, copper exposure has, however, been shown to induce a cellular stress response in fish (Hernández et al. 2006, Sappal et al. 2015b). However, this cellular response did not seem to affect the protein structure in cells. Nevertheless, several other mechanisms in response to copper exposure

might have been triggered, which could not be proven by these studies. Copper toxicity, including copper transport into the cells and its interactions with ion transport processes, depend on the valence and complex formation of copper ions, which are redox-active (Bogdanova et al. 2002). One possible response to copper is represented by the increased oxidative stress and the relative activity of the anti-oxidative enzymes which would counteract the increase of reactive-oxygen species (ROS) production and relative damage (Braz-Mota et al. 2017). While it has been shown that these mechanisms are activated within the cell in response to pollutants (Braz-Mota et al. 2017), this evidence could not be proven in the current study. Nonetheless, the heat stress response was not activated by the presence of copper at these concentrations suggesting that copper metal did not contribute to increase the protein improper folding in the current Study. Previously copper has been shown to induce Hsp expression in studies using higher copper concentration (Dorts et al. 2016) or different cyprinid species (Jing et al. 2013), thus, no response in our study indicates that the expession depends on the concentration of copper and species sensitivity.

The Hif-1α expression was also utilized to study the effect of copper exposure on the thermal phenotypic plasticity of fish. The expression of the Hif-1α was not increased in zebrafish exposed to copper in Study IV, and sticklebacks in Study III. The concentration of copper in the two studies was different (25 µg/L in zebrafish vs 100 µg/L in sticklebacks) but represented a sub-lethal concentration (Gravenmier et al. 2005). Sticklebacks exposed to copper for 1-week not only kept the expression of Hif-1α stable in both 4th or 7th day of exposure but were able to increase their upper thermal tolerance after 1-week exposure. This suggests that copper was somehow beneficial to those fish. The lack of Hif-1α activation, together with an improvement of the upper thermal tolerance, suggests that copper was not causing any physiological damage to the fish gills or other organs, and, most importantly, did not trigger a hypoxemic condition in fish tissues. However, these results need to be interpreted with caution due to the high levels of mortality, which may have resulted in selection for tolerant phenotypes. The molecular mechanisms underpinning the upper thermal tolerance improvement after copper exposure, however, is left uncovered. Nevertheless, I propose that Hif-1α, which has been suggested to be potentially involved in an improvement of the thermal capacity (Kawabe and Yokoyama 2012), is not actually directly responsible of such improvement, as shown also by Joyce and Perry 2020. On the other hand, as observed for the Hsp response, the Hif-1α activation might have been immediate, which in turns activate numerous pathways for keeping oxygen homeostasis into the tissues. Therefore, the lack of difference in Hif-1α concentration among the groups in sticklebacks might be due to ongoing cellular process, initially triggered by the

activation of Hif in the early part of the exposure, whose accumulation has been shown to disappear in 48h (Rissanen et al. 2006).

Another important aspect to take into consideration when studying the molecular response to environmental stressor, such as copper exposure, is that the response might be tissue specific. Previous studies have shown different pattern of the expression in tissues from the same organism (Iwama et al. 1999, Yoo and Janz 2003, Wang et al. 2007). The Hsps and Hif-1α were studied in white muscles in zebrafish, whereas in stickleback they were measured from liver. The difference of response between the two species from these studies might, therefore, be due to also tissue specific pattern of expression of these proteins or transcription factor.

4.2 Phenotypic plasticity of thermal tolerance under acute exposure

The aim in the Study II, III and IV, was to assess the phenotypic plasticity of thermal tolerance at individual level, before and after an exposure to a heat wave (Study II), copper exposure (Study III) in sticklebacks, and after a heat wave, copper exposure and combination of the two stressors in adult zebrafish (Study IV).

4.2.1 Heat wave (Study II and IV)

The survival of an individual under acute thermal extreme depends on many factors, among which is the thermal plasticity, i.e. the capacity to acclimate to elevated temperatures. In the current research, the thermal plasticity was assessed in two fish species belonging to different thermal niches. For this reason, the heat wave magnitude was shaped based on species-specific thermal windows and the environmental temperatures they might experience in nature (heat wave in sticklebacks: +10 ºC, heat wave in zebrafish: +7ºC), moreover the thermal ramps (CTmaxes) started from different acclimation temperatures, making comparison between species somewhat difficult. Nevertheless, both species were able to adjust their thermal tolerance in response to an acute thermal exposure, i.e., one-week long simulated heat wave. The plasticity of the upper thermal tolerance was therefore positive in all the individuals exposed to heat wave, and in both species (Fig. 4 B). Descriptions of such response have been documented in the previous literature and in different species, suggesting that fish are able to respond plastically to elevated temperatures (Fangue et al. 2006, Healy and Schulte 2012). However, most of the abovementioned studies focused on the acclimation response after longer exposure periods, (Underwood et al. 2012, Narum et al. 2013, Stitt et al. 2014, Penney et al.

