Plasticity and genetics shape thermal tolerance in non-native populations of the European green crab (*Carcinus maenas*)

Running title: Green crab thermal tolerance and plasticity

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Abstract

Some successful introduced species represent a genetic paradox: they are able to thrive in novel environments despite limited genetic diversity. The European green crab (*Carcinus maenas*) is a quintessential example of this paradox, having established across extensive introduced ranges in the northwest Atlantic and northeast Pacific in North America. Green crabs boast a broad thermal tolerance and exceptional thermal flexibility despite the loss of adaptive potential from repeated bottlenecks. Prior work has found a strong population-level association between an extended genomic region (supergene) and both sea surface temperature and cold tolerance. We conducted a series of three experiments to understand if this association translates to sublethal thermal tolerance and plasticity in righting response for individual adult crabs from multiple populations. While both crabs from Washington and Maine exposed to a 30ºC heat shock showed signs of stress, only the Maine population demonstrated different righting responses by supergene genotype. A second experiment exposing Washington *C. maenas* to repeated heat stress showed that crabs with prior heat exposure do not exhibit a stress response to a subsequent heat stress, irrespective of genotype. The third experiment examined short- and medium-term cold acclimation in *C. maenas* from Washington and Massachusetts. At 5ºC, crabs exhibited an acclimatory response where righting gradually sped up over the course of cold exposure. Some crabs in both populations failed to right at 1.5ºC. Failure to right could be indicative of dormancy employed to reduce energy consumption in colder conditions, but allow for opportunistic feeding and predator avoidance. While a higher proportion of Massachusetts crabs with the putatively warm-adapted supergene allele failed to right at 1.5ºC, this trend was not significant. We suggest that this subtle, context-specific impact of genotype on adult thermal tolerance is related to environmental temperature throughout life history shaping both supergene genotype distributions and thermal physiology without a direct causal link between supergene genotype and adult tolerance as measured in our experiments.

Adult crabs maintain robust thermal plasticity despite a substantial reduction in genetic diversity, likely contributing to the species' global success. Linking population-level genetic associations with individual-level physiology is complex, and reflects the impact of environmental conditions such as temperature throughout life history in shaping adult phenotype. Our work illustrates the importance of investigating population-level associations at the individual level to better understand the full range of mechanisms by which organisms respond to their environment.

Keywords: European green crab, acute stress tolerance, thermal plasticity, genotype, heat shock, heat priming, cold acclimation

Introduction

Temperature is a primary driver of physiology in marine invertebrates (Somero, Lockwood and Tomanek 2017), and substantial effort has focused on quantifying its impacts on these species (Yao and Somero 2014). Current research has identified mechanisms underpinning varied responses to thermal stress, including shifts in metabolic pathways, changes in lipid membrane composition, and differential gene expression (Pörtner 2002; Somero 2010, 2012). Understanding these mechanisms may aid in predicting organismal responses to ocean warming and more frequent thermal extremes (Calvin *et al.* 2023) across biological hierarchy, and uncover why certain species thrive in inherently stressful conditions (Somero 2010).

These physiological changes often arise from some combination of evolutionary adaptation and phenotypic plasticity (Fox *et al.* 2019). Evolutionary adaptation is studied at the level of populations, and depends on genetic differences that alter fitness in a given environment (Fox *et al.* 2019). Thermal extremes can influence the evolution of thermal sensitivity (Buckley and Huey 2016), and changes in critical thermal maxima can be related to genetic differences over time (Cuenca Cambronero *et al.* 2018). Phenotypic plasticity, or the ability for a single genotype to produce more than one phenotype, can also impact an individual organism's thermal tolerance (Hendry 2016; Fox *et al.* 2019). Several studies show that a prior exposure to heat stress in *Drosophila melanogaster* can improve responses to subsequent heat stress through a phenomenon called "heat priming" (Sejerkilde, Sørensen and Loeschcke 2003; Sørensen *et al.* 2008; Moghadam *et al.* 2019), and similar beneficial plasticity has been explored in marine invertebrates (Martell 2022; Glass *et al.* 2023; Abbas *et al.* 2024). However, acclimatory plasticity may not fully mediate negative impacts of thermal stress (Gunderson and Stillman 2015) or have long-term effects on thermal tolerance (Pottier *et al.* 2022).

Evolutionary adaptation and plasticity can also interact to shape organismal responses to stressors like temperature. Heterogeneous environments favor individual plasticity, and this plasticity is maintained as long as the benefits of plasticity outweigh the costs (Grenier, Barre and Litrico 2016; Hendry 2016). Plasticity can also have a genetic basis, arising from standing genetic variation (Handelsman *et al.* 2013; Landy *et al.* 2020). However, tradeoffs may exist between thermal tolerance and plasticity such that thermally-tolerant genotypes have lower plasticity (Barley *et al.* 2021; Griffiths *et al.* 2024). For example, experimental evolution of the copepod *Acartia tonsa* produced genotypes with a 2-5ºC increase in LD50, but plasticity of the same trait decreased over 60% (Sasaki and Dam 2021). Similarly, genotypes with high thermal tolerance and plasticity in the copepod *Tigriopus californicus* exhibited reductions in feduncity (Bogan *et al.* 2024).

If changes in thermal physiology are driven by selection and plasticity, then why do some species boast broad environmental tolerances with limited genetic diversity? Several introduced species thrive in novel environments even after genetic bottlenecks causing substantial loss of genetic diversity (Roman and Darling 2007; Estoup *et al.* 2016). Even so, population genomic research has identified specific genetic markers correlated with various environmental parameters in many introduced species (Schwander, Libbrecht and Keller 2014). Over 20 genes were associated with 11 temperature and climate variables in the fruit fly *Bactrocera dorsalis* (Zhang *et al.* 2023), and three regions of genomic markers were associated with heat tolerance in field and laboratory conditions in the insect pest *Thrips palmi* (Ma *et al.* 2024). While these studies suggest that adaptive genetic variation at specific markers can persist even after decreases in overall genetic diversity, it remains a challenge to link these population-level association studies with individual-level physiological mechanisms (Riginos *et al.* 2016; Lind and Lotterhos 2024). A few studies have explored this link, primarily in insects, with intriguing results. One group of markers associated with warmer and more arid conditions in the mosquito *Anopheles gambiae* was associated with increased larval thermal tolerance, but reduced pupae

survival (Rocca *et al.* 2009). Similarly, flies (*Drosophila suboscura*) with putatively warm-adapted markers had higher abundance of heat shock proteins than flies with cold-adapted markers (Calabria *et al.* 2012). To understand how broad environmental tolerance can be maintained in these species, it is important to test how genetics interacts with plasticity in populations with limited genetic diversity.

