Copper exposure improves the upper thermal tolerance in a sex-specific manner, irrespective of fish thermal history

Giovanna Mottola [*], Mikko Nikinmaa, Katja Anttila

Department of Biology, University of Turku, Vesilinnantie 5, Turku 20500, Finland

ARTICLE INFO

ABSTRACT

Ectotherms can respond to climate change via evolutionary adaptation, usually resulting in an increase of their upper thermal tolerance. But whether such adaptation influences the phenotypic plasticity of thermal tolerance when encountering further environmental stressors is not clear yet. This is crucial to understand because organisms experience multiple stressors, besides warming climate, in their natural environment and pollution is one of those. Here, we studied the phenotypic plasticity of thermal tolerance in three-spined stickleback populations inhabiting spatially replicated thermally polluted and pristine areas before and after exposing them to a sublethal concentration of copper for one week. We found that the upper thermal tolerance and its phenotypic plasticity after copper exposure did not depend on the thermal history of fish, suggesting that five decades of thermal pollution did not result in evolutionary adaptation to thermal tolerance. The upper thermal tolerance of fish was, on the other hand, increased by ~ 1.5 °C after 1-week copper exposure in a sex-specific manner, with males having higher plasticity. To our knowledge this is the first study that shows an improvement of the upper thermal tolerance as a result of metal exposure. The results suggest that three-spined sticklebacks are having high plasticity and they are capable of surviving in a multiple-stressor scenario in the wild and that male sticklebacks seem more resilient to fluctuating environmental conditions than female.

1. Introduction

Increased anthropogenic activities have caused global warming (Ficke et al., 2007). Global warming constitutes a threat to organisms, especially ectotherms, whose body temperatures are virtually identical to the environmental temperature (Ficke et al., 2007). However, ectotherms, such as fish, are able to respond to warming temperatures via evolutionary thermal adaptation or acclimation through phenotypic plasticity (Chen et al., 2018) resulting in improved upper thermal tolerance (Healy and Schulte, 2012). A commonly used proxy for upper thermal tolerance is the Critical Thermal Maximum (CT_{max}) (Rezende et al., 2011). The measure gives the temperature, which is acutely lethal to the animals. However, adaptational increase of CT_{max} may leave wild populations vulnerable to additional environmental stressors (Martinez et al., 2016). This is because there could be a variation in energy balance and, thus, a trade-off between temperature tolerance and an additional stressor (Martinez et al., 2016). Therefore, understanding whether warm-adapted individuals are able to maintain their upper thermal tolerance in the presence of an additional stressor is crucial for

predicting if populations with increased thermal tolerance will be favored or perish in a climate change scenario in contaminated environment.

One possible group of contaminants that fish encounter in nature are metals and, among those, copper is a major contaminant. It is mainly released in nature through mining activity and in wastewater discharges and has been found in surface water at concentrations up to 100 μg/L (Sanchez et al., 2005). Copper is a trace metal biologically important at low concentrations (Sappal et al., 2015), but if it bio-accumulates in higher than normal concentrations, it leads to impairment at different biological levels causing also mortality (Forouhar Vajargah et al., 2020). The toxicity of the copper, however, depends on other environmental variables as well. In previous studies, it has been, for example, observed that the copper-induced mortality was higher in zebrafish exposed to copper in softer water, compared to the ones in harder water with the same copper concentration (Pilehvar et al., 2020). This result suggest that the bioavailability of copper depends on the water characteristics, such as hardness (Newman, 2009). However, the bioaccumulation of copper might also be dependent on other physical features of

---

environment, like temperature. The interaction of copper with temperature is complex (Peruzza et al., 2021). Copper toxicity may be dependent on the species-specific thermal optimum. The temperatures above the optimum can lead to increased toxicity as a result of increased metabolic rate of animal (Kumar et al., 2018). On the other hand, pre-acclimation to high temperature might alleviate copper toxicity because high temperature can induce a stress response which in turn initiates a mechanism of cross-tolerance i.e. when the priming stressor initiates defense that offers protection to subsequent stress (Rodgers and Isaza, 2021).

Complexity of the interaction between copper and temperature in organism has so far prevented to make a conclusion whether these stressors have antagonistic or synergistic effects e.g. on thermal tolerance of animal. Earlier studies have focused on the physiological effect of acute co-exposure of copper and warm temperature on thermal tolerance of organisms, or on the role that a short thermal pre-acclimation has on copper toxicity (Pilehvar et al. 2019; Peruzza et al., 2021). However, there is still lack of studies focusing on the role that warm adaptation (through evolutionary changes) has on the thermal plasticity of fish under copper exposure. Such studies are needed to clarify whether warm adaptation affects the phenotypic plasticity in the presence of another stressor. For this purpose, discharge areas of nuclear power plants represent spatially replicated long-term "evolutionary laboratories" where fish exposed to a warmer temperature for generations might have adapted to warmer environment, giving the possibility to evaluate if evolutionary adaptation to increased temperature affects the response to metal exposure.

Here we took advantage of two thermally polluted environments from two nuclear power plants located along the Finnish coastline of the Baltic Sea to study the effect of exposure to a sub-lethal concentration of copper (100 µg/L) on the individual phenotypic plasticity of thermal tolerance in three-spined stickleback (*Gasterosteus aculeatus*). We compared their response with sticklebacks from four locations about 50 km away from the discharge areas. The water temperature of these locations follows the natural seasonal fluctuation compared to nuclear power plant areas, where the discharge water has been reported to increase the temperature of water in the nuclear power plant area by 2–5 °C compared to the inlet water areas (Ilus, 2009). Depending on the weather conditions, temperature increase can be observed at an approximate distance of 3–5 km from the discharge area. 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 winter in Finland, being maximally of around 7 km^2 (Ilus, 2009). Besides influencing the temperature conditions of the area, the changing in ice condition and generally high water temperature near nuclear power plants have caused modification in the oxygen concentration in those areas as a consequence of the biomass growth caused by warm temperature (Ilus, 2009). Therefore, organisms inhabiting these areas might have experienced also higher than normal oxygen levels during winter periods, potentially resulting from the lack of ice formation, or hypoxia as consequence of the high temperatures during summer, compared to those inhabiting pristine areas. Different thermal and oxygen regimes, as well as other characters of area like diversity of food web, which all change due to increased temperature, could constitute a selective pressure leading to a different capacity to handle environmental stressors, like exposure to copper. In this study, we acclimated fish from both nuclear power plant and control areas in common garden conditions and assessed the individual Critical Thermal Maximum (CT$_{max}$) using a dynamic thermal ramping test before and after the exposure to the sub-lethal copper concentration.