2021) although also few has measured a short term responses, like Vinagre et al. 2016. The magnitude of the plasticity, however, depends on many factors, like the

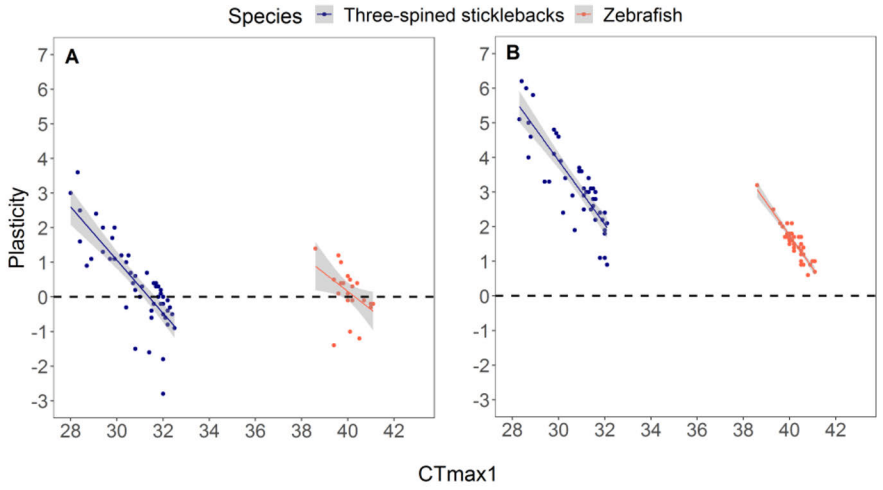

Figure 4. Relationship between initial upper thermal tolerance (CT_{max1}) and phenotypic plasticity of thermal tolerance ($CT_{max2} - CT_{max1}$) in three-spined sticklebacks (blue) and zebrafish (orange) after the exposure to handling control condition (A) and 1-week heat wave (B). The statistic was obtained running a linear regression model (LM).

time of exposure, intensity of thermal stress, speed of warming, as well as individual thermal history and life stage. In the current research the phenotypic plasticity was positive in both species (stickleback: +3.20 ºC, zebrafish: +1.06 ºC) after an acute thermal event; therefore, I propose that the acclimation mechanisms might occur also within few days of exposure, which contributes to lowering the occurrence of overheating, and consequently death, when temperature rises over a certain thermal window in acute manner (however, other study suggests that thermal acclimation requires at least three weeks, Hazel and Sellner 1980).

On the other hand, while three-spined sticklebacks increased their thermal tolerance also after the exposure to handling control conditions (plasticity: +0.6 ºC), this was not observed in zebrafish (plasticity: 0 ºC). Both species underwent the same measurement treatment; with the CTmax2 occurring after three weeks from the CTmax1. As mentioned before, the phenotypic plasticity is function of the exposure period and magnitude of the thermal increase, as well as the species. For instance,

the increase of the CTmax2 in three-spined sticklebacks might be the result of a thermal acclimation to the CTmax1 trial (Fig. 4 A). Such acclimation response after a thermal ramp has been previously observed in other species and it is named as heat hardening (Bilyk et al. 2012), which in this case seems to last after three-week recovery period. On the contrary, zebrafish lacked of heat hardening despite this has been previously observed in laboratory-reared zebrafish after one-week recovery from a CTmax measurement (Morgan et al. 2018). Acclimation is a rapid and reversible change in the phenotype (Weldon et al. 2011), and its reversibility has been shown to occur already after only one day in arthropod (Hoffmann et al. 2003, Weldon et al. 2011, Clemson et al. 2016, Sørensen et al. 2019). I, therefore, hypothesize that the lack of hardening in zebrafish in this study was the result of a reversible-type acclimation response occurring within the three-week recovery time. I also propose that the speed of reversibility is species-specific in fish since sticklebacks were not able to fully recover at same time as zebrafish. Although the magnitude of temperature change used during the CTmax measurement is considerably large, its duration is typically short (within hours). However, one-week heat wave used in this study triggered an acclimation response via phenotypic plasticity immediately after the exposure, suggesting again that the acclimation response is function of several factors, such as timing of exposure as well as recovery. I suggest future studies assessing the effect of longer thermal exposures (and recovery period) on the acclimation duration in fish.

There was a negative relationship between the initial thermal tolerance (CTmax1) and acclimation response to heat wave in both the species (Fig. 4 A, B). In other words, fish initially more thermal tolerant were less plastic after the heat wave (and handling control exposure). Such phenomenon has been previously found in many ectotherms as reviewed by Vinagre et al. 2016, after which a tolerance-plasticity trade-off hypothesis has been proposed (Barley et al. 2021, Sasaki and Dam 2021). Besides proving basis for tolerance-plasticity trade-off hypothesis, those studies also affirm that the mechanism for this hypothesis is species-specific, and it depends on demographic processes, as well as on genetic constrains limiting thermal acclimation (Donelson and Munday 2012, Seebacher et al. 2012). Therefore, organisms living in more seasonal/variable environment generally show lower thermal tolerance and higher acclimation capacity (plasticity), and organisms living in tropical, and more thermally stable areas, would show high thermal tolerance while being less plastic (Vinagre et al. 2016). I can confirm this hypothesis given that stickleback inner thermal tolerance was 30.7 ºC and its average acclimation breath was 3.2 ºC; while zebrafish had an inner thermal tolerance of 40.2 º C and their acclimation breadth was only 1.6 ºC. However, the trade-off slope in heat wave exposed fish was the same in both species (slope: -0.9, Fig. 4 B) despite the different

magnitude of the heat wave, meaning that the magnitude of the heat wave was proportional to each species thermal window.

