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Are you ready for the heat? Phenotypic plasticity versus adaptation of heat tolerance in three-spined stickleback

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Abstract

Heat waves constitute a challenge for aquatic ectotherms. However, the thermal tolerance of animals and their individual phenotypic plasticity to respond to heat waves may be influenced by thermal history. We tested these hypotheses by comparing the upper thermal tolerance and the individual capacities of three-spined sticklebacks from populations with different thermal histories to respond to heat waves. Two populations originated from thermally polluted nuclear power plant (NPP) habitats, while four locations represented geographically adjacent control areas. To disentangle the genetic adaptation from the phenotypic plastic response, we measured the individual upper thermal tolerance and the responses at molecular level in common-garden conditions before and after a laboratory-mimicked heat wave. We found that the sticklebacks exhibit considerable phenotypic plasticity in thermal tolerance since the heat wave increased fish upper thermal tolerance significantly. The individual plasticity to respond to the heat wave was also negatively correlated with initial thermal tolerance. On the other hand, neither the thermal tolerance nor the plastic responses differed between NPP and control sites despite detection of significant but low genome-wide divergence in 10 out of 15 pairwise comparisons. Our results suggest that five decades of NPP activity with warmer water have not resulted in a detectable evolutionary change in either the upper thermal tolerance or its plasticity in three-spined sticklebacks potentially rendering them sensitive to frequent heat waves.

KEYWORDS
critical thermal tolerance, evolution, heat shock proteins, heat wave, intraindividual variability, intraspecific variation, nuclear power plant, phenotypic plasticity

INTRODUCTION

Climate change causing global warming and extreme weather events poses a severe threat for a wide range of organisms (Till et al., 2019). Organisms and populations, however, are able to respond to a certain extent to rising temperatures via different behavioral, physiological, and evolutionary mechanisms. One of the potential outcomes of increased temperature at the population level is evolutionary adaption to the warmer habitat. This has been demonstrated

in a wide range of species living at different latitudes and/or thermal environments, the ones inhabiting warmer habitats usually possessing increased thermal performance (Pilakouta et al., 2020; Sandblom et al., 2016). However, not all species and populations from different latitudes and thermal environments show local adaptation to heat waves (Anttila et al., 2014). Furthermore, experimental evolution studies have shown that there is an upper boundary limit for thermal tolerance, which cannot be increased further via selection (Morgan et al., 2020). Such evolutionary experiments provide valuable insights on how phenotypic traits evolve in response to a specific environmental factor that is controlled in a laboratory (Merilä & Hendry, 2014). However, animals are living in complex environments where different factors could influence the thermal tolerance. Therefore, despite a body of work on thermal tolerance and adaptation in ectotherms (Moyano et al., 2020), it is still poorly known if and how aquatic vertebrates are able to adapt to rapid increase in water temperature in nature (Pilakouta et al., 2020). Besides evolutionary adaptation, animals are also able to respond to increasing temperatures via phenotypic plasticity, that is, via acclimation. The thermal tolerance of most animal changes in response to their recent thermal history through acclimation (Bilyk & DeVries, 2011). Indeed, it has previously been shown that fish species exhibit substantial phenotypic plasticity in the upper thermal tolerance in response to thermal acclimation (Bilyk & DeVries, 2011; Fangue et al., 2006; Healy & Schulte, 2012). The quantity of plasticity to increase the upper thermal tolerance, however, varies between fish species (Beitinger et al., 2000). Furthermore, among studies comparing the population variability in plasticity (Sasaki & Dam, 2019), only few have focused on intraspecific variation in thermal tolerance (Loughland & Seebacher, 2020) and within-individual variability. However, understanding the mechanisms driving intraspecific variation will be critical for predicting how organisms will respond to rapidly changing and novel environments (Nikinmaa & Anttila, 2019) and how rapid, widespread changes in biodiversity within species will impact communities and ecosystems (Roches et al., 2018). Moreover, individuals can differ in their response and much of their phenotypic variation is rooted in environmental variation (Dingemanse & Dochtermann, 2013; O'Dea et al., 2021). For example, earlier work has shown that populations from lower latitudes have higher thermal tolerance while exhibiting lower phenotypic plasticity (Sasaki & Dam, 2019). This supports the hypothesis of "concrete ceiling" of thermal tolerance (Sandblom et al., 2016) predicting that populations from warmer habitats may be already closer to their upper boundary limit of thermal tolerance than populations from colder habitats and therefore possess reduced plastic capacity to respond to further warming. To best of our knowledge, however, the presence of "concrete ceiling" of thermal tolerance has not been tested at individual level.

To study how thermal history of animals influences the genetic adaptation and individual plasticity of thermal tolerance, we took advantage of spatially replicated, coastal habitats of the Baltic Sea experiencing thermal pollution from nuclear power plant (NPP) cooling water during the last 50 years. We compared the thermal tolerance, and how it changes with exposure to heat wave (i.e., plasticity), of animals from NPP areas to those of animals from cooler control regions. The discharge water from the NPP has been reported to warm the temperature of water in these areas by 2–5°C compared with the inlet water areas (Ilus, 2009). Three-spined stickleback (*Gasterosteus aculeatus*) was selected as our study organism. The abundance of this species increased exponentially during the mid-1990s in the Baltic Sea (Nilsson et al., 2019). Moreover, although this pelagic species aggregates in coastal areas only for a few months during summer for reproduction (Nilsson et al., 2019), previous studies provide evidence for genomic divergence driven by local adaptation along thermal gradients in whole Baltic Sea, suggesting that gene flow is not constraining adaptive divergence, but rather, adaptive divergence may constrain gene flow (Defaveri et al., 2013; Defaveri & Merilä, 2013a; Guo et al., 2015). Therefore, we predicted that populations from NPP areas would show evidence for evolutionary local adaptation to warmer environment resulting in higher upper critical thermal tolerance. Moreover, we predicted, according to the hypothesis of "concrete ceiling," that individuals and populations with the highest initial thermal tolerance would show the smallest phenotypic increase in thermal tolerance after the experimental heat wave, that is, lowest phenotypic plasticity (van Heerwaarden & Kellermann, 2020; Sandblom et al., 2016). In order to uncover genetic divergence among studied populations, we also carried out genome-wide analysis of single nucleotide polymorphisms (SNPs) using high-throughput restriction site-associated DNA sequencing approach (RADseq). Furthermore, to shed light on possible cellular mechanisms behind population differences in tolerance to heat waves, we analyzed the expression of the heat shock proteins (HSP70 and HSP90) among populations during the simulated heat wave (Schoville et al., 2012).