Moreover, we studied the expression profile of the Heat Shock Proteins (HSP70, HSP90) and Hypoxia-inducible factor-1 (HIF-1) in order to uncover possible molecular mechanisms behind the change in CT$_{max}$. HSPs provide protection against cellular stress, caused by high temperature or presence of contaminants by preventing the aggregation or improper folding of other proteins (Currie and Tufts, 1997). Studying

the expression profile of HSPs is important to understand the magnitude of the stress-induced damage (Somero, 2020): therefore, we asked the question if it differed between populations from different thermal habitats. The hypoxia-inducible factor 1 (HIF-1), discovered by Semenza's group (Wang and Semenza, 1993), regulates hypoxic gene expression involved in energy metabolism, angiogenic signaling, growth factor signaling, apoptosis and embryonic development (Duarte-Gutiérrez et al., 2021) also in fish (Nikinmaa and Rees, 2005). High temperature activates HIF-1 (Rissanen et al., 2006) either directly or through hypoxemia, since the partial pressure of oxygen in the venous blood is reduced at high temperatures in fish (Ekström et al., 2016). Indeed, hypoxia and thermal tolerance are often correlated in fish (O'Brien et al., 2020) and in some fish species exposure to high temperature has been shown to induce expression of HIF-1 (Beers and Sidell, 2011), while in others HIF-1 expression has been unresponsive to high temperatures (Devor et al., 2016). Exposure to copper has also been shown to cause hypoxemia as a consequence of damage to the gill epithelium, which induced HIF-1 in rainbow trout (van Heerden et al., 2004). Moreover, copper up-regulated the HIF-1 transcript in the brain of crucian carp (*Carassius carassius*) (Zizza et al., 2016). Thus, HIF-1 may be induced by high temperature, copper exposure and their interaction. Here we tested whether HIF-1 was involved in plasticity of thermal tolerance of fish when exposed to a sub-lethal copper concentration, and if it was differentially activated in the sticklebacks inhabiting areas in the vicinity of nuclear power plants compared to control areas.

2. Material and methods

2.1. Study area and fish husbandry

Individuals of three-spined sticklebacks (*Gasterosteus aculeatus*) were caught from six different sites along the Finnish coastline of the Baltic Sea, in Gulf of Finland and Gulf of Bothnia in May 2018 (Fig. 1A). Two sites in each Gulf were discharge areas for cooling water systems of Finnish nuclear power plants (NPP): Loviisa (LOV, Gulf of Finland) and Olkiluoto (OLK, Gulf of Bothnia). We collected fish also from four control sites (CTRL), two around each NPP: Kotka (KOT) and Porvoo (POO) in Gulf of Finland and Pyhäranta (PYH) and Pori (POR) in Gulf of Bothnia, located about 50 km from the NPP areas (Fig. 1A). The construction of the nuclear power plants took place in 1971–1980. The reported average annual temperatures in NPP areas have been 2–5 °C higher than in control areas (Ilus, 2009). In order to monitor the water temperatures of the six areas, three temperature data loggers per area (HOBO Water Temp Pro v2 Logger, U22, Onset Computer Corporation, Bourne, MA, USA) were placed directly at the catching locations at the sea bottom (depth 1.5–2 m). The water temperature was recorded 17/5/2018 – 6/11/2018 from all the locations 4 to 24 times per day. The daily average temperatures for each location were, then, calculated by averaging the values of the three loggers per area (Fig. S1, Supplementary). The concentration of metals are monitored periodically by the Finnish Environmental institutions. The copper concentrations in control areas of Kotka and Porvoo in the Gulf of Finland were around 0.7 ± 0.08 µg/L, and in Loviisa around 2.8 ± 0.4 µg/L. Regarding the Gulf of Bothnia, the concentration of copper in Pori and Pyhäranta was around 0.7 ± 0.3 µg/L, and in Olkiluoto the concentration was around 1.6 ± 0.1 µg/L (https://merihavainnot.ymparisto.fi/merihavainnot/).

Fish (N = 100 per population) were caught using beach seine net and transported in large water containers to the University of Turku, Finland. During the transport, natural water temperature and oxygen saturation were maintained using air pumps and ice blocks. No mortality was observed during the transport. Some fish were used for other experiments (Mottola et al., 2022).

Fish were kept in common garden conditions in University of Turku in order to remove any differences due to acclimation to the original habitats of the fish. Fish were allowed to acclimate to laboratory conditions in six different 180 L tanks at +16 °C for two weeks. The
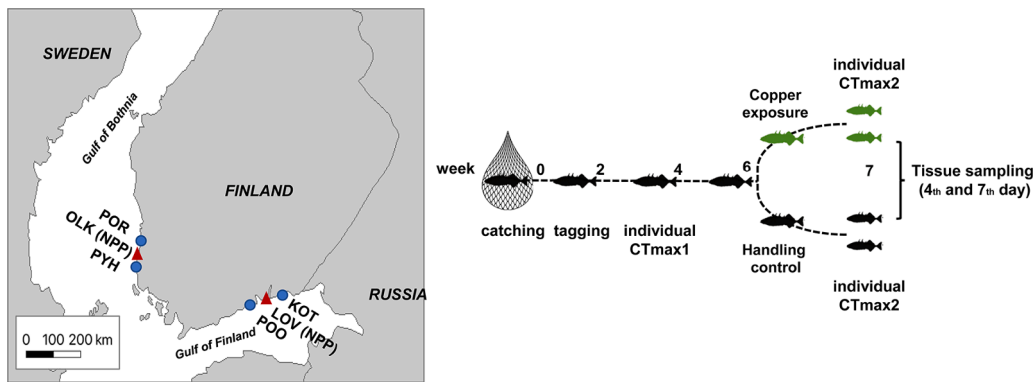
Fig. 1. Representation of the study area showing sampling locations (left). Red triangles indicate the nuclear power plant areas (NPP), while blue circles indicate the control locations (CTRL). Representation of the experimental design (right).

temperature choice was decided based on the environmental temperature recorded at the catching time in the six areas (Fig. S1, Supplementary). Water pH was kept at 8.0 and air saturation over 80%. Salinity (ppt) of natural water was slightly different in each location (LOV: 3.2 ± 0.2, POO: 4.3 ± 0.0, KOT: 1.7 ± 0.0, OLK: 5.3 ± 0.0, POR: 4.9 ± 0.0, PYH: 4.2 ± 0.4), therefore, we decided to keep water salinity in the common garden experiment at the average value of 4 ppt (filtered water with 76% NaCl; 20% MgSO$_4$; 3.5% CaCl$_2$; 0.5% KHCO$_3$). The water hardness was 55.2 fH. Photoperiod was set at 17L:7D that mimicked the natural photoperiod when the fish were caught. Fish were fed with frozen bloodworms (Delang & Ekman AB/ Akvarieteknik, Sweden) five times per week. One third of the water was changed once a week. Upon arrival, the fish were treated against nematodes using Nematol (Sera GmbH, Heinberg, Germany) according to the instructions of the manufacturer. In order to avoid potential tank effects, fish were tagged intraperitoneally with 1.35×7 mm RFID subcutaneous microchips (Loligo® Systems, Viborg, Denmark) under anesthesia (100 ppm MS-222 in 4 ppt brackish water buffered with 6 ppm NaHCO$_3$) after two weeks of acclimatisation. Thereafter, the populations were mixed into nine tanks (with density of 2 fish/L) and were let to recover for two weeks. All fish procedures were performed according to Finnish Animal Care permission (ESAVI/2867/2018).