4.2.2 Copper exposure alone and in combination with heat wave (Study III and IV)

Exposure to a sub-lethal concentration of copper showed species-specific response of the thermal tolerance. Unexpectedly, the exposure was increasing the thermal tolerance of sticklebacks, after one week of exposure (Fig. 5 A), resulting in a copper-induced thermal acclimation capacity. Zebrafish, on the other hand, did not increase their thermal tolerance after the exposure, suggesting that the copper triggered a different response in this species. To the best of my knowledge, the ability of sticklebacks to improve their thermal tolerance in response to copper exposure has never been reported before. Nevertheless, previous studies reported quite unexpected interactions between copper and other environmental variables (Pandolfo et al. 2010, Fitzgerald et al. 2016, Peruzza et al. 2021), suggesting that copper does not necessarily impair physiological functions. In the Study III, the concentration of copper used was 100 µg/L, which is considered a sub-lethal concentration for sticklebacks (Gravenmier et al. 2005), yet environmentally relevant (Sanchez et al. 2005). I propose several hypothesis explaining such thermal improvement after 1-week copper exposure in sticklebacks, and I try to compare the current with the opposite response found in zebrafish from Study IV.

Firstly, the exposure to copper could enhance the oxygen delivery capacity to the tissues by increasing blood parameters levels such as haemoglobin and haematocrit (Dethloff et al. 1999, Lai et al. 2006, Rankin et al. 2012, Prchal and Gordeuk 2021) by increased activity of hypoxia-inducible factor (Hif-1). As mentioned in the introduction chapter, Hif-1 activates molecular pathways regulating the oxygen homeostasis in response to hypoxia (low oxygen availability in the environment) or hypoxemia (low oxygen level in the blood stream). Therefore, if there is copper exposure which induces Hif activity, and the latter plays a significant role in fish thermal tolerance (Anttila et al. 2013, Morgenroth et al. 2021), increased oxygen uptake capacity triggered by copper exposure may also result in increased thermal tolerance.

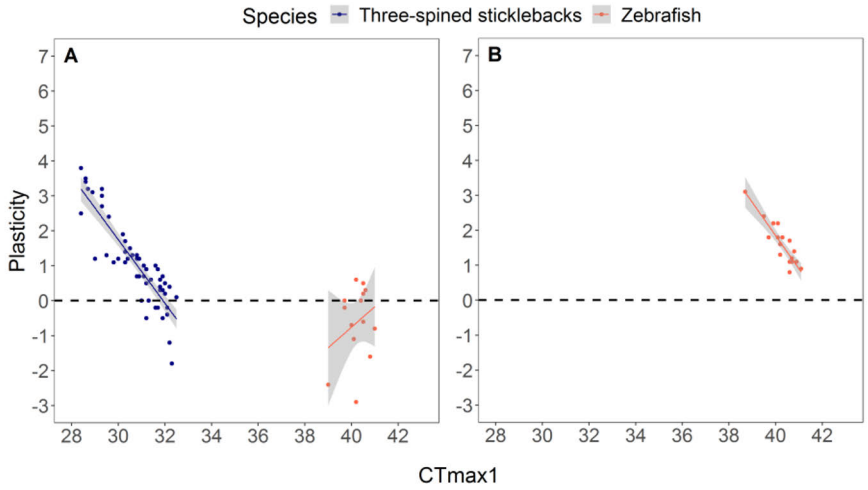

Figure 5. Relationship between initial upper thermal tolerance (CT_{max1}) and phenotypic plasticity of thermal tolerance ($CT_{max2} - CT_{max1}$) in three-spined sticklebacks (blue) and zebrafish (orange) after the exposure to copper (A) and 1-week heat wave + copper (B). The statistic was obtained running a linear regression model (LM).

Secondly, some other biochemical mechanisms are also involved in the regulation of thermal tolerance. Heat hardening in sticklebacks might constitute a warm pre-acclimation conditions that may share the same copper induced response pathways (cross-tolerance mechanism). Sappal et al. 2015, for example, showed beneficial effect on mitochondria efficiency in zebrafish exposed to copper after a warm acclimation. Moreover, another mechanism for copper-induced increase of thermal tolerance has been previously also reported in mussels after warm acclimation (Pandolfo et al. 2010). In Study III, sticklebacks were exposed to a short warm acclimation during the CTmax1 measurement. The acclimation response lasted for three weeks after the recovery (heat hardening seen in the handling control group, Study II and III), meanwhile sticklebacks that underwent copper exposure had two weeks of recovery from the CTmax1. Stickleback heat hardening might have served as an acute pre-acclimation which in turn enhanced the capacity of fish to handle the copper exposure, therefore resulting in increased thermal tolerance. While in zebrafish the CTmax1 measurements did not leaded to heat hardening and, thus, pre-acclimation, and there was no increase of thermal tolerance after the copper exposure.