METHODS

Study area and experimental design

In order to test whether heat adaptation has occurred in response to elevated water temperatures after five decades and to disentangle the potential genetic evolutionary changes from short-term nongenetic plastic

responses, we studied six populations of three-spined sticklebacks with different thermal histories. Two populations were sampled near Finnish NPP areas at Gulf of Finland and Gulf of Bothnia (500 km apart from each other) where the discharge waters have been reported to increase the temperature of water by 2–5°C compared to the inlet water areas (Ilus, 2009). The plants were built during 1971–1980, and they both use around 40–70 m^3 s^{-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 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). We also collected fish from two control sites (CTRL) around each NPP: Kotka (KOT) and Porvoo (POO) for Gulf of Finland and Pyhäranta (PYH) and Pori (POR) for Gulf of Bothnia. The sampling sites were about 50 km away from the LOV and OLK NPP areas, respectively (Figure 1a). The water temperature at studied locations was recorded with HOBO loggers (three per site) for 6 months (Figure 2). The daily average water temperatures for each location were, then, calculated by averaging the values of the three loggers per area (Figure 2). The average temperature and their minimum and maximum were as follows: KOT (average: 14.9 ± 0.05°C, min: 6.5°C, max: 25.6°C), POO (average: 16.3 ± 0.14°C, min: 3.2°C, max: 27.9°C), LOV, NPP (average: 18.3 ± 0.07°C, min: 9.9°C, max: 32.2°C), PYH (average: 16.3 ± 0.14°C, min: 3.8°C, max: 26.2°C), POR (average: 16.2 ± 0.16°C, min: 0.4°C, max: 28.8°C), and OLK, NPP (average: 15.3 ± 0.16°C, min: 3.2°C, max: 29.4°C) (Figure 2). Fish ($n = 100$ per population) were caught from the wild during May 2018 using beach seine net and transferred in large water containers immediately to the University of Turku. During the transfer, natural water temperature and oxygen saturation were maintained using air pumps and ice blocks. No mortality was observed during the transfer. The fish were kept in common-garden conditions in University of Turku after transfer in order to diminish the effects of acclimation to their original habitats and acclimated at 16°C (the average temperature of locations in early May, Figures 1b and 2). Similar protocol has been used for different populations of lacustrine three-spined sticklebacks in Dammark et al. (2018). Fish were let to acclimate to laboratory conditions in six different 180-L tanks for 2 weeks. 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, and 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% $CaCl_2$; and 0.5% $KHCO_3$). Photoperiod was set at 17L:7D to follow 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, Heinsberg, Germany) according to the instructions of the manufacturer. In order to reduce potential tank effects, fish were tagged intraperitoneally after 2 weeks of recovery 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 HCO_3). The populations were mixed in nine tanks (with density of 2 fish L^{-1}) and were let to recover for 2 weeks before further testing. In total, the fish had 4 weeks to recover from catching from wild and to acclimate to common-garden laboratory conditions, which is considered to be an adequate time period to ensure acclimation to new conditions (Dammark et al., 2018). All fish procedures were performed according to Finnish Animal Care permission (ESAVI/2867/2018).

RAD library preparation and sequencing, genotyping, and SNP calling

Genomic DNA was extracted from caudal fin of 165 individuals (around 30/each population) using a salt extraction protocol as described by Aljanabi and Martinez (1997), with some modifications (see Appendix S1). RAD library was prepared according to the protocol described by Baird et al. (2008). Briefly, 500 ng of genomic DNA was individually digested by using the restriction enzyme PstI (5′TGCAG 3′). Individual barcodes were ligated to the forward ends, and fragments were pooled into two libraries and size selected on Pippin HT (1.5% cassette). Amplification of libraries was performed using a PCR. Library construction was validated on Fragment Analyzer (High Sensitivity NGS kit), as well as by qPCR (ROCHE Light Cycler 480). Sequencing was performed on an Illumina NovaSeq 6000 using a NovaSeq Reagent Kit (100 cycles, single-end sequencing). Library preparation, sequencing, and demultiplexing were conducted at an external service provider (MGX—Montpellier GenomiX, Montpellier, France). Altogether, 745,126,832 reads were retained after quality filtering of sequencing 30 individuals per location. The obtained RAD data were analyzed using Stacks

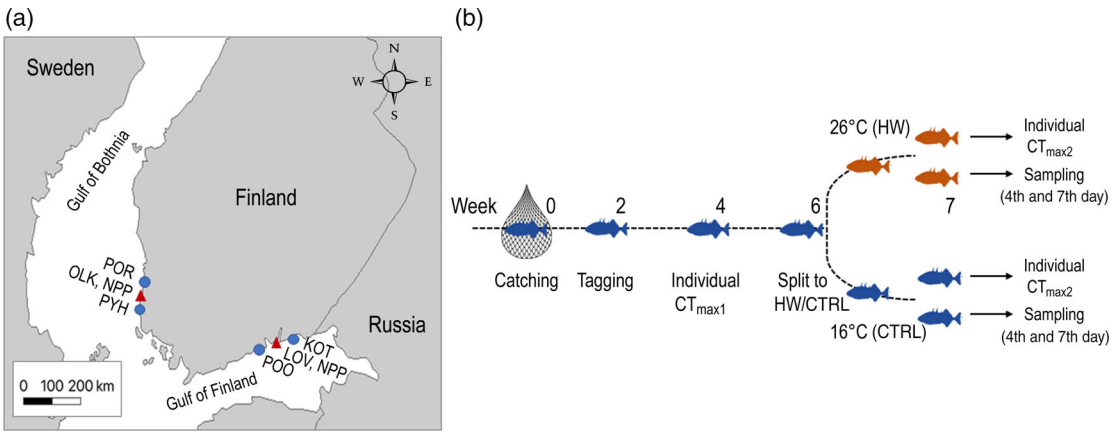

FIGURE 1 Representation of the study area showing sampling locations (a). Red triangles indicate the nuclear power plant areas (NPP), while blue circles indicate the control locations (CTRL). Abbreviations for areas are as follows: Loviisa (LOV), Olkiluoto (OLK), Kotka (KOT), Porvoo (POO), Pyhäranta (PYH), and Pori (POR). Representation of the experimental design (b). About 100 fish per site were caught and transported at University of Turku for acclimation at 16°C. After 2 weeks, they were pit-tagged intraperitoneally with 1.35 × 7 mm RFID subcutaneous microchips (Loligo Systems, Viborg, Denmark) under anesthesia. The initial upper critical thermal tolerance (CT_{max1}) of each individual was measured after 2 weeks of recovery from the tagging. Thereafter, the fish were split into two exposure groups for 1 week: heat wave (HW, 16°C → 26°C) and handling control (CTRL, 16°C → 16°C). After the exposure, a second CT_{max} (CT_{max2}) was performed on the same individuals. Liver samples and fin clips have been collected from other groups that have been exposed to heat wave and handling control only, without any measurement of the CT_{max}