The European green crab (*Carcinus maenas*) provides the perfect opportunity to study these interactions in genetically depauperate yet highly plastic populations. Like many non-indigenous species, *C. maenas* boasts a broad thermal tolerance and plasticity (Davidson, Jennions and Nicotra 2011; Zerebecki and Sorte 2011; Sorte *et al.* 2013). Green crabs inhabit conditions between 4-23ºC in the native range (Compton, Leathwick and Inglis 2010), and exhibit upper thermal limits higher and lower thermal limits lower than other crustacean species (Tepolt and Somero 2014; Tepolt 2024). This thermal tolerance is likely the result of the species evolving in variable estuarine environments in their native European range, promoting wide thermal optima range (Jost, Podolski and Frederich 2012). A variety of mechanisms may underpin this broad thermal tolerance. At warm temperatures, *C. maenas* may “dump” hemocyanin-bound oxygen to extend upper thermal limits (Jost, Podolski and Frederich 2012), increase heart (Tepolt and Somero 2014) and respiratory rates (McGaw and Whiteley 2024), and increase mitochondrial density and capacity (Rodrigues *et al.* 2015). Alterations to hemolymph magnesium content in colder temperatures can reduce the blockage of calcium channels that otherwise would impair movement (Frederich *et al.* 2000; Wittmann *et al.* 2010). Seasonal differences in thermal limits (Cuculescu, Hyde and Bowler 1998; Hopkin *et al.* 2006) and rapid homeoviscous adaptation (Chapelle 1978, 1986; El Babili *et al.* 1997) observed in *C. maenas* underscore the importance of phenotypic plasticity to *C. maenas* success across broad thermal gradients.

Green crabs in North America are characterized by substantial losses of genetic diversity, but still maintain broad thermal plasticity. Green crabs experienced an initial genetic

bottleneck upon establishment in the northwest Atlantic in the early 1800s, with populations in the northeast Pacific experiencing a second bottleneck after being introduced from a northwest Atlantic source (Carlton and Cohen 2003; Tepolt *et al.* 2009; Tepolt and Palumbi 2015). Crabs in the initial introduction to the northwest Atlantic came from the south-central European native range (Carlton and Cohen 2003; Roman and Palumbi 2004; Roman 2006; Darling *et al.* 2008). A second introduction of crabs from the northern part of the native range may have introduced genetic diversity associated with more robust cold tolerance, allowing the crabs to expand into Maritime Canada and Newfoundland (Roman and Palumbi 2004; Roman 2006; Blakeslee *et al.* 2010; Weihrauch and Mcgaw 2023). However, the genetic legacy of this second introduction has largely been restricted to the northern extent of the species' northwest Atlantic range. Despite this substantial loss of genetic diversity across much of their range, *C. maenas* populations in North America maintain broad thermal tolerance and plasticity, and in fact inhabit a broader thermal range than in their native range (Compton, Leathwick and Inglis 2010). Crabs from several introduced North American populations maintained extensive plasticity of both heat and cold tolerance, comparable to that of crabs in the native range (Tepolt and Somero 2014). Crabs from British Columbia were able to survive temperatures less than 5ºC for 18 weeks, and they are thriving in sub-arctic waters in Newfoundland (Kelley, de Rivera and Buckley 2013; Best, McKenzie and Couturier 2017).

Although overall genetic diversity is substantially reduced in much of North America, thermal plasticity in *C. maenas* may still have a genetic basis. Population genomics of *C. maenas* revealed a group of single nucleotide polymorphisms (SNPs) in strong linkage disequilibrium that were significantly associated with thermal tolerance on a population level; these SNPs likely represent an extended region of reduced recombination, or supergene (Tepolt and Palumbi 2020). Nearly all the genomic variation associated with temperature in a separate study in the northwest Pacific was found in this supergene, and a high proportion of these SNPs were predicted to be amino-acid changing mutations (Tepolt and Palumbi 2020; Tepolt *et al.*

2022). One gene in this region with two nonsynonymous SNPs is *hypoxia-inducible factor 1-alpha* (Tepolt and Palumbi 2020): as a key regulator of hypoxia, it may also be essential in marine invertebrate responses to temperature (Pörtner, Langenbuch and Michaelidis 2005; Pörtner, Bock and Mark 2017). Supergene allele frequency is strongly associated with temperature: as sea surface temperature increased, populations had a higher frequency of the putatively "warm-adapted" supergene version allele (Tepolt and Palumbi 2020). While this represents a strong link between genomic adaptation and population-level thermal tolerance, it is unclear if or how these differences translate to individual thermal plasticity in adult crabs. Supergene genotype had no impact on response to acute cold stress in adult *C. maenas* from several Gulf of Maine populations (Coyle *et al.* 2019); however, the crabs were only exposed to 4.5ºC for a maximum of six hours. Given the strong association between supergene allele frequencies and both cold tolerance and sea surface temperature (Tepolt and Palumbi 2020; Tepolt *et al.* 2022), and the complexities of linking population-level associations with individual physiology (Gagnaire and Gaggiotti 2016; Riginos *et al.* 2016; Tam *et al.* 2019; Lind and Lotterhos 2024), it is possible that the supergene may shape thermal tolerance across a different timescale.

Here, we used three experiments to better understand individual thermal tolerance and plasticity in adult crabs, and whether they are related to genotype at this temperature-associated supergene across multiple populations (**Figure 1**). In the first experiment, crabs were exposed to an acute heat shock to characterize physiological responses to warm temperatures. The second experiment involved repeated acute heat shocks to understand how "heat priming" impacts thermal plasticity. We investigated short- and medium-term responses to chronic cold temperature exposure in the third experiment. We expected that *C. maenas* exposed to repeated heat stressors in experiment 2 and those experiencing cold temperatures for longer than 24 hours in experiment 3 would exhibit evidence of thermal acclimation. Across experiments, we hypothesized that differences in supergene

genotype distributions would contribute to differences in thermal response between populations. Additionally, we predicted that genotype would influence individual crab responses in all experiments. We expected that crabs with at least one copy of the putatively warm-adapted supergene would have a muted response to heat stress in experiments 1 and 2. Conversely, we hypothesized that presence of the putatively cold-adapted supergene would confer benefits during short- and medium-term cold acclimation in experiment 3. Our results show how investigating thermal responses at the individual organism level can elucidate the context-specific role of genetic factors in shaping thermal tolerance and plasticity in genetically depauperate but highly successful populations.

Methods

General experimental setup

A series of experiments was conducted to assess the impact of short-term (~24 hours) and medium-term (94 hours) thermal stress on introduced *C. maenas* populations in both the northwest Atlantic and northeast Pacific. Crabs with orange-red or red integument color were not included in experiments; these colors indicate an extended time in intermolt and “red” green crabs have less robust tolerance to a range of physiological stressors (Styrishave, Rewitz and Andersen 2004). Experimental crabs were individually labeled using waterproof paper and superglue, and the propus and dactyl segments from the third walking leg on the right side (dorsal view) were preserved in 95% ethanol for subsequent genotyping. These joints were taken as the third walking leg has no significant role in crab righting behavior (Young, Peck and Matheson 2006). If the crab did not have a third walking leg on the right side, the third walking leg was taken from the left.