2.2. Critical thermal maxima and copper run-off simulation

In order to observe differences in the thermal tolerance of individual sticklebacks from NPPs compared to the control areas, we tested the initial Critical thermal maximum (CT_{max1}) after recovery from tagging (Fig. 1B). The CT_{max} was quantified for 109 fish (an average of 16 individuals per location) according to Sidhu et al., (2014). A VC/3 circulator-heater (Julabo Labortechnik GmbH, Seebach, Germany) was connected with two steel coils into a 45 L tank. Constant mixing of the water was assured by a pump and air saturation was kept over 80%. Around 50 individuals per time were placed in the ramping test setup (starting temperature 15.7 °C) and kept there for 1 h in order for the fish to familiarize to the new surrounding (Sidhu et al. 2014). Thereafter, water temperature was increased by 0.3 °C min^{-1} until 27 °C, above which the thermal ramp was slowed down to 0.1 °C min^{-1}. When an individual lost equilibrium (CT_{max}), individual tag and CT_{max} temperature were quickly recorded, and fish was transferred in a recovery tank. CT_{max} measurements were always performed at the same time of the day in order to minimize potential diel fluctuations. Post-trial mortality was followed for the two weeks of recovery.

To evaluate how industrial copper run-off affects the thermal tolerance and its plasticity in fish from different thermal environment, the fish that were used during the initial CT_{max} measurement were assigned to two groups: copper exposed (Copper, N = 56) and unexposed handling control fish (CTRL, N = 53) two weeks after initial CT_{max}

measurements (Fig. 1B). Copper and handling control fish were transferred to triplicate copper/control experimental tanks. After 1 h acclimation to the new tank condition, fish in the copper experimental tank were exposed to copper stress as previously described by Ruuskanen et al. (2020). Briefly, fish experienced one pulse Cu^{2+} exposure administered manually by adding copper (II) sulphate pentahydrate solution (nominal: 100 µg/L of CuSO$_4$•5H$_2$O, Merck, Darmstadt, Germany) to the experimental tanks. This concentration of copper represents environmentally relevant concentration encountered in polluted waters and was considered sub-lethal for sticklebacks (Sanchez et al., 2005; Ruuskanen et al., 2020). In order to measure the level of copper concentration in the water during the one-week-long exposure and compare it to the copper concentration in tanks with no added copper, water samples were taken both from exposure and control tanks (i) 2 h and (ii) 1 week after copper addition (water was not changed during the exposure). Fifty milliliters water samples from each copper tank and one handling control tank were placed in polypropylene Falcon tubes. In order to avoid metal precipitation, 1 ml of concentrated HNO$_3$/100 ml was added to the samples. The samples were kept at 4 °C before the analyses. Water samples were sent to SYNLAB Analytics & Services Finland Oy (Karkkila, Finland) which measured the copper concentration using inductively coupled plasma mass spectrometry (ICP-MS) (ThermoFisher Scientific, MA, USA). In the copper exposure and handling control tank, the measured concentrations of copper were 91, 101, 91 (copper tank) and 22 µg/L (control tank) after 2 h and 35, 48, 37 (copper tank) and 18 µg/L (control tank) after 1 week, respectively. According to chemical water analyses all the fish in current study were exposed to low concentration of the copper after they were brought to laboratory facilities. This is probably due to the technical purity of salts for producing brackish water. After 1-week exposure to sub-lethal copper concentration and handling control, fish were transferred into the CT_{max} set up and a second CT_{max} (CT_{max2}) was measured in order to evaluate how copper exposure affects the thermal tolerance of the fish and especially on the individual plasticity of thermal tolerance (i.e. change of CT_{max}, calculated as: CT_{max2} minus CT_{max1}). After the CT_{max} measurement, the sex of the fish was identified, and its mass and length were measured. We also counted the number of the lateral plates to evaluate potential morphological differences among populations (DeFaveri and Merilä, 2013).

2.3. Heat Shock Protein and hypoxia-inducible factor characterization

We characterized the molecular response of the fish to copper exposure at protein level. A group of fish was assigned to Cu (N = 66) and CTRL (N = 63) treatment for one week. A group of fish different from the one used for thermal tolerance evaluations was used since CT_{max} measurement could influence the expression of proteins. During the fourth (4D) and seventh (7D) day of copper/handling control

exposure, 10-30 fish per treatment (KOT = 25, LOV = 30, OLK = 23, POO = 20, POR = 10, PYH = 21) were sampled and sacrificed with cranial percussion (Fig. 1B). Twenty-nine fish (around 5 per population) were kept in the same rearing temperature without being transferred for the entire duration of the experiment (naive).

Immediately after fish were sacrificed, we recorded the tag, mass, length, condition factor, sex and number of plates of the fish. Thereafter, liver of each fish was dissected, weighed, and flash frozen in liquid nitrogen and stored at -80 °C until laboratory analyses. Protein characterization was performed according to Mottola et al. (2020). Around 25 mg of frozen liver tissue was 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), by using TissueLyser (Qiagen, Hilden, Germany) at 30 shakes s^{-1} for 2 min. 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). Twenty μg of protein of each sample was loaded in a TGX Stain-Free™ FastCast™ Acrylamide gels, 12% (BioRad, Cat#1610185). Proteins were separated by size at 200 V for 90 min. Thereafter, the gels were scanned for total protein analyses with ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). From the gels the proteins were transferred to a Whatman nitrocellulose membrane, pore size 0.45 μm (Perkin Elmer, Boston, MA, USA), at 100 V for 1 h at +4 °C and incubated in Tris-buffered saline (TBS) blocking solution containing 5% non-fat powdered milk. After that, membranes were incubated overnight with mouse monoclonal HSP90 beta (ab53497) primary antibody (1:10,000) (Abcam, Cambridge, UK), rabbit polyclonal anti-salmonid inducible HSP70 (AS05061A) primary antibody (1:10,000) (Agrisera, Vännäs, Sweden) and rabbit polyclonal HIF-1 alpha (ab2185) primary antibody (1:2,000) (Abcam, Cambridge, UK) in TBS-0.1% Tween-5% milk at +4 °C. The morning after, the membranes were incubated in TBS-0.1% Tween-5% milk with 1: 5,000 IRDye® 800CW Goat anti-Mouse IgG (Licor, Lincoln, NE, USA) and 1:10,000 Goat Anti-Rabbit IgG StarBright Blue 700 (Bio-Rad) secondary antibody for the detection of HSP90 and HSP70/HIF-1 alpha, respectively. After TBS-0.1% Tween membrane washing, the bands were visualized at 800 and 700 nm in ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA) (Supplementary Fig. S2). Densitometry was performed using ImageLab. Each gel contained a gel loading control sample to take gel-to-gel variation into account in calculations. For estimating the relative protein levels of HSP70, HSP90 and HIF-1 alpha, band intensities were divided with total protein gel band intensities.

2.4. Statistics

All the morphological, physiological and molecular data were initially tested for equal variance using Brown-Forsythe test, and for normality using Shapiro-Wilk test. If the assumptions were valid, one-way ANOVA and Tukey post-hoc test were used to assess the morphological difference among the populations in the mass, number of plates, and length. If data were not following the above-mentioned assumptions even after data transformation, non-parametric Kruskal-Wallis test with Dunn's post hoc test were applied instead. Since we found that mass was differing among the populations, we verified the relationship between fish mass and physiological response (CT_{max1}, CT_{max2}) using a Spearman's rank correlation analyses. Kruskal-Wallis test with Dunn's post hoc test was used to assess the differences in the CT_{max1} among populations and sex of the fish. Fish that did not survive after CT_{max1} were removed from the analyses. Regarding the molecular analyses, we primarily tested if there were any differences between the days of exposure to copper and handling control condition (4D vs 7D) using a Kruskal-Wallis test with days as factor.