In zebrafish the 1-week copper exposure on the contrary led to high mortality rate and low reproductive success. I suggest that the 2-weeks of recovery from the CTmax1 was not enough time for zebrafish to recover and, copper exposure might

have induced physiological damages, resulting in high mortality and a reduce the thermal tolerance in the surviving individuals. However, a third hypothesis behind the different response to copper found between sticklebacks and zebrafish is attributable to the physicochemical features of the experimental water used during each experiment. Water conductivity and salinity influence the availability of metals to fish. For instance, low water conductivity generally results in higher toxicity (Ebrahimpour et al. 2010). Sticklebacks in Study III were reared in artificially re-constructed brackish water, while zebrafish were in freshwater. The conductivity was therefore higher in Study III for sticklebacks, compared to zebrafish Study IV, suggesting a lower uptake of copper for sticklebacks. Therefore, one hypothesis is that the sub-lethal concentration of copper used in Study III was not leading to detrimental effect due to potentially reduced uptake resulting from elevated water conductivity. Thus, a potentially low-dose exposure to copper might have been beneficial for the fish, considering that copper is an essential metal for organism physiology. Although this hypothesis is speculative and needs further assessment to understand the routes of uptake and excretion of copper, the low conductivity of water (typical of Finnish waters) in Study IV, is a concern that should be addressed when interpreting the data.

For instance, copper exposure alone reduced the thermal tolerance of zebrafish, suggesting a physiological damage caused by copper exposure, but this result was observed only in fish that survived from the 77% mortality followed by copper exposure. Moreover, when copper exposure occurred in combination with heat wave, the thermal tolerance of zebrafish increased after 1-week. The phenotypic plasticity of thermal tolerance in this case was like the one induced after the heat wave exposure only, suggesting that the warm exposure was having a main effect on the thermal acclimation of fish, and that copper was not playing an antagonistic role in the thermal acclimation. However, also in this case, fish that were measured for the thermal tolerance were the ones that survived the copper (and heat wave) exposure (about 30% of the fish only). Therefore, those results need to be taken with caution, as they are likely to be relative to the acclimation capacity of more resistant phenotypes, and genotypes, selected for during the exposure. On the other hand, high temperature certainly increases fish metabolism, which in turn could allow for increasing uptake of unwanted substances via increased respiration rate (Rodgers 2021). At the same time, the physiological mechanisms of metal accumulation and detoxification are dependent on temperature (Sokolova and Lannig 2008). Although we did not measure the rate of detoxification, we cannot rule out that the lack of copper effect on the thermal tolerance was the result of an enhanced detoxification capacity of fish at higher temperatures. Again, this hypothesis is somehow speculative as I did not measure the accumulation rate of copper into zebrafish body, however, this may constitutes another explanation. Finally, another pitfall of the

Study IV is related to the presence of background copper in the control group, as a result of contaminated tap water in the University facility. Such contamination leaves the Study without a real control group. Therefore, since the copper exposure occurred also in the control group, it cannot be ruled out that a completely different response could have been resulted from fish reared in copper-free control water.

4.3 Sex-specific response to acute stress (Study II, III, IV)

The thermal capacity, both in response to heat wave and copper, was found to be sex-specific (Fig. 6). In Study II sex had a significant effect on the plasticity of the thermal tolerance after a heat wave exposure with male sticklebacks showing higher level of thermal plasticity compared to females. Males were able to improve their upper thermal tolerance by 1.1 ºC, compared to females whose thermal tolerance did not increase statistically significantly. Moreover, in Study III, the initial upper thermal tolerance of sticklebacks was sex specific, with females showing 1 ºC higher thermal tolerance than males. However, again the plasticity of the thermal tolerance after copper exposure was higher in males (+1.5 °C), compared to females (+0.6 °C). These results from sticklebacks in Study II and III suggest that generally males were more plastic than females when exposed to an environmental stressor. The ecological implications for this sex specific response are important, as if there is a sex imbalance at population level, this might constitute a threat for the survival of the population itself.

On the other hand, in Study IV, somewhat more complex trend was observed with zebrafish. Similarly, as with sticklebacks, female zebrafish in Study IV showed higher initial upper thermal tolerance than males (+ 0.36 ºC). Also, the males after the heat wave (and heat wave + copper) reached the same thermal tolerance as females (+ 1.6 ºC), i.e. showing in general higher plasticity to encounter high temperature. Nevertheless, one-week exposure to copper in control temperature was decreasing the male upper thermal tolerance (- 1.38 ºC), meaning that the effect of copper on thermal plasticity is sex-specific, and, when generalizing on all the Studies, the sex-specific response to copper might also depend on the species. However, when it comes to response to high environmental temperature both species had similar sex-specific effect, males showing higher plasticity. Currently it is still unknown why and how males display higher plasticity, but it could e.g., be related to reproduction status, the mature females investing more energy for egg-production and thus, trading-off the energy from plasticity. Furthermore, at least stickleback