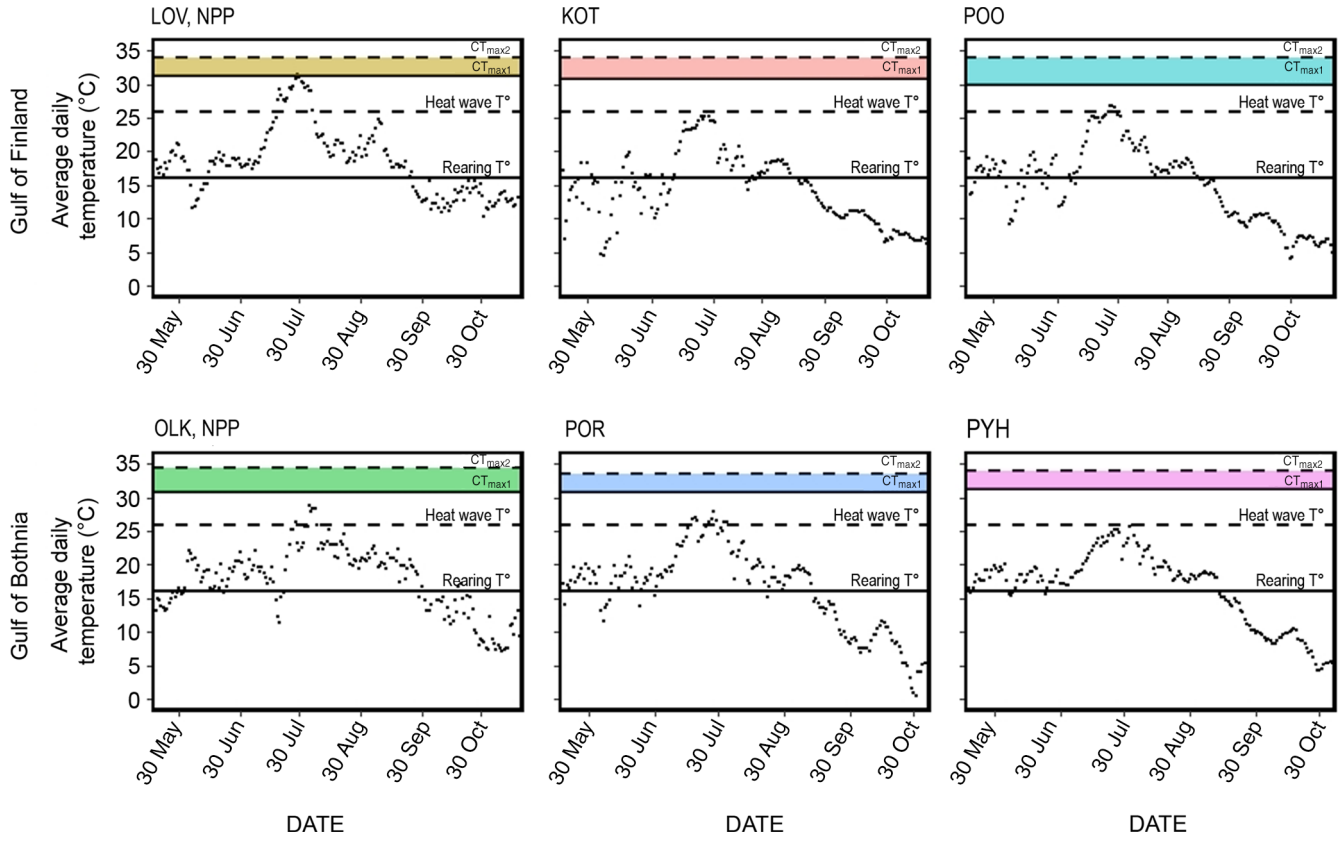

FIGURE 2 Average daily temperature (°C) in the different sampling locations during the period 17 May–20 November 2018 recorded 4–24 times per day and at depth 1.5–2 m using HOBO Water Temp Pro v2 Logger. Solid lines indicate the temperature where fish were reared and the first critical thermal maximum (CT_{max1}). Dashed lines indicate the heat wave temperature and CT_{max2}, respectively. Colored area indicates the plasticity range relative to each population. Abbreviations: Loviisa (LOV), Olkiluoto (OLK), Kotka (KOT), Porvoo (POO), Pyhäranta (PYH), Pori (POR), nuclear power plant (NPP)

(version 2.41) (Catchen et al., 2011, 2013). Demultiplexing, quality filtering (q), and cleaning (-c) were conducted using the process_radtags module of Stacks v2.0 (Catchen et al., 2013). Thereafter, reads were realigned to the latest three-spined stickleback reference genome (Nath et al., 2021) with bwa mem algorithms (Li & Durbin, 2009), and sorting and indexing bam files were done using SAMTOOLS (Li et al., 2009). This genome assembly was enhanced using long-read Pacific Biosciences sequencing technique, leading to a fivefold improvement in continuity (76% of gaps filled) over the previous version of the reference genome (Peichel et al., 2017).

Single nucleotide polymorphism calling was carried out with Stacks v2.0 (Catchen et al., 2013) using gstacks module with default parameters. Detected loci were filtered with populations module, setting options -r to 0.9, -p 6, --min-maf 0.05, and --max-obs-het 0.5. Hence, the resulting SNP dataset contained all markers that featured less than 10% missing allele calls, present in all six sampling sites, minor allele frequency at least 0.05, and observed heterozygosity less than 0.5.

In order to exclude markers associated with sex determination in three-spined stickleback as the analyzed 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) that incorporates EIGENSTRAT method to test for association while correcting for population structure. 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 F_{ST} estimator (Weir & Cockerham, 1984) in the StAMPP R package (Pembleton et al., 2013; Pembleton & Pembleton, 2020). Significance of F_{ST} values and 95% CI were computed using bootstrapping as implemented in the package. In order to visualize population structure, a discriminant analysis of the principal components (DAPC) was performed with R/adegenet (Jombart & Ahmed, 2011). The optim.a.score function was used to choose the optimum number of PCs to retain.