Linear mixed-effect model was used to test whether CT_{max1} and

CT_{max2} were different after fish were exposed to copper run-off or handling control. The model was also run to assess potential physiological divergences at population level. Therefore, the model included the trial (CT_{max1} vs CT_{max2}) and the populations as fixed factors. Moreover, since sex could have a major effect on fish thermal tolerance and its plasticity, we decided to include sex and its interaction with trial into the model also as a fixed factor. Individual fish identity was included as random factor in order to account for repeated measures of the same individual. The model was run as following: *Temperature ~ Trial + sex + Trial:sex + Population + (1 | fish identity)*.

Since we did not find any significant effect of the days on the expression pattern of our protein of interest, we decided to run a Linear mixed effect model for HSP70, HSP90 and HIF-1alpha including the effects of the treatment (copper vs handling control and naive), the population and sex as fixed factor and the day of exposure (4th and 7th day) as random factor in order to account for different sampling time. Thus, the model structure was: *HSP70-HSP90-HIF-1 ~ Exposure group + Population + Sex + (1|Day of sampling)*.

To evaluate the degree of variability between CT_{max}s and its plasticity in both handling and copper exposed groups, and also in the expression of the HSPs and HIF, we performed an F-test.

Models were run using the function *lmer* in the package "lmerTest", and model output were visualised using a type III Analyses of variance using Satterthwaite's method. Post-hoc test of the model has been performed using the function *glht* into the "multcomp" package. All the statistical analyses and plotting were performed using RStudio version 3.6.1 (R Core Development Team, 2019).

3. Results

Statistical analyses conducted on morphological features showed a divergence among the populations in mass (chi-squared = 35.8, *p-value < 0.001*), length (chi-squared = 11.73, *p-value < 0.001*) and number of lateral plates (chi-squared = 27, *p-value < 0.001*). Major morphological divergences have been observed between populations inhabiting different Gulfs, with fish populations living in the Gulf of Finland showing lower mass and length, but higher number of plates compared to the populations living in the Gulf of Bothnia (Table 1). Correlation analyses showed no statistically significant relationship between fish mass and CT_{max1} (ρ = 0.1, *p-value = 0.3*) or CT_{max2} (ρ = 0.1, *p-value = 0.3* in handling control; ρ = 0.2, *p-value = 0.2* in copper).

In order to assess whether fish showed different initial upper thermal tolerance among the populations after 4 weeks acclimation to common

Table 1

Average of mass, length and number of lateral plates for adult individual of three-spined sticklebacks (*Gasterosteus aculeatus*) inhabiting six Finnish coastline areas of the Baltic Sea. Values are expressed as mean ± standard error.

	KOT	LOV (NPP)	POO	POR	OLK (NPP)	PYH
MASS	1.2 ± 0.06	1.4 ± 0.09	1.3 ± 0.1	2.0 ± 0.1	1.9 ± 0.1	1.8 ± 0.1
LENGHT	5.5 ± 0.08	5.8 ± 0.1	5.6 ± 0.2	6.6 ± 0.2	6.4 ± 0.1	6.3 ± 0.1
PLATES	11 ± 0.9	12.5 ± 0.6	9.4 ± 1.3	6.2 ± 0.8	7.5 ± 0.9	7 ± 1.0
CT_{max1}	31.3 ± 0.2	30.3 ± 0.2	29.8 ± 0.5	31.0 ± 0.3	31.1 ± 0.2	30.6 ± 0.2
CT_{max2}_CTRL	31.5 ± 0.3	31.1 ± 0.2	31.2 ± 0.3	31.3 ± 0.4	31.1 ± 0.3	31.2 ± 0.2
CT_{max2}_Cu	32 ± 0.2	31.6 ± 0.1	32.6 ± 0.1	31.7 ± 0.3	31.8 ± 0.2	31.8 ± 0.2

Abbreviations: Kotka (KOT), Loviisa nuclear power plant (LOV (NPP)), Porvoo (POO), Pori (POR), Olkiluoto nuclear power plant (OLK (NPP)), Pyhäranta (PYH). Critical thermal maximum 1 (CT_{max1}), Critical thermal maximum 2 after handling control (CT_{max2}_CTRL), Critical thermal maximum 2 after copper exposure (CT_{max2}_Cu).

garden conditions, we measured their CT_{max1}. Average CT_{max1} was 30.7 ± 0.1 °C and was not depending on population (chi-squared = 8.0, *p-value* = 0.2). However, it depended on sex (chi-squared = 13.1, *p-value* < 0.001), with female fish showing higher upper thermal tolerance than male ones (F = 31.2 ± 0.2 °C; M = 30.2 ± 0.2 °C, Fig. 2).

Since fish were individually tagged, we could make a comparison between the CT_{max1} and the CT_{max2} in copper-exposed and handling control fish. Linear mixed effect model showed a slight increase of the upper thermal tolerance after handling control conditions, given by the effect of the Trial (CT_{max1} vs CT_{max2}) (F = 10.1, *p-value* < 0.01) (Fig. 3). No direct sex effect was observed in the plasticity of the thermal tolerance (F = 0.5, *p-value* = 0.5) in the handling control group. However, the interaction between sex and Trial was found to be statistically significant (F = 6.5, *p-value* = 0.01). Indeed, a hardening effect in the handling control was observed to be higher in male fish, which increased their upper thermal tolerance by 1.2 °C (30.1 °C ± 0.3 → 31.3 °C ± 0.2) in the latter CT_{max} measurement. In female fish such effect was not seen (31.2 °C ± 0.3 → 31.2 °C ± 0.2). No population effect (F = 0.9, *p-value* = 0.4) was found in handling control group.

When the same model was run for the copper exposed fish group, we also found a significant plasticity in the thermal tolerance (F = 32.5, *p-value* < 0.001; i.e. change in plasticity). Moreover, a significant effect of sex on the plasticity after copper exposure was seen (F = 7.9, *p-value* < 0.01). The interaction between sex and Trial was found to be significant also in the copper exposed fish group (F = 4.9, *p-value* < 0.05), where male fish were able to increase their upper thermal tolerance by 1.5 °C (30.3 °C ± 0.3 → 31.8 °C ± 0.1), while in females the increase was 0.6 °C (31.3 °C ± 0.2 → 31.9 °C ± 0.1) (Fig. 3). No effect of the population was observed (F = 0.5, *p-value* = 0.74). We also found that the inter-individual variability of the CT_{max} decreased from first to second determination both in the handling controls (F = 4.2, *p-value* < 0.001) and copper-exposed fish (F = 5.7, *p-value* < 0.001). When testing the variability of the $CT_{max}s$ in different sexes the inter-individual variability of the CT_{max} decreased in females in the handling control group (F = 3.3, *p-value* < 0.01), but not in copper-exposed group (F = 2.0, *p-value* = 0.10). In contrast, the inter-individual variability of male fish decreased from CT_{max1} to CT_{max2} in both handling control (F = 5.5, *p-value* < 0.001) and copper-exposed (F = 8.8, *p-value* < 0.001) group.