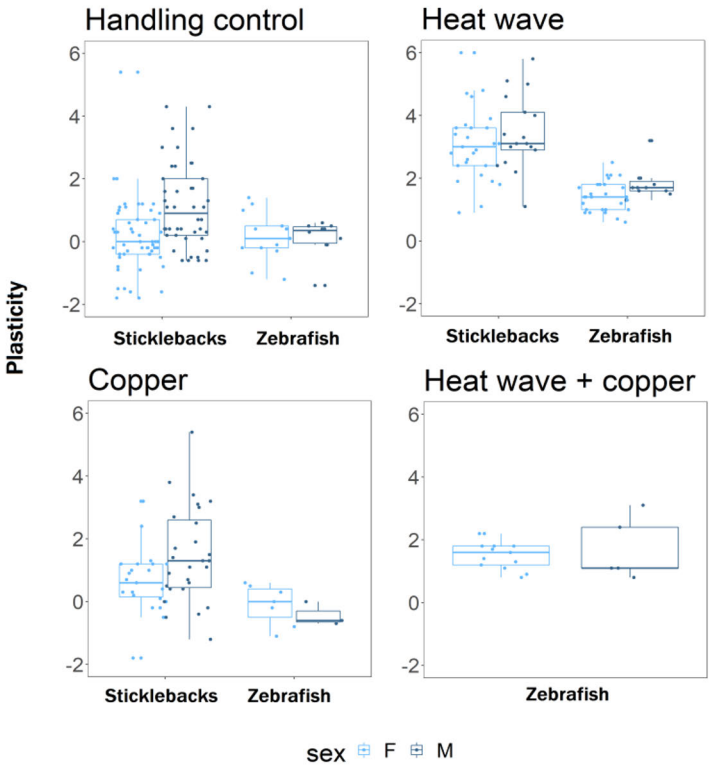

Figure 6. Boxplot representing the sex-specific differences in phenotypic plasticity of thermal tolerance ($CT_{max2} - CT_{max1}$) in three-spined sticklebacks and zebrafish exposed to handling control (Study II, III, IV), heat wave (Study II, IV), copper (Study III, IV) and combination of both (Study IV). Light blue boxplot and dots represent the female, while dark blue boxplot and dots represent male fish.

males might have evolved coping strategies with varying environmental temperatures as they nurse the eggs in shallow shoreline and according to our measurements the temperature fluctuates even 10 ºC within one day in those areas. This is not the first study showing sex-specific response of thermal tolerance. For instance, it has been observed that the compensatory cardiac mechanisms under heat stress are sex-specific in rainbow trout (Ekström et al. 2017). Moreover, sex-specific differences in thermal tolerance have been previously reported in many species (Jeffries et al. 2012, Madeira et al. 2012, Sasaki and Dam 2019, Missionário et al. 2022). In the current thesis, even though the influence of sex on the trends observed was not the main aim, the results came out to be one of the ecologically most

important ones. So far most publications do not even mention the sex of study individuals, as reviewed by Edmands 2021 (Ignatz et al. 2021). However, the results from the current thesis, as well as the previous ones, suggest that sex plays extremely important role in thermal tolerance and its plasticity, and differences between sexes must be considered when addressing thermal tolerance questions.

4.4 Developmental and parental effects after an acute environmental change (Study IV)

In Study IV, I focused on the phenotypic and developmental plasticity of thermal tolerance in both zebrafish adult and embryos, but also on parental effect occurrence on thermal tolerance in offspring from exposed parents to understand whether the parental condition influences the capacity of offspring to survive sudden extreme events. To disentangle the parental from the developmental effect, offspring were reared in both matched and unmatched parental environments.

4.4.1 Developmental plasticity

Embryos exposed to heat wave, copper, and the combination of both were differently affected according to each condition. Life-history traits (mortality, hatching success and growth) were affected by both the temperature increase and the copper exposure. The mortality increased with copper concentration and with high temperature exposure, however, copper exposure during the heat wave treatment seemed to cause higher embryo mortality than the single stressor exposure. Similarly, the hatching success decreased when copper exposure was combined with heat wave and the larvae at 5dpf were also smaller in co-exposure. Similar results have been shown also by Johnson et al. 2007, in similar experimental conditions and in Fitzgerald et al. 2016 under copper and hypoxia exposure. Since the mortality was especially high in embryos which had not yet hatched, it is likely that the combination of both stressors inhibits the hatching process thereby leading to embryo death. Embryos exposed to heat wave were smaller than the control ones. This might be since those embryos were already above their optimal growth temperature, therefore more energy is allocated to thermal physiological adjustments to compensate for the high temperature (Koban 1986), which in turn leads to less growth.

The occurrence of malformations was mostly caused by the high temperatures, with increased temperature causing oedemas and spinal deformity occurrence, except for swim bladder inflation that was affected by both heat wave and copper.

This suggests that the occurrence of heat wave during the development is highly detrimental and will have long term effects if the fish are able to survive. For instance, a malfunction of the swim bladder prevents fish from swim properly (Lindsey et al. 2010), therefore fish have less chances for survival (Battaglene and Talbot 1992).

The upper thermal tolerance of 5dpf larvae was mostly influenced by the heat wave exposure, with individuals exposed to heat wave during the development having lower upper thermal tolerance than the control and copper exposed. Copper, on the other hand, did not seem to affect the upper thermal tolerance of embryos. Similar effect of temperature have been reported in Drosophila species (Kellermann and Sgrò 2018). The results are, however, opposite as compared to adults that were able to increase their tolerance. This is showing that embryos are much more vulnerable to sudden environmental changes, probably due to fact that they are investing large amount of energy for growth and organ development, and they do not have any scope to encounter stressors. Moreover, in the current study the inter-individual variance was higher under heatwave condition, suggesting that the phenotypic response to heat stress becomes larger with increasing rearing temperature, which in turns might allow some individuals to cope better with heat than others.