Crabs were placed in 21 cm x 26 cm x 41 cm (22.386 L) tanks filled with 800 micron filtered raw seawater from Great Harbor, MA, USA (Experiments 1 and 3) or 50 micron filtered

raw seawater from Vineyard Sound, MA, USA (Experiment 2). Each tank had an air stone (Quickun Aquarium Air Stone) attached to a 10L aerator (Whisper® Aquarium Air Pumps, Tetra®) and an aquarium filter (MARINA i25). Crabs were distributed to balance sex, carapace width, and integument color among tanks. Tanks were checked for mortalities and fed daily. Each day, every tank was given ½ teaspoon of commercial crab food (Crab Cuisine, Hikari USA Inc.) if tank temperature was ≥ 15ºC, and ¼ teaspoon of food if tank temperature was ≤ 5ºC. These quantities were sufficient to satiate crabs since there was always a small amount of uneaten food the next day. Particulate waste was removed prior to feeding. Every third day, ammonia concentrations were tested using an API® Ammonia Test Kit prior to removing particulate waste from tanks and performing a 60% water change. A conditioner (½ tsp Kordon Amquel Plus Aquarium Water Conditioner) was added to tanks during water changes if the ammonia concentration was above 1.0 ppm. Filter cartridges were replaced every five days A 12-hour:12-hour light:dark cycle was maintained for all tanks (Tepolt and Somero 2014).

Crabs were initially kept in acclimation conditions for at least five days (see details below). Water temperature was maintained during the acclimation period using either a flowing water bath or environmental chamber manipulation. Water temperature in each tank was recorded every 15 minutes using two HOBO Data Loggers (Onset, USA), one at the water surface and one at the bottom of the tank. Warming (Experiments 1, 2, and 3) was performed using 300-Watt Deluxe Titanium Heating Tubes (Finnex, USA) with an external 1650-Watt temperature controller (bayite, China). Cooling (experiment 3) was performed in an environmental chamber with 0ºC as a set point. A non-parametric Kruskal-Wallis test (`kruskal.test` from R `stats` package (R Core Team 2024) was used to confirm that temperature treatments were statistically different from each other and that replicate tanks were not different from each other.

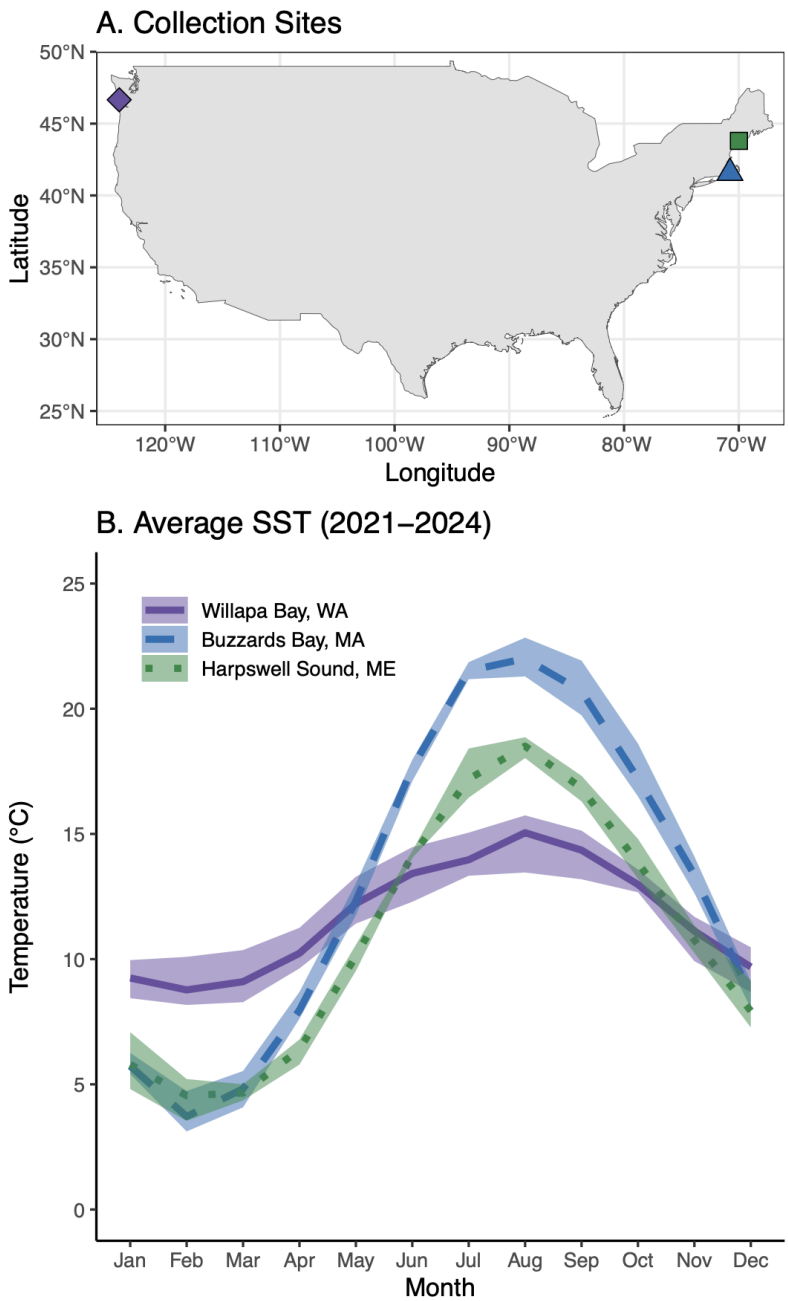

Figure 1. **A**) Green crab collection sites for experiments (purple diamond = Willapa Bay, WA; blue triangle = Buzzards Bay, MA; and green square = Harpswell Sound, ME). Crabs from ME and WA were used for experiment 1 (2024), crabs from WA were used for experiment 2 (2023), and crabs from MA and WA were used for experiment 3 (2024). **B**) Average sea surface temperature (SST) for 2021-2024 near collection sites (solid purple line = Willapa Bay, WA; long blue dashes = Buzzards Bay, MA; small green dashes = Harpswell Sound, ME). SST data were obtained from NOAA's OI SST V2 High Resolution Dataset provided by the NOAA PSL, Boulder, Colorado, USA, from their website at https://psl.noaa.gov (Huang *et al.* 2021). A four-year mean, SST minimum, and SST maximum were calculated for each month. Solid lines represent four-year averages, while ribbons represent ranges.