We also evaluated whether copper exposure affected the expression profile of HSP70, HSP90 and HIF-1. We did not find any differences in HSP70 protein level among the three different exposure groups (naïve, handling control and copper exposed) (F = 0.3, *p* = 0.7), or among

populations (F = 1.2, *p* = 0.2) and between sex (F = 2.8, *p* = 0.1). Lack of difference was found also for the HSP90 among the treatment groups (F = 1.4, *p* = 0.3), population (F = 1.4, *p* = 0.2) and sex (F = 0.5, *p* = 0.5), and in HIF-1 among the three groups (F = 3.0, *p* = 0.05) populations (F = 0.9, *p* = 0.4) or sex (F = 0.8, *p* = 0.4) (Fig. 4; Supplementary Table S2, S3). All the statistical results of the models about CT_{max} and protein levels are presented in Supplementary Tables S1, S2, S3.

4. Discussion

Assessing whether evolutionary adaptation to global warming affects the phenotypic plasticity of fish under a second stressor has so far remained unresolved, even though in nature fish experience multiple stressors. Here, we used fish inhabiting thermally polluted habitats near nuclear power plants to reveal whether potential adaptation to high environmental temperatures, or secondary effects of high temperature – like changed water oxygen levels or composition of food webs, affects their phenotypic plasticity to respond to a mimicked copper run-off, compared to fish inhabiting thermally unpolluted areas. We found, against our hypothesis, that the initial upper thermal tolerance (CT_{max1}) did not depend on the population of origin, probably suggesting that the increase in water temperature during five decades long activity of the nuclear power plants did not result in evolutionary change of the upper thermal tolerance in fish collected nearby those areas. However, independent of the population origin, the copper exposure increased the upper thermal tolerance of the fish in sex specific manner, with males showing higher plasticity than females. This would suggest that 1-week exposure to copper might have triggered a defense response, which also resulted in improved thermal tolerance, probably via shared pathways of response. The results also suggest that the three-spined stickleback populations living in the Baltic Sea might be able to cope with a multiple-stressor environment without losing their thermal capacity. Especially male sticklebacks seem to have high resistance to fluctuating environmental conditions.

There are a few possible reasons why the upper thermal tolerance, and the response to copper, did not differ between populations. First reason is related to variability of CT_{max}. The fish in the vicinity of NPPs necessarily experience high temperature spikes more frequently than fish inhabiting areas without thermal pollution. We found that the variability of CT_{max} decreased from first to second measurement in handling control fish, i.e., experiencing extremely high temperature affects mostly the differences in thermal tolerance between individuals even if the mean CT_{max} does not improve much. Consequently, an increase in temperature in nature (i.e. near NPPs) may decrease the capability to increase temperature tolerance via adaptation i.e. average CT_{max} does not increase near NPPs, and, thus, all the populations were having similar upper thermal tolerance values in the current study. These evidences confirm the "concrete ceiling" hypothesis which predict low potential for improvement of the upper thermal tolerance in population experiencing increasing environmental temperatures (Sandblom et al., 2016).

The second reason could be that the fish in NPP areas have been behaviorally thermoregulating and, thus, there have not been selections for high CT_{max}. Temperature in the areas in the vicinity of the nuclear power plant have been really close to the upper thermal limits of sticklebacks, i.e. temperature rose to 32.2 °C in Loviisa NPP during a heat wave in July 2018 (Fig. S1, Supplementary material). However, fish might have escaped the stressful conditions choosing cooler microhabitats. Such phenomenon could have caused an evolutionary inertia, thus not leading to thermal adaptation. However, although the adults might be able to escape from a stressful condition, this is not an option for stickleback embryos laid along the shoreline during the reproductive period. Three-spined sticklebacks, indeed, build their nest near the shoreline where shallow water tends to become warmer during early summer, when males provide parental care to the nest (e.g. oxygenation of the nest) (Ostlund-Nilsson et al., 2006). The warm temperature in the
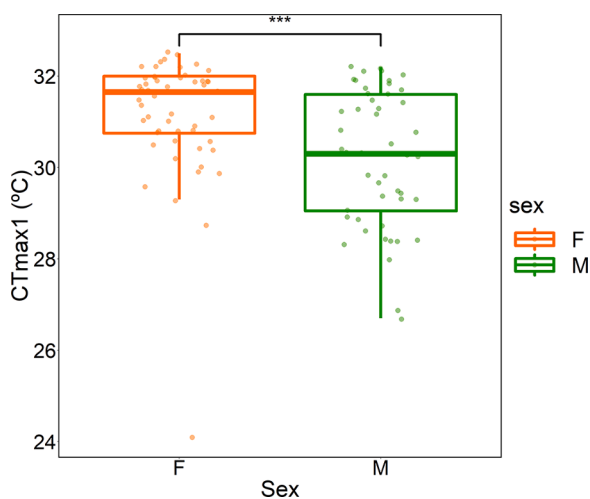

Fig. 2. Boxplot showing the differences between male (N = 48) and female (N = 52) in the initial upper thermal tolerance (CT_{max1}, °C) in three-spined sticklebacks. Green plot represents male fish, and orange one is representing female fish. *** *p-value* < 0.001.
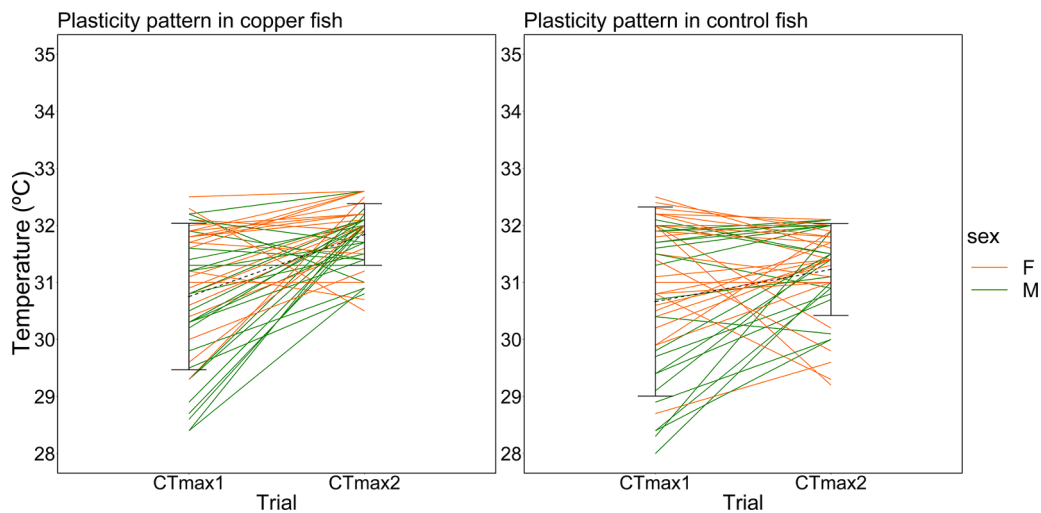
Fig. 3. Individual CT_{max1} and CT_{max2} after one week of exposure to handling control (A, N = 53) condition and laboratory mimicked copper run-off (100 ug/L) (B, N = 50). Different colors represent different sex. Black dot and whiskers indicate the average CT_{max1} and CT_{max2} ± SE, while dashed black line indicates the mean slope of plasticity.
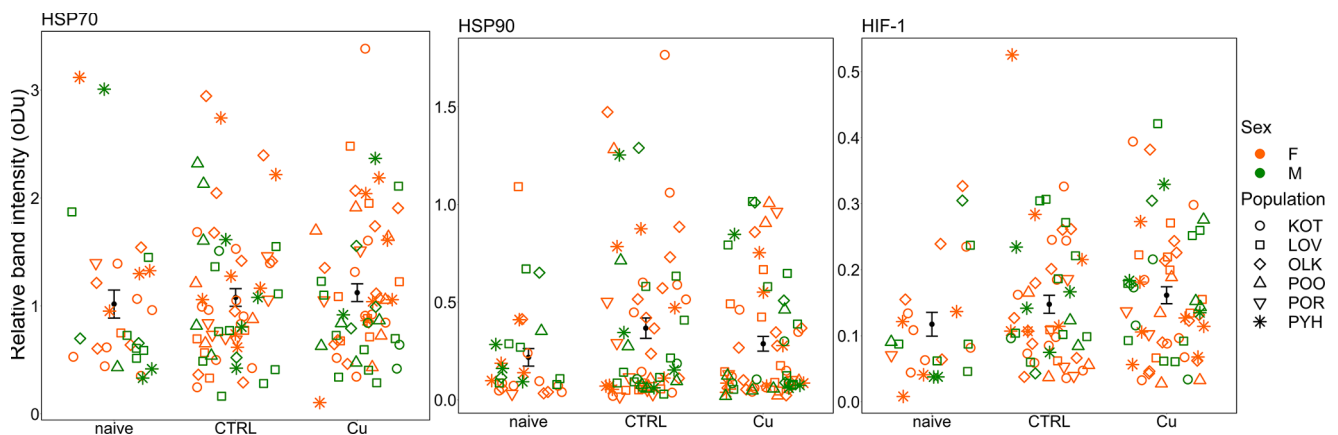