4.4.2 Parental effects on embryo plasticity

Copper exposure caused a decrease in the reproductive performance as has been previously observed (Cazan and Klerks 2015, Driessnack et al. 2017), yet large clutch in response to copper exposure at 33 ºC was unexpected. Large clutches with smaller eggs have been previously reported for female sticklebacks reared at high temperature (Shama and Wegner 2014). In this study, female zebrafish exposed to heat wave alone produced smaller eggs compared to control female, suggesting a temperature-induced plasticity in egg size (Bownds et al. 2010, Liefting et al. 2010, Shama and Wegner 2014). It has previously shown that female fish reared at elevated temperature produced smaller offspring in order to maximize their own fitness in favour of fecundity (Shama and Wegner 2014), a mechanisms called “selfish maternal effect”.

Embryos born from exposed parents were generally showing different features and performance compared to the ones born from not exposed parents. Embryos born from heat wave exposed parents were generally smaller and maintained reduced size over the development. Moreover, these embryos were showing higher mortality (~30%) compared to control (~4%) embryos even in the case when the offspring were kept in control condition after the reproduction. However, heat wave + copper

parental exposure did not increase offspring mortality compared to parental heat wave exposure only, suggesting that parental copper does not have a synergistic effect on offspring mortality. Hatching success also decreased in offspring from parental heat wave (91%) compared to control (99%), but not in those born from parental heat wave + copper group (97%), perhaps due to lack of maternal transfer of copper. However, it is important to take into consideration that the offspring born from heat wave + copper exposed parents were from one set of parents only, as the other adult fish were either not able to reproduce or they died during the exposure period. Therefore, the low mortality rate and high hatching success of these offspring might be the result of the selection of more resistant adult phenotypes (and also genotypes), which have been selected during the heat wave + copper exposure. The genetic background of these embryos is the result of a selection towards potentially more resistant individuals and it is unlikely that it reflects the response of the entire population.

The occurrence of malformations was also influenced by the parental exposure, and again heat wave seemed to be the main factor affecting the presence of malformation. Although there was a tendency of the heat wave and copper parental exposed embryos having higher level of malformation, these were not statistically significant from the embryo born from heat wave exposed parents, suggesting that the temperature experienced by the parent resulted in decreased quality of the eggs which was also shown by the size of the eggs.

The upper thermal tolerance of the larvae at the 5dpf was slightly lower compared to the one recorded in control parents. Moreover, the thermal tolerance seemed to be variable in the offspring generation from heat wave exposed parents, compared to the other group, suggesting that heat wave exposed parents' triggered high variation in the offspring thermal tolerance and produce more diverse phenotypes. This result is extremely important from an ecological point of view, as high variation in the population phenotypic response allows for selection depending on the environmental conditions. This result was unexpected as it has previously shown that the warm parental environment was shaping the thermal tolerance of the offspring towards higher limits compared to the parents (Donelson et al. 2018), therefore resulting in a pre-conditioning effect. In this thesis, it might also be that the heat wave exposure was highly detrimental, therefore inducing a lack of anticipatory parental effect but, on the other hand, inducing a bet-hedging strategy (Morrongiello et al. 2012).

4.4.3 Plasticity in matching parental environment and anticipatory parental effect

The evolution of adaptive phenotypic plasticity is relying on cues that could trigger phenotypic adjustment in order to match the environment (Uller et al. 2013). Therefore, if the parents experience a certain condition, they might inform the offspring generation, which in turn become more resistant to that condition. This mechanism is called "anticipatory parental effect", and it is extremely important to assess because it helps to understand whether some traits are adaptive or not.

In Study IV, the embryos born from heat wave exposed parents and in turn exposed to heat wave were showing higher mortality, compared to the embryos exposed to heat wave, but born from control exposed parents (14% mortality in heat wave exposed from control parent vs 33% mortality in heat wave exposed from heat wave exposed parents). Moreover, embryos born from heat wave exposed parents and exposed to the same parental condition (again heat wave) were not showing an improved survival capacity compared to the ones born from heat wave parents and reared in control condition. This suggests that the parental exposure did not result in an anticipatory parental effect on the offspring. Furthermore, parental exposure was negatively affecting the survival of the offspring. In co-exposure with copper the output was following similar pattern as the mortality of embryos from unexposed parents but exposed to heat wave and copper was around 42%; the unexposed embryos born from exposed parents were having 33% mortality and the exposed embryos born from exposed parents were showing 50% mortality. These results suggest that the mortality of embryos depended more on the copper exposure during the development but the parental exposure seemed to worsen the offspring survival. However, this result needs to be taken with caution as the copper exposure of the parents was high enough to cause mortality, therefore preventing any potential adaptive responses that might occur at lower concentrations.

The hatching success was quite high in the heat wave matched parental environment (95%), and it was similar to the ones obtained from embryos exposed to heat wave from unexposed parents (86%). Also, the embryos reared in control conditions, but born from parents exposed to heat wave showed high hatching success (91%). This suggests no extreme effect of the parental or rearing condition on hatching success. When copper was added, this tendency changed, as the embryos exposed to heat wave + copper born from unexposed parents showed only 40% hatching success vs the ones exposed to heat wave + copper and born from parents exposed to the same condition (75%). The hatching success was instead high in unexposed embryos coming from parents exposed to heat wave + copper condition (97%). Since the hatching success in embryos reared in control condition but born

from exposed parents was extremely high, the result suggests that again copper exposure is resulting in toxic effects when present during the developmental phases.