Measuring thermal tolerance with time-to-right

Time-to-right (TTR) was used in all experiments to assess sublethal thermal tolerance. TTR was defined as the number of seconds *C. maenas* spent on righting behavior (defined in (Young, Peck and Matheson 2006)), including the “hesitation time” before moving the fifth pereopod pair after crabs are placed dorsal side up. Individual crabs were acclimated for one minute, then placed with their dorsal side on the bottom surface of the tank. Crabs were then given 90 seconds to right. If a crab did not right within 90 seconds, it was manually righted and recorded as “not righting” for that trail. Crabs were rested for 10 seconds before repeating the TTR trial twice for a total of three TTR measurements per crab per trial. To control for other factors that may influence righting response, crabs were weighed, measured, sexed, and assessed for integument color and missing legs after each TTR trial.

Supergene genotyping

Crabs were genotyped at a single-nucleotide polymorphism (SNP) in the *structural maintenance of chromosomes protein 3* (SMC) gene to understand how individual variation at the putative supergene impacts thermal tolerance. This SNP is part of a group of SNPs in very strong linkage disequilibrium in the putative supergene, and is diagnostic of supergene extended genotype (Tepolt & Palumbi 2020). DNA was extracted from ~4 mm^2 of leg joint tissue using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA) following manufacturer’s instructions. Incubation with the ATL Lysis Buffer and Proteinase K occurred for at least two hours. The final eluate was pipetted back onto the spin column, incubated, and re-eluted to increase DNA yield.

Genotyping was done with one of two equivalent methods. For Experiment 2, a 126 bp region of the SMC gene containing a C/T single nucleotide polymorphism (SNP) diagnostic for supergene allele was amplified using the SMC F and R primers and methods specified in Coyle

et al. (2019) (**Table S1**). Amplified product was sent to Sequegen (Worcester, MA, USA) for Sanger sequencing. Resultant chromatograms were edited and aligned using Sequencher v.5.4.6 (Gene Codes, Ann Arbor, MI, USA). Samples from Experiments 1 and 3 were genotyped using a restriction digest assay. A 690 bp region of the SMC gene containing the same known C/T SNP was amplified using SMC_long F and R primers (**Table S1**). Amplified product was digested with 0.5 µL of AluI enzyme (Promega, USA or ThermoFisher, USA) at 37ºC for one hour. The recognition site includes a CG dinucleotide at 88 bp in the PCR product, meaning that digestion only occurs for C alleles There is an additional non-target cut site at 477 bp that is digested for all genotypes. Digested product was run out on a 1.5% agarose gel with GelRed® Nucleic Acid Gel Stain (Biotium, Inc, Fremont, CA, USA) at 90 V for at least 65 minutes then imaged. A 477 bp band indicated TT homozygotes, a 389 bp band indicated CC homozygotes, and both bands indicated CT heterozygotes (**Figure S2**). Both CC and CT genotypes had also an 88 bp band. All genotypes had a 213 bp band due to digestion at the non-target site (**Figure S2**). The C allele is found in the cold-adapted supergene allele, while the T allele is found in the warm-adapted supergene allele.

Experiment 1: Heat tolerance after heat shock across populations

This experiment tested whether *C. maenas* response to an acute heat shock varied by population, genotype, and parasite infection intensity. Crabs were sourced from Grays Harbor, WA, USA by Washington Fish and Wildlife on June 26, 2024 (n_{WA} = 30; carapace $width_{WA}$ = 49 mm ± 6 mm, $weight_{WA}$ = 25.11 g ± 11.35 g), or trapped at Harpswell, ME, USA by Quahog Bay Conservancy on July 18, 2024 under Maine Department of Marine Resources Green Crab Landing Licenses 11915 and 36329 (n_{ME} = 33; carapace $width_{ME}$ = 51 mm ± 4 mm, $weight_{ME}$ = 28.09 g ± 7.44 g). The WA and ME crabs were placed in tanks with 15-17 crabs each, separated by site. All crabs were acclimated at room temperature (~22-24ºC) for at least five days. After the acclimation period, tanks were set to 30ºC. Crabs experienced a heat ramp of

~1ºC/hour over 6 hours, then a ~30ºC heat shock for 22 hours (**Figure 2AB**; WA: July 11-12, ME: July 25-26).

TTR was assessed before and after the heat shock. Prior to analysis, replicate TTR measurements for each individual crab at each time point were averaged and log-transformed. Trials where the crab did not right itself within 90 seconds were excluded from averaging. After experiments were concluded, crabs were sacrificed and dissected to count the number of parasitic trematode metacercarial cysts per Blakeslee et al. (2015), with the modification that all hepatopancreas tissue was examined on 12.5 cm x 7.5 cm squash plates. No other parasites were identified in these populations.

A series of linear mixed effects models (`lmer` from R `lme4` package; (Bates *et al.* 2015)) was used to evaluate how various factors impacted log-transformed average righting response time with crab ID used as a random effect to correct for repeated measurements of the same animals. First, a null model was created including timepoint and parasite intensity as explanatory variables. Timepoint was modeled as a categorical variable because it directly corresponded with heat shock (hour 0 = no heat shock, hour 22 = heat shock). Parasite intensity was calculated by dividing metacercarial cyst count by crab carapace width. To control for other factors that may explain variation in righting response, crab sex, integument color, carapace width, weight, and whether or not a crab was missing legs were used as explanatory variables in all linear mixed models. The influence of these factors on righting response was not tested.An Analysis of Variance (ANOVA; `anova` from R `stats` package (R Core Team 2024)) was used to perform a likelihood ratio test and compare the null model with a model testing the influence of a specific factor (*ie*. only timepoint or parasite infection intensity). *P*-values were corrected for multiple comparisons using a Bonferroni correction. Factors were considered significant when adjusted $p < 0.05$. Normality and homoscedasticity were verified visually. Significant factors (*ie*. timepoint, parasite intensity, or both) identified by ANOVA tests were then included in a second

null model. This “no genotype” null model was compared to three separate models containing these significant factors, and either genotype, presence of the C allele, and presence of the T allele. Another set of ANOVA tests were used to discern if these genotype-specific factors significantly impacted log-transformed average righting response. Pairwise tests were conducted with `emmeans` v.1.11.0 for any significant factor (Lenth *et al.* 2018).

Experiment 2: Heat tolerance after repeated heat shocks

The goal of this experiment was to understand how repeated heat shocks influenced individual heat tolerance in *C. maenas*. A total of 24 crabs (carapace width = 47 mm ± 6 mm, weight = 24.18 g ± 8.28 g) were collected from Grays Harbor, WA, USA by Washington Fish and Wildlife and received at Woods Hole Oceanographic Institution on June 22, 2023. Crabs were labelled and sorted by integument color, then held at room temperature for 39 days until genotyping analysis was finished (see *Genotyping assays*). Crabs were split between four tanks to ensure even distribution of sex, carapace width, integument color, and genotype, and acclimated at 15ºC for one week using a flowing water bath.