Fig. 4. Relative expression of the HSP70, HSP90 and HIF-1 in naïve, handling control and copper exposed fish, respectively. Black dot and whisker represent the average value ± SE. Different colours represent different sex, meanwhile different shapes represent different populations. Kotka (KOT), Loviisa (LOV), Porvoo (POO), Pori (POR), Olkiluoto (OLK), Pyhäranta (PYH).

nuclear power plant, or during a heat wave, might then cause thermally driven developmental effect or mortality at larval stage as a result of males abandoning the nest. Moreover, even though previous study by Guo et al. (2015) has shown that stickleback populations are genetically divergent in the whole Baltic Sea, and at least the fish from the different Gulfs are different both genetically (DeFaveri and Merilä, 2013; Guo et al., 2015) and morphologically (as seen also in the current study), there might not have been yet genetic divergence between the NPP populations and their respective control populations. Indeed, our previous companion study conducted on the same fish, showed low genetic divergence among the populations, suggesting a gene flow or the lack of selective pressure in response to warm temperature in the nuclear power plant areas (Mottola et al., 2022). The absence of a population-specific response in the upper thermal tolerance might be, therefore, driven by the genetic homogeneity of the populations. The physiological response was confirmed at molecular level, where we have not found any population-specific pattern of expression of either HSPs or HIF-1. Importantly, however, the tolerances and responses were similar in both Gulfs, suggesting a consistent pattern of the response to copper exposure, which was replicable.

Even though our first hypothesis about population differences was rejected, we found several novel results. First of all, one-week long

exposure to sub-lethal copper concentration increased the individual upper thermal tolerance by ~1.5 °C. These results are environmentally relevant since the copper concentration used in the common garden experiment was sub-lethal for sticklebacks and represented a concentration that has been found from marine environments where it is considered as high concentration (Sanchez et al. 2005). As with initial thermal tolerance, the phenotypic responses to copper were similar in all the populations, suggesting that the responses to copper are not population-specific but repeatable regardless of the origin of the fish. This suggests that the finding is general.

To our knowledge, this is the first study where an increase in the upper thermal tolerance following a copper exposure has been reported. There are several possible explanations for the acquired result. The improved thermal capacity might have e.g. result from enhanced oxygen delivery to tissues after copper exposure. Some studies are showing that sub-lethal copper exposure increases the blood oxygen delivery to tissues via increased hemoglobin and hematocrit levels (Dethloff et al., 1999). Furthermore, copper exposure has been shown to enhance the expression of HIF-1alpha in fish, which enhances the capillary density (Nikinmaa and Rees, 2005) and, thus, oxygen delivery to tissues. Recently it has been shown that ligation of coronary blood flow to heart reduces the thermal tolerance of fish (Morgenroth et al., 2021), meaning

that oxygen supply via capillaries plays a significant part in thermal tolerance of fish. However, we do not know if the improvement of thermal capacity in fish exposed to copper in our study was the result of enhanced oxygen delivery capacity, especially because the increase was not associated with a change of HIF-1alpha level. Furthermore, a recent study has shown that by knocking out HIF-1alpha in zebrafish, the plasticity of thermal tolerance is not impacted (Joyce and Perry, 2020). Thus, the different responses to copper exposure may represent species-specific differences. One aspect to take into account is that the fish from this study have been acclimated to artificially constructed brackish water (4 ppt), which has a high concentration of dissolved ions (i.e. the water hardness was 55.2 fH). The water hardness has been previously observed to influence the copper bioavailability in fish and other organisms (Pilehvar et al., 2020). Therefore, one hypothesis is that the sub-lethal concentration of copper used in the current study was not resulting in detrimental effect on the thermal tolerance because of potentially reduced bioaccumulation given by elevated water hardness. Thus, the exposure to copper might have been beneficial for the fish, being an essential metal for organism physiology. Moreover, the copper concentration in the filtered water used during the study was moderately high (about 20 µg/L). It is previously showed that lower sensitivity to copper exposure was followed by copper pre-acclimation in *Daphnia magna* (Bossuyt and Janssen, 2003). Therefore, we cannot rule out the hypothesis that fish might have benefitted from a potential pre-exposure to the baseline copper concentration via acclimation, and opposite outputs might have resulted from the absence of pre-acclimation.

Similar to the lack of response in HIF-1, also the level of the HSP70 and 90 seems to not be influenced by the presence of copper, although earlier studies suggest that an exposure of organisms to metals initiates the heat shock response (Jing et al. 2013). HSPs are also increased in heat shock, thus, it could have been the mechanism linking the two stressors and possibly explaining the increased thermal tolerance after copper exposure. It is possible that the copper concentration used in our study did not cause enough protein damage to cause HSP induction. Alternatively, copper may have caused HSP induction at the onset of exposure, but the response may have waned during the week-long exposure. Therefore, we cannot rule out the hypothesis that high expression of the HSPs and HIF and, similarly a different response of the CT_{max2}, might have resulted if these were tested during the early part of the exposure i.e. fish would not been able to acclimate to these conditions. Moreover, the group tested for the upper thermal tolerance experienced an initial thermal stress given by the CT_{max1}, potentially triggering molecular pathways involved into the increased expression of stress proteins, or HIF-1. On the other hand, the fish utilized for the study of the HSPs and HIF-1 were not exposed to any initial thermal change, merely to copper exposure. Thus, the molecular mechanism underpinning the improved upper thermal tolerance by copper exposure could not be revealed with our study design if the improvement of thermal tolerance was driven also by initial thermal stress of CT_{max1} and not merely by the copper exposure.