The growth was influenced both from parental and rearing exposure in heat wave and heat wave + copper conditions, with embryos in matching parental environment being smaller than the one from embryos exposed to heat wave but born from unexposed parents (3.42 mm vs 3.61 mm, respectively). Also, the growth of the embryos reared in control condition but born from parents exposed to heat wave was high, with the larvae average length being 3.66 mm.

The occurrence of malformations (swimming bladder inflation occurrence, spinal deformities, and presence of oedema) was mostly driven by the rearing condition of the embryos. Indeed, embryos with the highest occurrence of malformation were the one exposed from control parents and exposed from exposed parents, suggesting that the parental exposure does not result in higher occurrence of malformations in the next generations, and this was mostly the result of developmental effect of the heat wave and copper exposure.

In the heat wave exposure only, the upper thermal tolerance in embryos reared in the matching parental heat wave environment was not changing from the one reared in the unmatched parental environment suggesting that the parental exposure did not result in higher thermal capacity when embryos encountered the same condition as their parents. However, in the heat wave and copper matching parental environment, both the parental and exposure condition influenced the upper thermal tolerance, which worsened after the exposure, resulting in low thermal tolerance. This result is in contrast, at least when considering the heat wave exposure only, with previous studies which found an enhanced thermal capacity in the offspring born from parents exposed and reared in matching parental environment (Donelson et al. 2018). However, there has been also shown weak evidence of anticipatory parental effect when parental and offspring environments are matched (Uller et al. 2013).

4.4.4 Limitations of Study IV and recommendations for future work

In Study IV, the filtered tap water used in the experiment contained mild concentration of background copper (~ 25 µg/L). This issue resulted in a lack of proper control groups, therefore rendering the results of the experiments only partially reliable. The response obtained by the used control might therefore be more attributable to the response that fish might have if they were exposed to mild copper concentrations. If this is the case, the lack of mortality, as well as the lack of heat hardening in the control group, occurs at mild copper concentrations, suggesting a copper concentration-dependent tolerance.

Another limitation of the Study, was the about 70-77% mortality and only one successful reproduction in adult fish exposed. This might have caused the selection of more resistant phenotypes, therefore suggesting that the thermal performance of the exposed adults, and their offspring, is relative only to this resistant group and might not represent the population response. The high mortality was most probably given by the low water conductivity (typical of the Finnish water). Such water feature indicates how different water sources and features might influence the toxicity of metals in natural and laboratory populations.

Based on the above, I recommend using different types of water when assessing the effect of contaminants on fish physiology, such as reconstituted or reverse osmosis water. Further measures preventing the occurrence of unexpected issues with water quality are represented by the frequent testing of the water parameters during the experimental step, especially when the use of reconstituted water is not possible and one has to rely on the filtered tap water. This is because the water providers might use different water sources which might differ in the amount of ions in the water. This represents an issue when addressing eco-toxicological questions, as a change in ion content compromise the metals uptake, and therefore their toxicity.

Another point that is worth to address is the frequent handling of the adult individuals during the experiment. Given the nature of the experimental design, whose purpose was to study the thermal tolerance of each individual for more than one time, fish have been exposed to short, yet frequent handling. This might have caused a stress in the fish, which can be related to the high level of Hsps in the adult fish. The presence of a naïve control group (neither used for measurement nor exposures) would have constituted a reliable handling control.

Despite the limitations, Study IV still constitutes a new approach when studying the effect of a multiple-stressor exposure on the thermal tolerance of fish, as well as offers future perspectives when addressing the evolution of the thermal tolerance in a more reliable environmental scenario, where several stressors are occurring simultaneously.

4.5 Lack of evolutionary adaptation in populations from thermally polluted environment (Study II, III)

The thermal tolerance and its phenotypic plasticity can be influenced by the thermal history of organisms. Therefore, disentangling the environmental and genetic effect by laboratory common garden acclimations is a valuable way to

determine if a correlation between thermal tolerance and plasticity is shaped by environmental factors. One of the aims of the current thesis was to assess whether 50-years long exposure to warm temperature from nuclear power plants resulted in evolutionary warm adaptation in population living those areas. This would have potentially resulted in higher thermal tolerance compared to fish living in pristine areas and hypothetically a different response to a heat wave and/or exposure to pollutants. In the Study II and III, the wild populations of three-spined sticklebacks originated from 50-years thermally polluted environments (nuclear power plants) and pristine areas of the Baltic Sea and were reared in common garden conditions, after being exposed to acute stress. The measure of the CTmax before the exposure was similar among all the population studied. This suggest that the thermally polluted environment in the nuclear power plant areas was not causing any adaptation to warm temperature in three-spined sticklebacks, and that most probably the thermal history was similar across each individual. Similar results have been found in previous studies (Dammark et al. 2018), confirming that the evolution of the thermal tolerance in wild populations might be absent in the 50-years temporal window, or has been prevented by other mechanisms.