Critical thermal maxima and heat wave simulation

The upper critical thermal tolerance of fish was measured using critical thermal maximum (CT_{max}) method. The initial CT_{max1} of each individual was tested after 2 weeks of recovery from tagging (Figure 1b). The CT_{max} was quantified for 141 fish (~16 individuals per location) according to Sidhu et al. (2014). For testing the CT_{max}, a VC/3 circulator–heater (Julabo Labortechnik GmbH, Seelbach, Germany) was connected with two metal steel coils inserted into a 45-L tank. There was a constant mixing of the water, and the air saturation was kept over 80% with an air pump. Around 50 individuals per time were placed in the setup (starting temperature + 15.7°C) and kept there for 1 h in order for the fish to familiarize to the new surrounding and reduce stress (Sidhu et al., 2014). Thereafter, water temperature was increased by 0.3°C min^{-1} until 27°C, above which the heating 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 removed from the setup and placed in a recovery tank. CT_{max} was measured every day at the same time in order to minimize potential diel fluctuations (Lydy & Wissing, 1988). Posttrial mortality was followed for the 2 weeks of recovery.

Fish were given 2 weeks to recover from CT_{max1} before they were assigned to two groups: heat wave-exposed (HW, $n = 47$) and handling control (CTRL, $n = 50$) (Figure 1b). Two weeks was considered to be long enough to recover from heat stress since earlier studies have shown that recovery could happen even in 24–32 h or maximally in 1 week (Maness & Hutchison, 1980; Morgan et al., 2018). Heat wave fish were transferred to duplicate heat wave experimental tanks. The water temperature of tanks was increased by 1°C every 30 min until reaching +10°C higher than normal rearing temperature (i.e., 26°C) and kept under that condition for 7 days. This heat wave temperature was chosen in order to exert physiological and molecular effects on the fish without causing mortality. Moreover, the temperature of 26°C was environmentally relevant since it represented the actual heat wave water temperature in Southern Finland in catching locations of the fish (Sinclair et al., 2019) (Figure 2). Control group was similarly transferred to duplicate experimental tanks and kept in normal rearing temperature for 1 week (handling control). After 1-week heat wave, a second CT_{max} (CT_{max2}) was measured in order to analyze the individual phenotypic plasticity of CT_{max}. The starting temperature for the CT_{max2} was the acclimation temperature of the fish (Morgan et al., 2020). Thus, there was a 30-min difference in experiment durations for warm- and cold-acclimated fish. After the measurements, the sex of the fish was identified and mass and length of the fish were measured. We also counted the number of the lateral plates to evaluate potential morphological differences among populations (DeFaveri & Merilä, 2013b).

Heat shock protein characterization

In order to characterize the molecular response of the fish to heat wave at protein level, a group of fish ($n = 149$) was assigned to heat wave (HW) ($n = 57$) and control (CTRL) ($n = 63$) treatment for 1 week. About 25 fish per treatment (KOT = 32, LOV = 29, OLK = 23, POO = 19, POR = 11, and PYH = 31) were sampled and sacrificed with cranial percussion during the fourth day of heat wave/control temperature (4D) and at the end of the exposure (7D) (Figure 1b). Twenty-nine fish (about five per population) were kept in the same rearing temperature without being transferred for the entire duration of the experiment (untreated control group).

After tag reading, mass, length, condition factor, sex, and number of plates were measured before dissecting the liver of the fish. Liver was 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, and pH 6.8) 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 (PerkinElmer, Turku, Finland). Twenty micrograms of protein of each sample was loaded in a TGX Stain-Free FastCast Acrylamide gels, 12% (Bio-Rad, 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 (PerkinElmer, Boston, MA, USA), at 100 V for 1 h at +4°C and incubated in Tris-buffered saline (TBS) blocking solution containing 5% nonfat powdered milk. After that, membranes were incubated overnight with mouse monoclonal HSP90 beta (ab53497) primary antibody (1:10,000) (Abcam, Cambridge, UK) or rabbit polyclonal antisalmonid inducible HSP70 (AS05061A) primary antibody (1:10,000) (Agrisera, Vännäs, Sweden) in TBS-0.1% Tween-5% milk at +4°C. Next morning, the membranes were incubated in TBS-0.1% Tween-5% milk with 1:5000 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, 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). 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 of HSP70 and HSP90, band intensities were divided with total protein gel band intensities, that is, giving HSP levels per total protein amount of samples.

Statistics

The equal variance and normality of the data were tested with Brown-Forsythe test and Shapiro-Wilk test, respectively. Kruskal-Wallis test with Dunn's post hoc test was used to evaluate the CT_{max1}, mass, length, Fulton's condition factor, and number of plate differences among populations and between Gulfs. The phenotypic plasticity, from now on called "thermal plasticity," was calculated subtracting CT_{max1} from the CT_{max2}. The same calculation was also done for the control fish in order to evaluate whether handling caused any effect on plasticity.

Linear mixed-effect model (Model 1) was used to test whether the heat wave or handling control was having an effect on the individual CT_{max}, and whether such a change was similar in individuals belonging to the same population. The model was run using the function *lmer* in the package "lmerTest" (Kuznetsova et al., 2017), and individual fish identity was included as random factor, accounting for a repeated measure of the same individual. "Trials" (CT_{max1} and CT_{max2}) and "population" were included as fixed factors, while "mass" was used as covariate. Furthermore, "sex" and its interaction with mass were included in the model in order to evaluate whether males and females with different population origins had different thermal tolerances and its plasticity. Model 1 structure was $Temperature \sim Trial + Population + Mass + Sex + Mass{:}Sex + Trial{:}Population + (1 \mid Fish.ID)$. Model output was visualized using a type III ANOVA with Satterthwaite's method.

In order to evaluate whether the populations had different plasticity of thermal tolerance, we fit a linear model (Model 2) separately for phenotypic plasticity of CT_{max} (i.e., $CT_{max2} - CT_{max1}$). The Model 2 structure was $Plasticity \sim Population + Sex + Mass$. Besides this, we fitted linear Model 3 to test for the effect of the "CT_{max1}" (as continuous variable) and "population" (as fixed factor) on individual thermal plasticity of fish as following: $Plasticity \sim CT_{max1} + Population$. Multiple comparisons for all the Models were performed using the function *glht* into the "multcomp" package (Bretz et al., 2021) and taking into account the covariates (mass) and the interactions (Hothorn et al., 2008), when needed.