One other possible hypothesis we believe is worth exploring in further studies is whether the copper is initiating a resistance to heat stress, or vice versa, thanks to a cross-protection interaction (Rodgers and Isaza, 2021). It has been already documented that the exposure to a primary stressor is improving the capacity to cope with further stressor in fish species (Fitzgerald et al., 2016). However, so far it has been unclear what the mechanisms behind the cross-protection are. Therefore, identifying whether there are shared molecular pathways which contribute to the enhancement of the thermal performance in copper exposure would represent a valuable next goal.

One of the clearest results of the present study was the sex-dependent differences in the thermal tolerance. The sex-specific differences can be expected for sticklebacks, as males and females differ in their behavior markedly (King et al., 2013). However, it is not clear if the differences are beneficial or not in the thermally- or copper-polluted environments. We found that the initial upper thermal tolerance was 1 °C-higher in

female fish than in males. On the other hand, the phenotypic plasticity after the exposure to copper was higher in male fish compared to females. In our study, assessing the phenotypic plasticity of thermal tolerance after an exposure to 1-week long heat wave on the same populations we found similar response with males showing higher capacity of acclimation to high temperature than female (Mottola et al., 2022). These observations suggest a better performance of male sticklebacks under environmental perturbations. We cannot explain the physiological mechanism underpinning such difference, but this might be related to the maturation stage of the fish. The fish in our study were all adults, but their reproductive stage could have been somewhat different from each other (especially some females could have been more mature than others) during the exposure to copper and the CT_{max} measurements. This could have led to differences in sensitivity to copper, and, therefore, less consistent response of females to copper exposure in terms of temperature tolerance. Females could have, e.g., had higher energy expenditure than males to produce eggs and there could have been high variability between females depending on maturation stage. Copper exposure also leads to increased energy expenditure (De Boeck et al., 1997; Sanchez et al., 2005), thus, females could have been more sensitive and, thus, not able to increase their thermal tolerance. However, this is just a hypothesis and further studies are needed to evaluate the dependency of the thermal tolerance from maturation stage during copper exposure. The exposure to copper seems also to decrease the variability of the upper thermal tolerance among the individuals, especially in males. This result is important when assessing the capacity of a population to respond to environmental selective pressure (Nikinmaa and Anttila, 2019).

In conclusion, the present study assessed the phenotypic plasticity of the thermal tolerance in three-spined sticklebacks inhabiting different thermal environments in the wild and exposed to an environmentally relevant copper concentration after acclimation to common-garden conditions. We show that the thermal tolerance of males and females was different, that 1-week copper exposure increased the individual upper thermal tolerance of fish, and that some responses to copper exposure were sex-specific. We also showed that the different thermal regimes in the vicinity of nuclear power plants have not resulted in either increased initial upper thermal tolerance or different thermal plasticity after the exposure of fish to copper. Further studies are, however, required to reveal at molecular level, why copper exposure enhanced the capacity of fish to respond an acute temperature challenge.

CRediT authorship contribution statement

Giovanna Mottola: Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Mikko Nikinmaa:** Formal analysis, Writing – review & editing. **Katja Anttila:** Conceptualization, Investigation, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Medicine (KA) and Kone Foundation (KA).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2022.106145.

Reference

Beers, J.M., Sidell, B.D., 2011. Thermal tolerance of Antarctic notothenioid fishes correlates with level of circulating hemoglobin. Physiol. Biochem. Zool. 84, 353–362.

Bossuyt, B.T.A., Janssen, C.R., 2003. Acclimation of Daphnia magna to environmentally realistic copper concentrations. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 136, 253–264.

Chen, Z., Farrell, A.P., Matala, A., Narum, S.R., 2018. Mechanisms of thermal adaptation and evolutionary potential of conspecific populations to changing environments. Mol. Ecol. 27, 659–674.

Currie, S., Tufts, B.L., 1997. Synthesis of stress protein 70 (Hsp70) in rainbow trout (Ocorhynchus mykiss) red blood cells. J. Exp. Biol. 200, 607–614.

De Boeck, G., Vlaeminck, A., Blust, R., 1997. Effects of sublethal copper exposure on copper accumulation, food consumption, growth, energy stores, and nucleic acid content in common carp. Arch. Environ. Contam. Toxicol. 33, 415–422.

DeFaveri, J., Merilä, J., 2013. Variation in age and size in Fennoscandian three-spined sticklebacks (Gasterosteus aculeatus). PLoS One 8, e80866.

Dethloff, G.M., Schlenk, D., Khan, S., Bailey, H.C., 1999. The Effects of Copper on Blood and Biochemical Parameters of Rainbow Trout (Oncorhynchus mykiss). Archives of Environmental Contamination and Toxicology 36, 415–423. https://doi.org/10.1007/PL00006614.

Devor, D.P., Kuhn, D.E., O'Brien, K.M., Crockett, E.L., 2016. Hyperoxia does not extend critical thermal maxima (CT$_{max}$) in white-or red-blooded Antarctic notothenioid fishes. Physiol. Biochem. Zool. 89, 1–9.

Duarte-Gutiérrez, J., Peregrino-Uriarte, A.B., Gómez-Jiménez, S., Mata-Haro, V., Yepiz-Plascencia, G., 2021. HIF-1 is involved in the regulation of expression of metallothionein and apoptosis incidence in different oxygen conditions in the white shrimp Litopenaeus vannamei. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 262, 111072.

Ekström, A., Brijs, J., Clark, T.D., Gräns, A., Jutfelt, F., Sandblom, E., 2016. Cardiac oxygen limitation during an acute thermal challenge in the European perch: effects of chronic environmental warming and experimental hyperoxia. Am. J. Physiol. Regul. Integr. Comp. Physiol. 311, R440–R449.

Ficke, A.D., Myrick, C.A., Hansen, L.J., 2007. Potential impacts of global climate change on freshwater fisheries. Page Rev. Fish Biol. Fish. 17, 581–613.

Fitzgerald, J.A., Jameson, H.M., Dewar Fowler, V.H., Bond, G.L., Bickley, L.K., Uren Webster, T.M., Bury, N.R., Wilson, R.J., Santos, E.M., 2016. Hypoxia suppressed copper toxicity during early development in zebrafish embryos in a process mediated by the activation of the HIF signaling pathway. Environ. Sci. Technol. 50, 4502–4512.

Forouhar Vajargah, M., Mohamadi Yalsuyi, A., Sattari, M., Prokić, M.D., Faggio, C., 2020. Effects of copper oxide nanoparticles (CuO-NPs) on parturition time, survival rate and reproductive success of guppy fish, poecilia reticulata. J. Clust. Sci. 31, 499–506.

Guo, B., DeFaveri, J., Sotelo, G., Nair, A., Merilä, J., 2015. Population genomic evidence for adaptive differentiation in Baltic Sea three-spined sticklebacks. BMC Biol. 13, 1–18.