After the acclimation period, two tanks (treatment group) were exposed to a ~30ºC heat shock for 20 hours, while the other two tanks (control group) were maintained at 15ºC. After the initial heat shock, all tanks were maintained at 15ºC for 48 hours. Both the control and treatment groups were then exposed to a second heat shock of ~30°C for 24 hours.

Two whole-organism physiological metrics were used to evaluate crab response to thermal stress. Righting response was measured for all crabs after the acclimation period (day 0), at the end of the first heat shock (day 1), and at the end of the second heat shock (day 4). Measurements taken for crabs experiencing heat shocks were conducted at 30ºC. A series of linear mixed effects models was used to assess the impact of treatment, time, the interaction of treatment and time, genotype, presence of the C allele, and presence of the T allele on log-transformed average TTR (see *Experiment 1* for specific methods). For this experiment,

timepoint was modeled as a categorical predictor, as it was a proxy for heat shocks. Pairwise tests were conducted with `emmeans` v.1.11.0 for any significant factor (Lenth *et al.* 2018). Oxygen consumption was also measured after the day 4 heat shock for six crabs from the control and four crabs from the treatment group. See the Appendix S2 for more details on oxygen consumption methodology and analysis.

Experiment 3: Cold tolerance across populations in response to short- and medium-term cold exposure

This experiment investigated how short- and medium-term exposure to cold temperatures influenced thermal plasticity in green crabs from MA and WA. Crabs (n = 59; carapace width = 47 mm ± 8 mm, weight = 25.26 g ± 15.53 g) from Buzzards Bay, MA, USA were obtained from a bait shop in New Bedford, MA, USA on June 6, 2024. Additional crabs (n = 28; carapace width = 44 mm ± 8 mm, weight = 20.63 g ± 11.63 g) were trapped in Eel Pond, MA, USA between June 7, 2024 and June 15, 2024 under MA Special Permit 174763. Since New Bedford and Eel Pond are both part of Buzzards Bay, MA, USA, crabs from these locations were treated as one MA population. Crabs (n = 98; carapace width = 53 mm ± 4 mm, weight = 32.85 g ± 7.62 g) from Grays Harbor, WA, USA were trapped by Washington Fish and Wildlife on June 26, 2024. All crabs were sorted by integument color, labelled, and acclimated to ~15ºC in an environmental chamber for two weeks. The MA *C. maenas* were split between six tanks, and the WA crabs were split between eight tanks, with 12-15 crabs per tank. After acclimation, the environmental chamber was set to 0ºC and half of the tanks decreased to a target temperature of 1.5ºC over 12 hours (MA) or 18 hours (WA). The remaining tanks were outfitted with a 300-Watt Deluxe Titanium Heating Tube (Finnex, USA) with an external 1650-Watt temperature controller and ramped down to 5ºC over 10 hours (MA) or 12 hours (WA).

At hours 0, 4, 22, 28, 46, and 94, righting response was assessed for at least seven crabs per tank. Sample size decreased over the course of the experiment due to one crab per tank being dissected at each timepoint for future analyses. A series of linear mixed effects models was used to assess the impact of treatment, time, the interaction of treatment and time, genotype, presence of the C allele, and presence of the T allele on log-transformed average TTR (see *Experiment 1* for specific methods). For this experiment, timepoint was modeled as a continuous predictor. Pairwise tests were conducted with `emmeans` v.1.11.0 for any significant factor (Lenth *et al.* 2018). To obtain pairwise differences between different timepoints, timepoint was re-estimated as a categorical variable.

Cold temperatures can impair movement, leading to failure to right within the given 90 seconds. A second set of models were used to evaluate how various factors impacted failure to right. For these models, TTR for each crab was coded as a “success” (righting within 90 seconds) or “failure” (no righting within 90 seconds). Binomial linear mixed effects models with crab ID as a random effect were used to understand how temperature, time, the interaction between temperature and time, genotype, presence of the C allele, and presence of the T allele impacted binary righting response. The impact of sex on failure to right was also assessed given previous research demonstrating sex-specific differences in cold tolerance in the species (Coyle *et al.* 2019). An ANOVA (`anova` from R `stats` package (R Core Team 2024)) was used to conduct likelihood ratio tests to compare the null model with a model testing the influence of a specific factor. Factors were considered significant at $p < 0.05$ after correction. Crab sex, integument color, carapace width, weight, and whether or not a crab was missing legs were used as explanatory variables in all models.

To compare responses to temperature conditions between populations, separate linear mixed effects models were created to evaluate the impact of population and significant variables from the population-specific models on average time to right, or on failure to right. Generalized

linear models (`glm` from R `stats` package (R Core Team 2024)) were also used to compare the effect of population on failure to right at each timepoint for crabs in the colder temperature treatment. All *P*-values were corrected for multiple comparisons using a Bonferroni correction.

Results

Experiment 1: Genotype mediates response to heat stress in some populations

Crabs from both WA and ME were exposed to a 22 hour heat shock to understand short-term responses to high temperatures. WA crabs were exposed to 30.04ºC ± 0.30ºC (**Figure 2A**), and ME crabs were exposed to 29.91ºC ± 0.30ºC (**Figure 2B**). No WA crabs died during the acclimation period or experiment, while four ME crabs died during the acclimation period. Three of these deaths were CT crabs, while the fourth was a TT crab. Hepatopancreas tissue was dissected to characterize parasite infections. Parasites were not found in crabs from WA, while ME *C. maenas* contained 0-105 metacercarial cysts (intensity = 0.46 cysts/mm ± 0.56 cysts/mm). Parasite intensity did not impact righting response (χ^2 = 0.06, *P*-value = 1).