Differences in the expression pattern of HSP70 and HSP90 between populations, treatment, and sampling time points were assessed using one-way ANOVA and Kruskal-Wallis test, respectively. Graphs were plotted using "ggplot2" (Wickham, 2016). All the statistical analyses and plotting were performed using RStudio version 3.6.1 (R Core Development Team, 2019).

RESULTS

Genotyping, filtering, and genetic differentiation

The average RADseq coverage depth per individual was 7.9×. After filtering, 19,303 SNPs were retained to perform a genome-wide association studies (GWAS) analysis for sex determination. Ten SNPs were significantly associated with sex in this data set, and therefore, they were discarded from further analyses (Appendix S1: Figure S1). Subsequently, 101 SNPs were excluded because of deviations from Hardy-Weinberg equilibrium and a high-quality data set of 19,191 SNPs was used to perform pairwise genetic differentiation and structure analyses.

The pairwise F_{ST} between gulfs of Bothnia and Finland was 0.00105 ($p < 0.0001$). The six sampling sites yielded 15 possible pairwise combinations, of which 10 were significant (p value <0.05) and ranged from 0.005 (POO vs. LOV) to 0.0015 (PYH vs. KOT) (Table 1). All three pairwise comparisons within the Gulf of Bothnia showed lack of significance, revealing no distinguishable genetic differences among these three sampling sites. Among sampling sites in Gulf of Finland, one pairwise comparison was nonsignificant (KOT vs. LOV: $F_{ST} = 0.0000$, $p = 0.4108$), while the other two suggested weak genetic structuring (POO vs. LOV: $F_{ST} = 0.0005$, $p = 0.0203$; KOT vs. POO: $F_{ST} = 0.0004$, $p = 0.0545$). All pairwise comparisons between sites of the two gulfs were significant, corresponding to the first axis of DAPC plot (Figure 3).

Morphological divergence

Analyses among populations ($n = 186$) showed significant differences in terms of mass ($\chi^2 = 66.8$, $p = 4.665e{-}13$), length ($\chi^2 = 74.6$, $p = 1.084e{-}14$), and number of lateral plates ($\chi^2 = 51.5$, $p = 4.275e{-}10$), but not in the Fulton's condition factor ($\chi^2 = 1.8$, $p = 0.88$) (Appendix S1: Table S1). Overall, studied sticklebacks from Gulf of Bothnia were larger than the ones from Gulf of Finland (length: $\chi^2 = 74.1$, $p = 1.1e{-}14$; mass: $\chi^2 = 66.3$, $p = 4.7e{-}10$). Fish from Gulf of Bothnia also possessed lower number of lateral plates compared to Gulf of Finland individuals (average lateral number of plates: 7.5 vs. 12.6, $\chi^2 = 52.5$, $p = 4.3e{-}10$) (Appendix S1: Table S1).

Upper thermal tolerance and phenotypic plasticity

No significant differences were observed in CT_{max1} ($\chi^2 = 8.2$, $p = 0.14$) when the initial differences in the CT_{max1} among populations were tested before dividing the fish into the exposure groups. The average CT_{max1} across the populations was 30.7 ± 0.12°C.

In heat wave-exposed group, in the linear mixed-effect Model 1 the Trial ($p = 2e{-}16$) had significant effect on CT_{max}. Indeed, the average CT_{max2} was 33.9 ± 0.1°C (Figure 4a), and therefore, the heat wave caused significant 3.2 ± 0.2°C increase in CT_{max} across all the individuals. The populations did not show significant differences in CT_{max} (Figure 4a). In Model 2, the population did not affect the plasticity of thermal tolerance either ($F = 1.3$, $p = 0.3$). The sex or mass of the fish did not influence the

TABLE 1 Genetic differentiation expressed as pairwise F_{ST} (Weir & Cockerham, 1984) between sampling locations of Gulfs of Bothnia and Finland

	POO	KOT	LOV	POR	PYH
KOT	0.0004 ($p = 0.0545$)				
LOV	**0.0005 ($p = 0.0203$)**	0.0000 ($p = 0.4108$)			
POR	**0.0011 ($p = 0.0002$)**	**0.0010 ($p = 0.0004$)**	**0.0006 ($p = 0.0396$)**		
PYH	**0.0009 ($p = 0.0000$)**	**0.0015 ($p = 0.0000$)**	**0.0009 ($p = 0.0000$)**	0.0000 ($p = 0.4739$)	
OLK	**0.0011 ($p = 0.0000$)**	**0.0014 ($p = 0.0000$)**	**0.0009 ($p = 0.0000$)**	−0.0006 ($p = 0.9607$)	−0.0001 ($p = 0.6669$)

Notes: Data were obtained from RADseq analyses of 165 individuals (~30 individuals/population). p value <0.05 was considered statistically skignificant. Significant divergence is shown in boldface.

Abbreviations: KOT, Kotka; LOV, Loviisa nuclear power plant; OLK, Olkiluoto nuclear power plant; POO, Porvoo; POR, Pori; PYH, Pyhäranta.