One mechanism preventing local adaptation proposed in this thesis is the behavioural thermoregulation: fish might escape an unfavourable thermal condition, simply choosing cooler environments when exposed to too warm condition. This mechanism, also known as Bogert effect, could therefore prevent the fish from acclimating physiologically to high temperatures and form more thermal tolerant phenotypes. The lack of physiological response to a stressor might prevent the individual to develop physiological or molecular strategies, which in turns lead to absence of cues for the offspring generations (lack of parental and/or multigenerational effect). Overall, this would potentially reduce the selection pressure on thermal phenotypic traits, like the thermal tolerance. However, since only the upper thermal tolerance and molecular thermal responses have been studied in these two chapters, it cannot be ruled out that other traits, not directly connected to temperatures, might have been selected.

Another hypothesis supporting the low phenotypic divergences in thermal tolerance is the lack of genetic divergence among populations nearby each nuclear power plant, which might be due to high gene flow. Three-spined sticklebacks become pelagic during wintertime, returning to the coastal habitats only for the reproduction (Bergström et al. 2015). It is, therefore, possible that the population found in the nuclear power plant during the sampling do not belong to to the same area during the wintertime.

It is, however, crucial to investigate if fish are able to adapt to future climate change conditions and nuclear power plant areas provide a nice setting for such studies as temperatures has been high for several decades. However, for studying

such effect in those areas the species dispersal abilities should be more limited than in three-spined sticklebacks and e.g., studying the thermal tolerance of more sessile species could provide us the answers in the future

5 Conclusions

The current thesis focused on the phenotypic plasticity of thermal tolerance and how this is shaped by different environmental conditions utilizing both wild and laboratory-reared fish species. Moreover, the assessment of the phenotypic plasticity was put in an evolutionary context, as the potential for evolution of the phenotypic plasticity was assessed also in relationship to the fish thermal history. This research represents an important step forward in the study of the thermal tolerance as it addresses its phenotypic plasticity in response to heat waves, which has been considered as one of the most detrimental stressors for fish physiology. Moreover, the phenotypic thermal responses have been assessed in a scenario where copper co-exposure occurred, constituting one of the first study utilizing a multiple-stressor effect approach for addressing the thermal tolerance as well as the possible parental effects in future generations, despite the limitations of the study. The approach used in the current thesis was multilevel, spanning from the biochemical response to the evolutionary potential for adaptation, therefore representing a broad focus on the phenotypic plasticity of thermal tolerance. Generally, fish were able to respond to a stressful warm condition in a plastic way, and the level of plasticity was depending on the species thermal window and the exposure timing, as well as the magnitude of the heat stress and, unexpectedly, these were influenced by sex. These results suggest that fish may be able to respond to a 1-week long heat wave event in nature by increasing their thermal tolerance, which would result in an effective survival strategy in response to a stressful event. Although the fish used in the current experiment showed a positive acclimation response, the current thesis does not consider the effect of longer heat waves, or the occurrence of multiple ones, which can have a different impact on fish thermal capacity in nature. Therefore, despite the positive outputs brought from these experiments, the effect of more severe and frequent heat waves might not result in improved thermal capacity of fish in nature. Moreover, the sex-dependent vulnerability to high temperature and copper could

result in a sex-biased survival and the further related ecological consequences for wild populations.

At molecular level, the response mechanisms presented in the Study I underlined the importance of studying the heat shock response not only at transcriptional level but also at protein level, therefore suggesting the study of the protein expression as an important tool to understand the mechanisms of phenotypic plasticity of the thermal response at molecular level. However, what emerged from Study I was not replicated in the further studies, where an increase in water temperature did not result in increase of the heat shock proteins, suggesting that the link between the physiological response and the molecular pathways is not straightforward and that other molecular mechanisms, that have not been considered in this thesis, might be involved. When addressing the parental effects on thermal tolerance, the study, although with some limitations, showed that the beneficial effect of the parental exposure on the thermal tolerance of the offspring generation might not occur. This result highlight that the parental effect occurrence might be dependent on many factors, such as the length of the heat stress as well as its magnitude, suggesting that further research is needed to assess how the mode of exposure is influencing the parental effect. The implications for natural populations of these results are that, despite the heat wave exposure was resulting in an improvement of the acclimation capacity within a generation, the offspring might be negatively influenced. This could lead to population decline in long run.

When the study of the thermal tolerance has been put in a more evolutionary context, I found that the 50 years of exposure to higher temperature from the nuclear power plant did not result in evolutionary adaptation to higher temperatures suggesting that fish require more generations for the adaptation to occur, or that the selection pressure has not been high enough to result in an adaptation mechanism. This evidence, together with the lack of parental anticipatory effect results highlight that populations in the natural environment might not be able to handle stressful events via parental effect, or evolutionary mechanisms, leading to the phenotypic plasticity (within a generation) as the only physiological strategy to cope with climate change. While helping the wild populations in handling stressful events, the phenotypic plasticity strategy might not be effective to counteract repetitive thermal extremes which will become more frequent in the near future. Moreover, as in the natural environment the stressors are rarely occurring in isolation, it is important to consider that the phenotypic response to temperature increase might be further shaped by the presence of other stressors, such as pollutants, which may or may not interact with the coping stress mechanisms of fish. It is therefore important to find mitigation strategies which would reduce the load of contaminants released in nature, as well as find thermal refugia from heat waves.

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