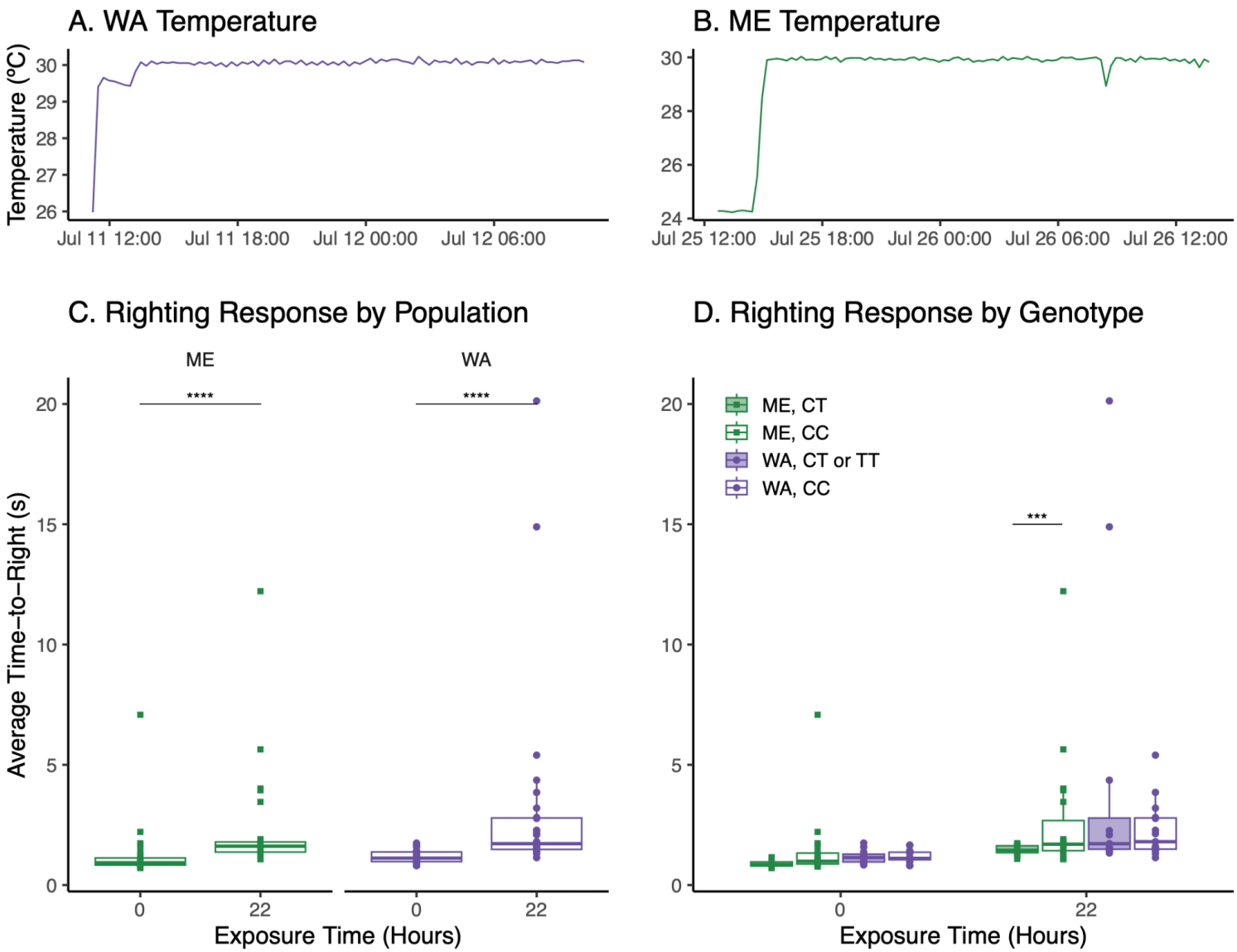

Figure 2. Experimental temperature conditions for **A**) WA (purple) and **B**) ME (green) experiments; HOBO loggers were added to the tanks after the heat ramp had started. Righting response before (0 hours) and after (22 hours) heat shock by **C**) population and **D**) population and genotype. In all panels, green squares represent TTR for ME *C. maenas* and purple triangles for WA *C. maenas*. Significantly different pairwise comparisons are indicated on the figure (*** = *P*-value < 0.001, **** = *P*-value < 0.0001).

Righting response was evaluated before and after heat shock to understand the factors that impact short-term heat tolerance (**Figure 2**). Timepoint had a significant impact on righting response in both populations (**Figure 2C**; WA comparison: $\chi^2 = 21.69$, *P*-value < 0.0001; ME comparison: $\chi^2 = 19.20$, *P*-value < 0.0001). Crabs from both WA and ME had significantly slower

TTR after heat shock ($mean_{WA,\ 22\ hours}$ = 3.22 s ± 4.22 s; $mean_{ME,\ 22\ hours}$ = 2.22 s ± 2.15 s) than prior to heat shock ($mean_{WA,\ 0\ hours}$ = 1.16 s ± 0.26 s; $mean_{ME,\ 0\ hours}$ = 1.23 s ± 1.15 s).

The impact of supergene genotype on short-term response to heat shock was also tested (**Figure S2A-B**). Crabs with a CC genotype in WA showed a non-significant trend towards a faster righting response after heat shock ($mean_{WA,\ 22\ hours,\ CC}$ = 2.29 s ± 1.16 s) than CT ($mean_{WA,\ 22\ hours,\ CT}$ = 4.34 s ± 6.45 s) and TT crabs ($mean_{WA,\ 22\ hours,\ TT}$ = 4.22 s ± 5.97 s). Neither genotype (χ^2 = 0.29, *P*-value = 1), presence of the C allele ($\chi^2 = 5.47 \times 10^{-4}$, *P*-value = 1), nor presence of the T allele (χ^2 = 0.22, *P*-value = 1) had a significant impact on average TTR in WA *C. maenas* (**Figure 2D**). Only CC and CT crabs were represented in our ME samples.After heat shock, CT crabs had a significantly faster righting response ($mean_{ME,\ 22\ hours,\ CT}$ = 1.46 s ± 0.20 s) than CC crabs ($mean_{ME,\ 22\ hours,\ CC}$ = 2.67 s ± 2.62 s; comparison: χ^2 = 8.72, P-value = 0.02) (**Figure 2D**; t = 2.79, *P*-value = 0.008). Crab ID explained 0.54% and 13.94% of the variation in final WA (time only) and ME models (time and genotype), respectively.

Experiment 2: Prior heat exposure, but not genotype, impacted crab response to heat stress

The righting response of *C. maenas* that experienced a prior heat shock was compared to the response of crabs naïve to heat stress in order to understand how repeated exposures impact thermal plasticity (**Figure 3A**). We note that there were small temperature differences between tanks for the second heat shock (**Table S2**; **Figure 3B**). Because of this experimental difference, tank was used as a random effect in subsequent analyses to account for significant temperature differences.