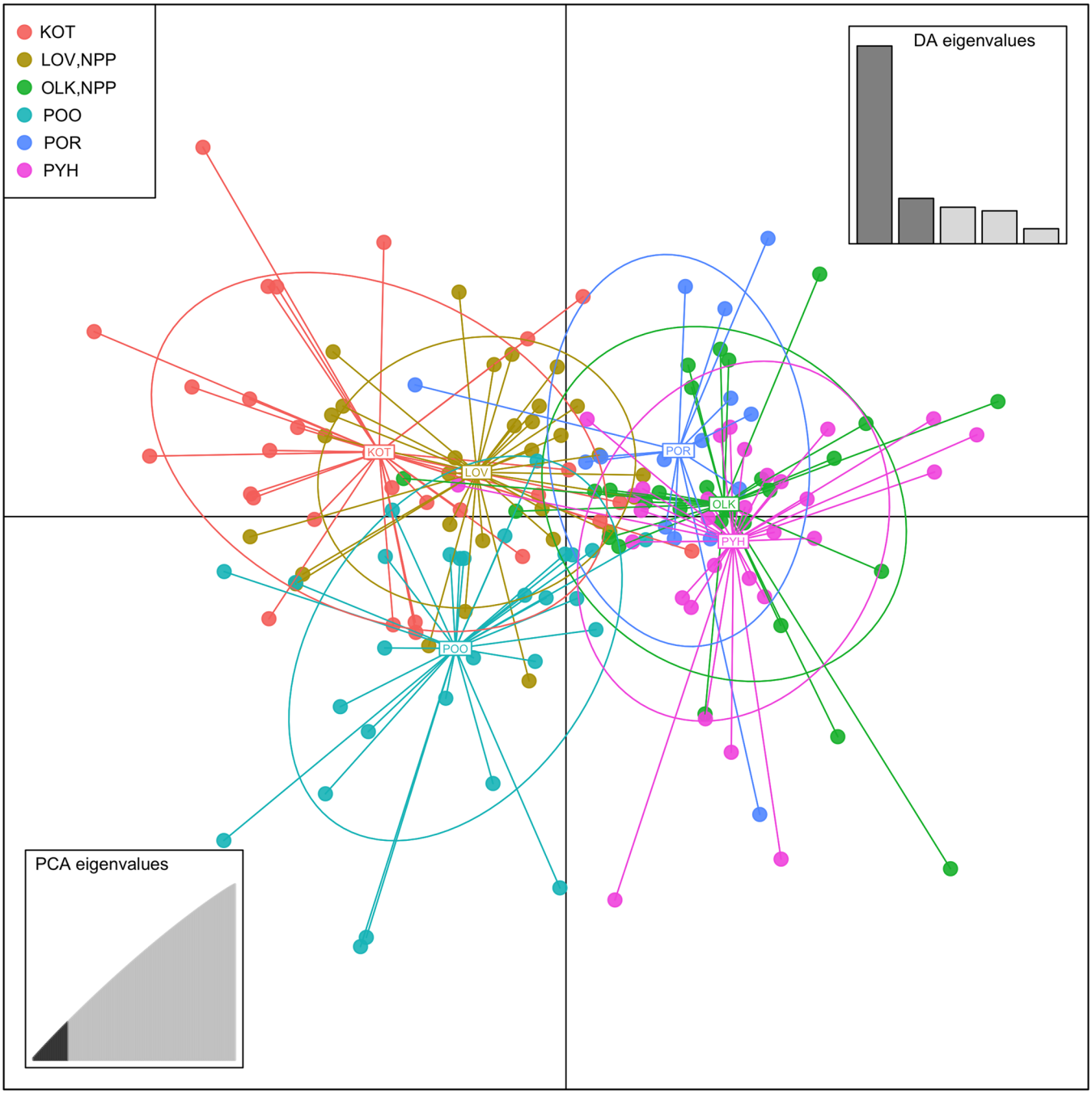

FIGURE 3 Discriminant analysis of principal components (DAPC) of genetic differentiation performed on three-spined sticklebacks. Individuals from different populations are represented by different colors. Inset graph at the top right corner: A bar plot of eigenvalues of the discriminant analysis (DA eigenvalues) for the first five principal components. The bars shown in dark gray are those represented in the plot. At the bottom left corner, the inset represents the number of principal components retained in dark gray (28), according to a score optimization. Abbreviations: Loviisa (LOV), Olkiluoto (OLK), Kotka (KOT), Porvoo (POO), Pyhäranta (PYH), Pori (POR), Nuclear power plant (NPP)

CT_{max} (Model 1, $p = 0.2$, $p = 0.5$, respectively) or plasticity (Model 2, $F = 2.8$, $p = 0.09$, respectively). In Model 3, the initial level of thermal tolerance (CT_{max1}) had a strong effect on thermal plasticity. We observed a significant negative relationship between initial CT_{max1} and individual plasticity (R^2: 0.7364, $F = 21.5$, $p = 5.9e{-}11$) (Figure 5). The fish having low CT_{max1} were able to increase the CT_{max} by over 6°C after the heat wave, while the fish having high CT_{max1} only marginally increased CT_{max2} (<0.5°C).

There was a slight but significant increase of CT_{max} also in the handling control fish (0.6 ± 0.2°C; Model

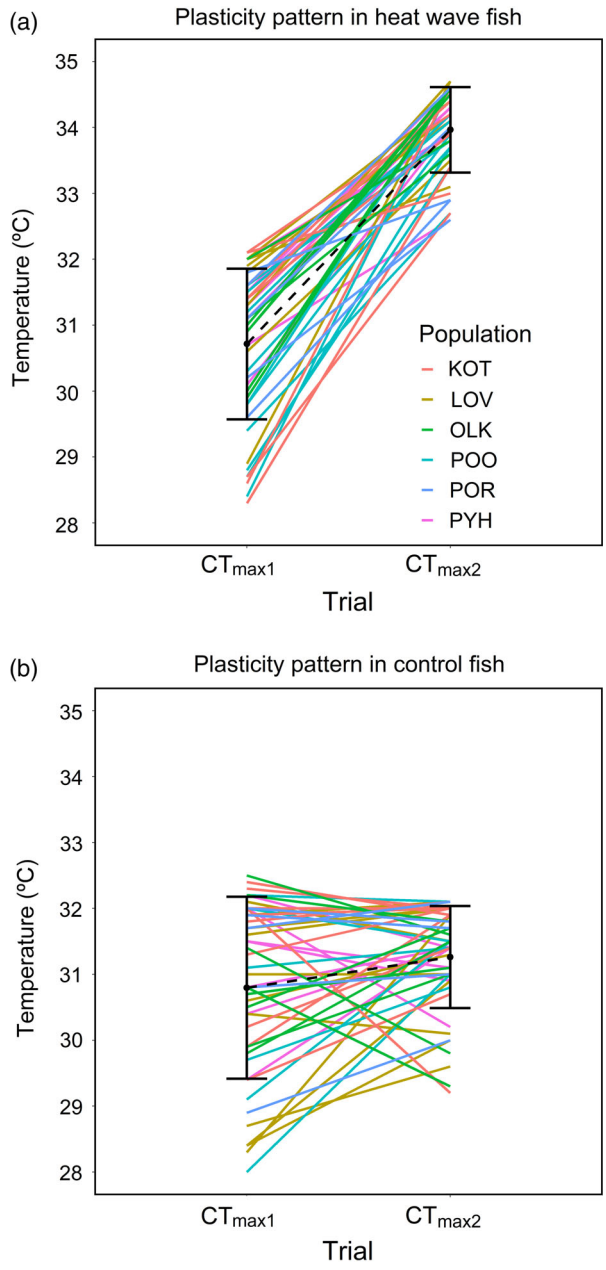

FIGURE 4 The individual first and second critical thermal maximum (CT_{max1} and CT_{max2}) after 1-week exposure to laboratory-mimicked heat wave (increase of +10°C) ($n = 47$) (a), and individual CT_{max1} and CT_{max2} in handling control fish ($n = 53$) (b). Black dot and whiskers indicate the average CT_{max1} and $CT_{max2} \pm$ SE, while dashed black line indicates the mean slope of plasticity. Abbreviations for areas are as follows: Loviisa (LOV), Olkiluoto (OLK), Kotka (KOT), Porvoo (POO), Pyhäranta (PYH), and Pori (POR)