Healy, T.M., Schulte, P.M., 2012. Factors affecting plasticity in whole-organism thermal tolerance in common killifish (Fundulus heteroclitus). J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 182, 49–62.

Ilus, E. 2009. Environmental effects of thermal and radioactive discharges from nuclear power plants in the boreal brackish-water conditions of the northern Baltic Sea. Radiation and Nuclear Safety Authority, Helsinki (2009).

Jing, J., Liu, H., Chen, H., Hu, S., Xiao, K., Ma, X., 2013. Acute effect of copper and cadmium exposure on the expression of heat shock protein 70 in the cyprinidae fish tanichthys albonubes. Chemosphere 91, 1113–1122.

Joyce, W., Perry, S.F., 2020. Hypoxia inducible factor-1α knockout does not impair acute thermal tolerance or heat hardening in zebrafish: thermal tolerance in Hif-1α-/-zebrafish. Biol. Lett. 16. https://doi.org/10.1098/rsbl.2020.0292.

King, A.J., Fürtbauer, I., Mamuneas, D., James, C., Manica, A., 2013. Sex-differences and temporal consistency in stickleback fish boldness. PLoS One 8, 31–36.

Kumar, N., Krishnani, K.K., Brahmane, M.P., Gupta, S.K., Kumar, P., Singh, N.P., 2018. Temperature induces lead toxicity in Pangasius hypophthalmus: an acute test, antioxidative status and cellular metabolic stress. Int. J. Environ. Sci. Technol. 15, 57–68.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Martinez, E., Porreca, A.P., Colombo, R.E., Menze, M.A., 2016. Tradeoffs of warm adaptation in aquatic ectotherms: live fast, die young? Com. Biochem. Physiol. Part A Mol. Integr. Physiol. 191, 209–215.

Morgenroth, D., McArley, T., Gräns, A., Axelsson, M., Sandblom, E., Ekström, A., 2021. Coronary blood flow influences tolerance to environmental extremes in fish. J. Exp. Biol. 224, jeb239970. https://doi.org/10.1242/jeb.239970.

Mottola, G., Kristensen, T., Anttila, K., 2020. Compromised thermal tolerance of cardiovascular capacity in upstream migrating Arctic char and brown trout—are hot summers threatening migrating salmonids? Conserv. Physiol. 8 (1) https://doi.org/10.1093/conphys/coaa101.

Mottola, G., Lopez, M.E., Vasemägi, A., Nikinmaa, M., Anttila, K.. Are you ready for the heat? Phenotypic plasticity vs adaptation of heat tolerance in three-spined stickleback.Ecosphere. 10.1002/ecs2.4015.

Newman, M.C., 2009. Fundamentals of Ecotoxicology. CRC press.

Nikinmaa, M., Anttila, K., 2019. Individual variation in aquatic toxicology: not only unwanted noise. Aquat. Toxicol. 207, 29–33.

Nikinmaa, M., Rees, B.B., 2005. Oxygen-dependent gene expression in fishes. Am. J. Physiol. Regul. Integr. Comp. Physiol. 288, 1079–1090. https://doi.org/10.1152/ajpregu.00626.2004.

O'Brien, K.M., Rix, A.S., Grove, T.J., Sarrimanolis, J., Brooking, A., Roberts, M., Crockett, E.L., 2020. Characterization of the hypoxia-inducible factor-1 pathway in hearts of Antarctic notothenioid fishes. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 250, 110505.

Ostlund-Nilsson, S., Mayer, I., Huntingford, F.A., 2006. Biology of the Three-Spined Stickleback. CRC press.

Peruzza, L., Thatje, S., Hauton, C., 2021. Acclimation to cyclic hypoxia improves thermal tolerance and copper survival in the caridean shrimp Palaemon varians. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 259, 111010.

Pilehvar, A., Cordery, K.I., Town, R.M., Blust, R., 2020. The synergistic toxicity of Cd(II) and Cu(II) to zebrafish (Danio rerio): effect of water hardness. Chemosphere 247, 125942.

Pilehvar, A., Town, R.M., Blust, R., 2019. The effect of thermal pre-incubation and exposure on sensitivity of zebrafish (Danio rerio) to copper and cadmium single and binary exposures. Aquat. Toxicol. 213, 105226.

R Core Development Team, 2019. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, 2012URL. https://www.R-project.org.

Rezende, E.L., Tejedo, M., Santos, M., 2011. Estimating the adaptive potential of critical thermal limits: Methodological problems and evolutionary implications. Funct. Ecol. 25, 111–121.

Rissanen, E., Tranberg, H.K., Sollid, J., Nilsson, G.E., Nikinmaa, M., 2006. Temperature regulates hypoxia-inducible factor-1 (HIF-1) in a poikilothermic vertebrate, crucian carp (Carassius carassius). J. Exp. Biol. 209, 994–1003.

Rodgers, E.M., and D.F.G. Isaza. 2021. Harnessing the potential of cross-protection stressor interactions for conservation : a review. Conserv. Physiol. 9,1–27. coab037.

Ruuskanen, S., Mottola, G., Anttila, K., 2020. Experimental copper exposure, but not heat stress, leads to elevated intraovarian thyroid hormone levels in three-spined sticklebacks (Gasterosteus aculeatus). Ecotoxicology 29, 1431–1440. https://doi.org/10.1007/s10646-020-02278-1.

Sanchez, W., Palluel, O., Meunier, L., Coquery, M., Porcher, J.M., Aït-Aïssa, S., 2005. Copper-induced oxidative stress in three-spined stickleback: relationship with hepatic metal levels. Environ. Toxicol. Pharmacol. 19, 177–183.

Sandblom, E., Clark, T.D., Gräns, A., Ekström, A., Brijs, J., Sundström, L.F., Odelström, A., Adill, A., Aho, T., Jutfelt, F., 2016. Physiological constraints to climate warming in fish follow principles of plastic floors and concrete ceilings. Nat. Commun. 7, 1–8.

Sappal, R., Fast, M., Stevens, D., Kibenge, F., Siah, A., Kamunde, C., 2015. Effects of copper, hypoxia and acute temperature shifts on mitochondrial oxidation in rainbow trout (Oncorhynchus mykiss) acclimated to warm temperature. Aquat. Toxicol. 169, 46–57.

Sidhu, R., Anttila, K., Farrell, A.P., 2014. Upper thermal tolerance of closely related Danio species. J. Fish Biol. 84, 982–995.

Somero, G.N., 2020. The cellular stress response and temperature: function, regulation, and evolution. J. Exp. Zool. 333, 379–397.

van Heerden, D., Vosloo, A., Nikinmaa, M., 2004. Effects of short-term copper exposure on gill structure, metallothionein and hypoxia-inducible factor-1α (HIF-1α) levels in rainbow trout (Oncorhynchus mykiss). Aquat. Toxicol. 69, 271–280.

Wang, G.L., Semenza, G.L., 1993. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc. Nat. Acad. Sci. U.S.A. 90, 4304–4308.

Zizza, M., Canonaco, M., Facciolo, R.M., 2016. Neurobehavioral alterations plus transcriptional changes of the heat shock protein 90 and hypoxia inducible factor-1α in the crucian carp exposed to copper. Neurotoxicology 52, 162–175.