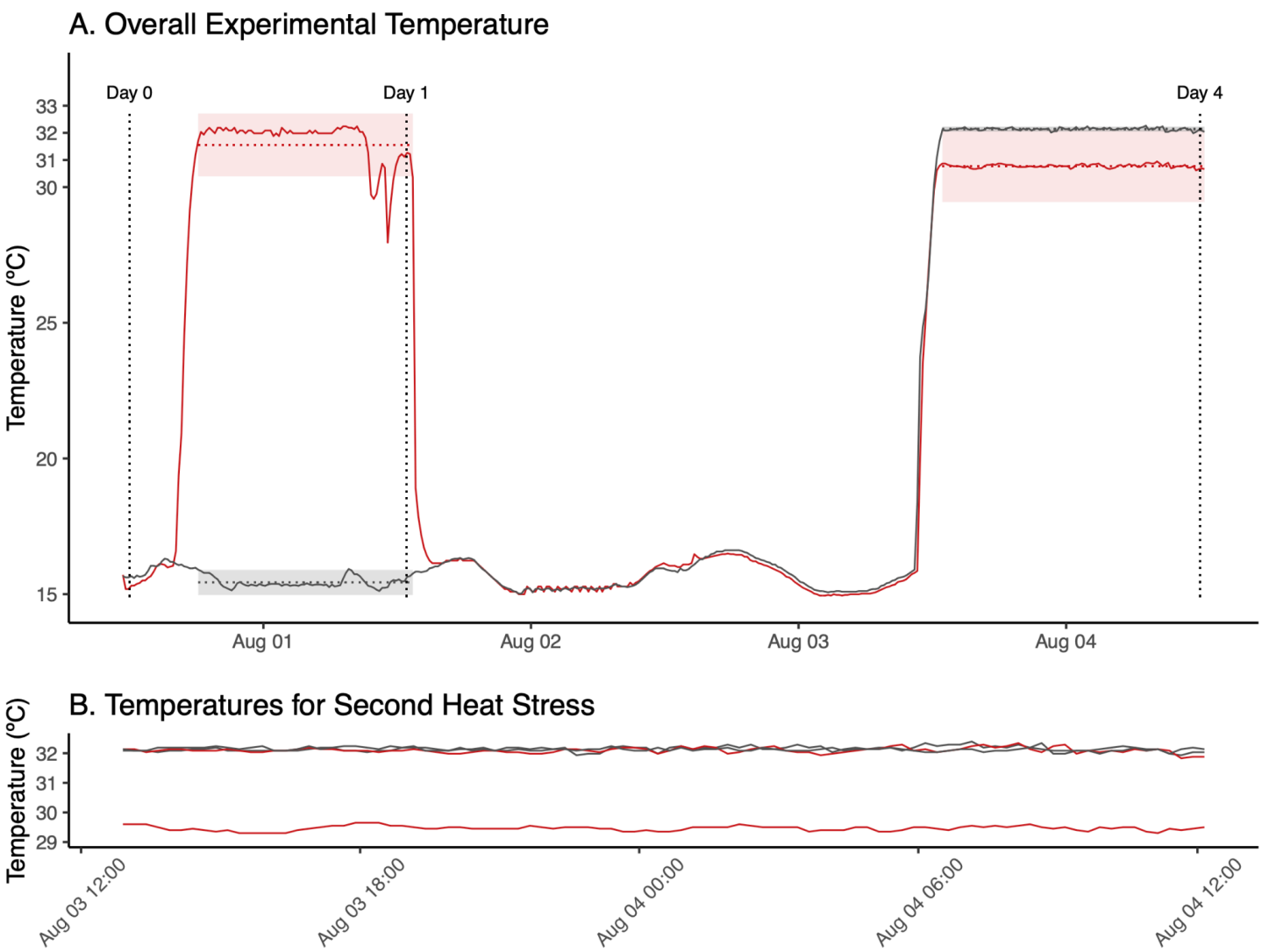

Figure 3. **A**) Water temperatures for Experiment 2. Vertical lines indicate when TTR was measured (day 0, day 1, or day 4). Dashed grey horizontal lines indicate temperature conditions for the control crabs averaged across two replicate tanks, while dashed red horizontal lines indicate temperature conditions for treatment crabs averaged across two replicate tanks. Standard error during the second heat stress is larger for the treatment crabs due to slight differences in temperature conditions between replicate tanks. **B**) Tank-specific temperatures during the second heat stress. One treatment tank had a significantly lower temperature than the other three tanks. In all panels, red indicates treatment tanks and grey indicates control tanks.

Average TTR for an individual crab was significantly impacted by treatment (χ^2 = 19.23, *P*-value = 0.001), time (χ^2 = 21.74, *P*-value = 0.001), and their interaction (χ^2 = 14.56, *P*-value = 0.004) (**Figure 4A**). These significant differences were driven by differences in TTR between control and treatment crabs at the end of the experiment. On day 4, *C. maenas* exposed to a

prior heat shock had faster and less variable righting times ($mean_{treatment}$ = 1.18 seconds ± 0.62 seconds) than their counterparts that did not experience a prior heat shock ($mean_{control}$ = 4.78 s ± 4.98 s) (**Figure 4A**; $t_{control\text{-}treatment}$ = 3.87, *P*-$value_{control\text{-}treatment}$ = 0.002).

Additionally, crabs that did not experience a prior heat shock had significantly slower righting response on day 4 than they did on days 0 or 1 (**Figure 4A**; $t_{day1\text{-}day5}$ = -3.68, *P*-$value_{day1\text{-}day5}$ = 0.002; $t_{day2\text{-}day5}$ = -4.67, *P*-$value_{day2\text{-}day5}$ < 0.0001). Interestingly, crabs that experienced a prior heat shock had similar average TTR after the first ($mean_{treatment,\ day\ 1}$ = 1.26 s ± 0.68 s) and second ($mean_{treatment,day\ 4}$ = 1.18 s ± 0.62 s) heat shocks, while control crabs showed an increase in TTR after their only heat shock relative to their earlier control TTR (**Figure 4A**). Crab ID and tank accounted for 12.40% or 0% of the variance in TTR in the final model (treatment, time, and their interaction only), respectively. There was also no impact of priming on oxygen consumption (**Figure S2**).

The impact of supergene genotype on thermal response and priming was also assessed (**Figure S2C**). Genotype (χ^2 = 4.24, *P*-value = 0.72), presence of the C allele (χ^2 = 1.37, *P*-value = 1), and presence of the T allele (χ^2 = 3.43, *P*-value = 0.39) had no significant impact on average TTR in the full model. Even though these trends were not statistically significant, crabs with at least one T allele had slower TTR than their counterparts without a T allele, irrespective of prior heat shock exposure (**Figure 4B**; $mean_{treatment,day\ 4,\ T}$ = 1.13 s ± 0.68 s, $mean_{treatment,day\ 4,\ no\ T}$ = 0.80 s ± 0.06 s, $mean_{control,\ day\ 4,\ T}$ = 6.17 s ± 6.01 s, $mean_{control,\ day\ 4,\ no\ T}$ = 2.83 s ± 2.44 s).

Figure 4. **A**) Average TTR across all timepoints for control (grey) and treatment (red) crabs. The inset shows data for crabs whose TTR was < 4 seconds. Timing of the heat shocks are indicated by transparent red boxes. Day 1 measurements were taken after the first heat shock for the treatment crabs, and day 4 measurements were taken after the second heat shock experienced by all crabs. Pairwise differences between days are indicated within control (grey uppercase) and treatment (red lowercase) treatments. **B**) Average TTR by genotype.

Experiment 3: Cold acclimation differs by population with potential genotype-associated trends

Crabs from MA and WA were ramped down to target temperatures of 1.5ºC or 5ºC and held there for several days to understand the factors that govern short- and medium-term cold acclimation (**Figure 5**). All tanks reached experimental temperatures by hour 22 of the 94 hour experiment. Actual temperatures experienced were slightly higher than the targets (**Table S2**).