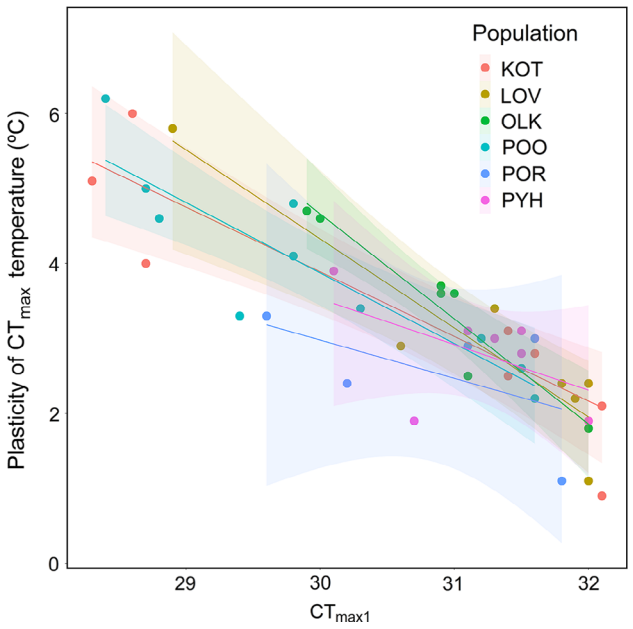

FIGURE 5 Relationship between first upper thermal maximum (CT_{max1}) and plasticity (calculated as: Plasticity = $CT_{max2} - CT_{max1}$) in heat wave-exposed group. Black line and gray ribbon indicate linear regression line (adjusted R^2: 0.7364) and 95% CI, respectively. Abbreviations for areas are as follows: Loviisa (LOV), Olkiluoto (OLK), Kotka (KOT), Porvoo (POO), Pyhäranta (PYH), and Pori (POR)

1, $F = 5.4$, $p = 0.02$) indicating heat hardening by testing (Figure 4b). There were not population differences in CT_{max} (Model 1, $F = 0.4$, $p = 0.8$) or plasticity (Model 2, $F = 1.7$, $p = 0.2$) in control fish. The sex had significant effect on plasticity (Model 2, $F = 6.9$, $p = 0.01$). Males showed higher level of thermal plasticity than females (males: $+1.1 \pm 0.3$°C, females: -0.06 ± 0.2°C). Mass did not had effect on CT_{max} or plasticity ($p = 0.6$ and $p = 0.97$, respectively).

Heat shock proteins

In general, we found no differences in HSP70 protein levels between time points (Appendix S1: Table S2) for either the heat wave-exposed fish or the handling control fish among the populations ($F = 1.15$, $p = 0.33$). Similarly, no differences were found in the expression of HSP90 between sampling time points ($\chi^2 = 5.1$, $p = 0.27$) or populations ($\chi^2 = 8.1$, $p = 0.15$).

DISCUSSION

The capacity of animals to respond to thermal extremes might depend on their thermal history and population-level adaptation to warmer habitat but also on the individual plasticity of upper thermal tolerance. Those mechanisms constitute important strategies for organisms to counteract climate change. However, evolutionary adaptation to high temperature might also reduce the

individual phenotypic plasticity, therefore lowering the individual capacity to increase the thermal tolerance. In the current study, the heat wave increased the upper thermal tolerance of fish, but we did not observe population differences either in upper thermal tolerance or its plasticity. These findings suggest that five decades of warmer water temperature in the discharge area of the NPPs have not resulted in a significant change in thermal tolerance or its plasticity in the studied stickleback populations. Below, we discuss the physiological, population genetic, and ecological interpretations, and evolutionary implications of our findings in the face of ongoing climate change.

The lack of population divergence in thermal tolerance between stickleback populations despite of 50 years of thermal pollution in the discharge area of the NPPs was unexpected, at least at a first glance. In fact, we anticipated populations from the NPPs to show evidence of local adaptation through increased upper thermal tolerance compared to populations from unaffected habitats given that thermal performance and tolerance-related traits are expected to be partly heritable (Muñoz et al., 2015) and controlled by many genes (Quinn et al., 2011). However, the lack of significant differences in CT_{max} between populations experiencing different thermal regimes has been observed in several earlier studies. For example, the small differences in CT_{max} (31.4–32°C) observed among three-spined stickleback populations in four Danish lakes did not show clear relationships with water temperatures, despite large differences in mean water temperature (up to 8.8°C) (Dammark et al., 2018). Similarly, studies on marine and freshwater sticklebacks have shown a lack of significant differences in heat tolerance, the average CT_{max} being around 31–32°C (Barrett et al., 2011; Metzger et al., 2016; Wuitchik et al., 2021), which is similar to our estimates of CT_{max1} (30.7°C). Moreover, a lack of CT_{max} divergence has been also observed among populations from NPP and control areas of killifish (*Fundulus heteroclitus*) (Drown et al., 2021). Thus, our results are consistent with earlier estimates and variability of upper thermal tolerance and suggest that adaptation with respect to upper thermal tolerance is not widespread in three-spined stickleback or killifish.

Yet, it is more challenging to identify specific mechanisms underlying the observed patterns in thermal tolerance and there are several mutually nonexclusive explanations for our findings. Firstly, the openness of habitat surrounding the NPP may enable behavioral thermoregulation of fish. Indeed, regulatory behaviors often dampen the impact of environmental variation on organisms, thereby minimizing the intensity of selection on traits, a phenomenon known as "Bogert effect" (Huey et al., 2003; Muñoz & Losos, 2018). This could contribute to reducing the selection pressure on physiological traits (Gunderson & Stillman, 2015) like the upper thermal tolerance. Therefore, sticklebacks from the current study might have chosen cooler microhabitats (i.e., suitable refugia in deeper waters), rather than shifting their physiology toward more thermally extreme phenotypes. Secondly, low or absence of genetic divergence between studied sample sites suggests that a high level of migration and gene flow may limit the adaptation to local thermal regimes. Thus, gene flow from adjacent areas can counteract changes in gene frequency caused by selection, imposing a limit on local adaptation to extreme temperature regimes. Marine species are often viewed as having open populations that are interconnected by high gene flow (Sanford & Kelly, 2011), which can constrain local adaptation (Defaveri & Merilä, 2013a). Indeed, although the warmer environment from NPP areas might constitute a strong gradient driving local adaptation, this gradient might act on a finer scale compared to home range and movement of three-spined sticklebacks. For example, previous population genetic studies on three-spined stickleback have found evidence for differential selection along thermal gradient in the Baltic Sea (Defaveri & Merilä, 2013a; Guo et al., 2015). However, since the spatial resolution in these previous investigations was broader compared to the current study, putative signatures of selection in these studies may reflect adaptation at larger spatial scale. Thirdly, it is possible that high thermal plasticity in three-spined stickleback reduces the selection pressure for CT_{max}. Indeed, as compared to many other fish species, three-spined sticklebacks have high level of phenotypic plasticity in response to high environmental temperatures as seen with the current results as well (Beitinger et al., 2000). The previous environmental conditions that the fish have experienced in nature could also have an effect on interindividual variation in thermal tolerance, that is, developmental effect (e.g., Jonsson & Jonsson, 2019) that cannot be ruled out in the current experimental design and future studies should also test the adaptation and plastic responses in laboratory-reared populations.

The hypothesis of the presence of trade-off between thermal tolerance and plasticity predicts that species with high thermal tolerance are less likely to have high phenotypic plasticity of thermal tolerance (van Heerwaarden & Kellermann, 2020). In our study, the individual plasticity was negatively correlated with high temperature tolerance of fish. Low potential for phenotypic plasticity in organisms with initially high temperature tolerance organisms has previously been reported in crabs (Stillman, 2003) and laboratory zebrafish (Morgan et al., 2018). We found this trend in wild population of

sticklebacks at individual level. In the present study, fish having high initial thermal tolerance were able to increase their thermal tolerance after the heat wave only by <0.5°C, that is, about as much as the control fish not subjected to heat wave. At the same time, fish initially showing low upper thermal tolerance were able to increase their thermal tolerance by almost 6°C. This result is confirming the presence of a ceiling for the upper thermal tolerance (Sandblom et al., 2014) at the individual level and, on smaller scale, the trade-off hypothesis, which predicts that organisms with highest thermal tolerance will have the lowest plasticity (Comte & Olden, 2017; van Heerwaarden & Kellermann, 2020). It should be noted that some of this plasticity could be due to "heat hardening," meaning that prior exposure to heat stress (i.e., CT_{max}) could increase the second measurement of thermal tolerance as results of acclimation to thermal challenge (Morgan et al., 2018). There was small increase in CT_{max2} in our control fish (0.6°C), but this increase with hardening was significantly lower than the general response to heat wave (3.2°C). Alarmingly, the water in Loviisa NPP reached temperatures higher than fish initial CT_{max} during the exceptionally warm summer of 2018. This suggests that fish could already face the limits of their thermal capacity near the NPP areas if they do not respond to such extremes through a mechanism of behavioral thermoregulation.

Although behavioral thermoregulation could be one way to respond heat waves for adult individuals, it could represent a limiting factor for egg survival and hatching success. This may be the case especially in cases if male sticklebacks have built the nest on shallow waters near NPP where heat wave might be severe. Interestingly, we found significant effect of sex on plasticity in the handling control group, with males increasing their CT_{max} by +1.1, compared to −0.06°C of females. Higher capacity of males to acclimate to thermal challenge has previously been observed in zebrafish (Morgan et al., 2018). This suggests that male sticklebacks may be more resilient to thermal fluctuations and therefore can potentially be able to stay at the nesting site during short heat fluctuations (Hopkins et al., 2011). However, more prolonged heat wave results in a weakening of this capacity since the effect of sex was lost in the heat wave group, where male plasticity was only +0.5°C higher than female.

In addition to studying the performance at whole individual level, we also aimed to shed light on molecular mechanisms behind thermal tolerance. We expected that the fish from NPP areas induce less HSPs after exposure to heat wave as compared to fish from control populations (Narum et al., 2013). Interestingly, we found that heat wave did not induce any detectable increase of HSPs. This was unexpected since heat waves usually cause the induction of HSP synthesis (Narum et al., 2013). However, the induction of the genes involved in the heat shock response usually occurs in the early part of the exposure (Podrabsky & Somero, 2004). Therefore, one possible reason why we did not find an increase of HSP level as response to heat wave is that the fish were sampled 4 and 7 days after starting the exposure to heat wave. Hence, the sticklebacks might have been already acclimated to higher temperature before sampling and the level of HSPs may have returned back to normal level.

To conclude, despite differences in their thermal histories the three-spined stickleback populations showed similar upper thermal tolerance and individual plasticity to respond to a laboratory-mimicked heat wave. The results suggest that an increase of habitat temperature for 50 generations has not increased the upper thermal tolerance nor its plasticity. At the individual level, we found that phenotypic plasticity depends on the initial upper thermal tolerance of the fish, with fish which initially show high temperature tolerance exhibiting low phenotypic plasticity. Together, these results suggest that stickleback populations from those areas might be vulnerable to climate change-driven extreme events since they did not increase their upper thermal tolerance above their boundary limit with plasticity and we did not observe any local adaptation of thermal tolerance. However, to further understand the evolution of thermal tolerance and its fitness consequences, controlled selection experiments are needed in future.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Giovanna Mottola, Katja Anttila, Mikko Nikinmaa, and Anti Vasemägi conceived the ideas of the study and designed the methodology. Giovanna Mottola, Katja

Anttila, and Anti Vasemägi sampled the fish. Giovanna Mottola and Katja Anttila performed the physiological and molecular analyses. María E. López and Giovanna Mottola performed the analyses on genetic data. Giovanna Mottola performed the statistical analyses and drafted the manuscript. All the authors read, commented, and gave the final approval for publication.

DATA AVAILABILITY STATEMENT

Genomic data from RADSeq (Mottola et al., 2021a) are available from Dryad: https://doi.org/10.5061/dryad.x69p8czjd; temperature and physiological data (Mottola et al., 2021b) are available from Dryad: https://doi.org/10.5061/dryad.wm37pvmn9.

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