Differences in temperature conditions between these two experiments were likely due to the additional tanks used for WA and higher ambient temperatures in July 2024 versus June 2024.

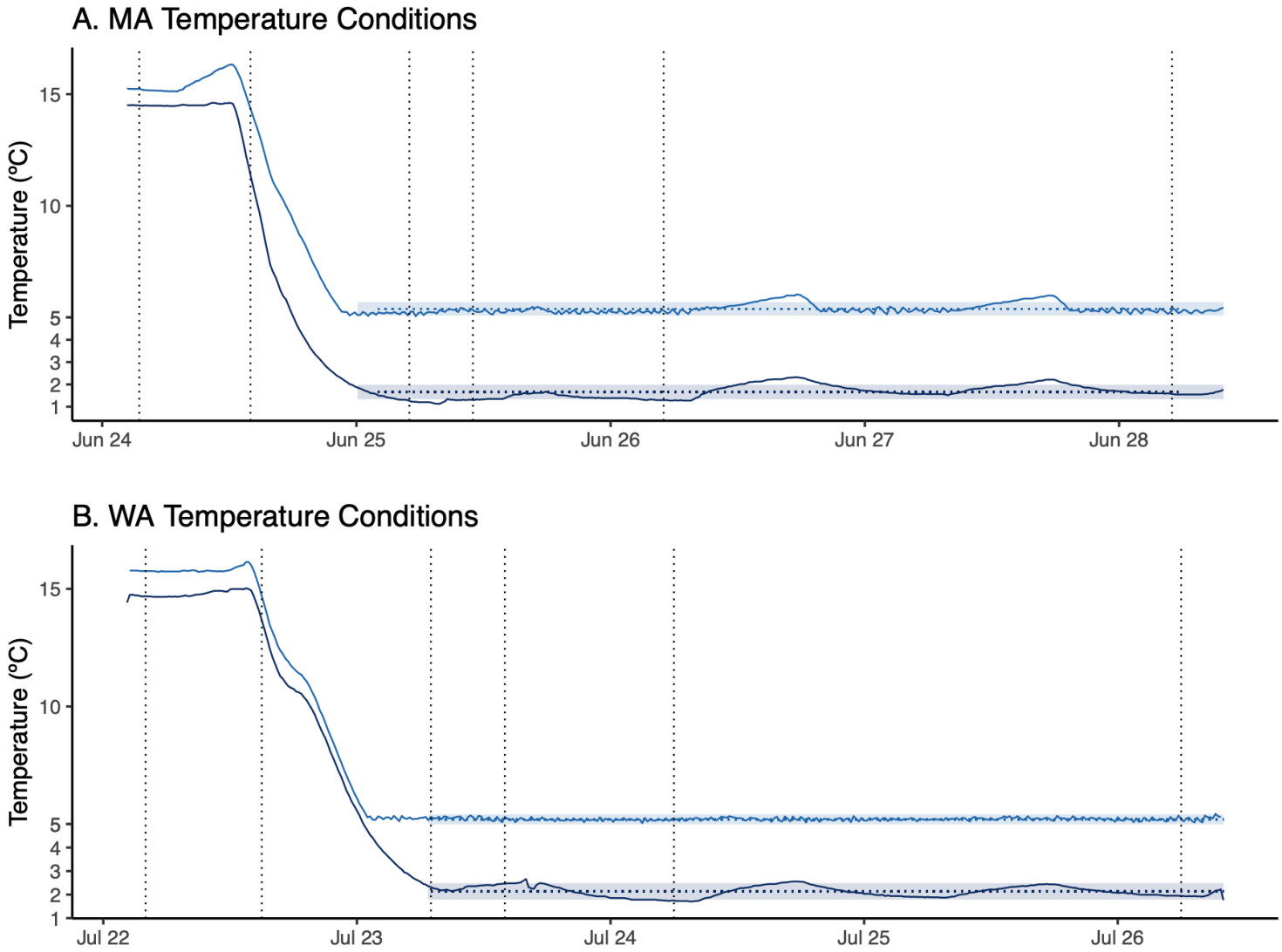

Figure 5. Temperature conditions for **A**) MA and **B**) WA *C. maenas* used in experiment 3. In both panels, average temperatures experienced by crabs across replicate tanks in cold (light blue; MA = 5.39ºC ± 0.30ºC; WA = 5.20ºC ± 0.22ºC) and colder (dark blue; MA = 1.66ºC ± 0.32ºC; WA = 2.14ºC ± 0.35ºC)) treatments are indicated by the solid lines. Ribbons depict standard errors. TTR sampling points are indicated by vertical lines. Experiments were conducted in 2024.

Temperature treatment (χ^2 = 75.54, *P*-value < 0.0001), time (χ^2 = 164.45, *P*-value < 0.0001), and their interaction (χ^2 = 49.83, *P*-value < 0.0001) significantly impacted the average

righting response of MA crabs (**Figure 6A**). Crabs at 1.5ºC had significantly slower TTR than crabs in 5ºC at all timepoints except for hour 0, when all crabs were at their common 15ºC acclimation temperature (**Table S3**). Additionally, there were significant differences in TTR between timepoints for each treatment (**Figure 6A; Table S4**). Crabs at 1.5ºC had an average TTR of 39.92 s ± 30.69 s at hour 22, with TTR values decreasing to 33.09 s ± 22.58 s 94 hours after the experiment began. Crabs in 5ºC similarly had slower TTR 22 hours into the experiment (6.44 s ± 7.20 s) with TTR improving after 94 hours (3.83 s ± 2.94 s). The impact of supergene genotype on righting response was also explored (**Figure S2D**). There was no significant impact of genotype (χ^2 = 1.22, *P*-value = 1), presence of the C allele (χ^2 = 0.84, *P*-value = 1), or presence of the T allele (χ^2 = 0.31, *P*-value = 1) on average TTR in MA *C. maenas*. Crab ID explained 10.8% of the variance in the final model (temperature, time, and their interaction only).

Throughout the experiment, some *C. maenas* from MA failed to right at 1.5ºC, but not at 5ºC. Shortly after first reaching 1.5ºC at hour 22, 90% of crabs failed to right, while 64.3% failed to right at the end of the experiment (**Figure 6B**). Failure to right was significantly associated with temperature (χ^2 = 53.20, *P*-value < 0.0001) and time (χ^2 = 63.24, *P*-value < 0.0001), but not their interaction (χ^2 = 0, *P*-value = 1) nor crab sex (χ^2 = 3.41, *P*-value = 0.52). Of the crabs that failed to right at 22 hours, 77.8% had at least one T allele, while 66.7% of those that failed to right at 94 hours had at least one T allele (**Figure 6C**). The impact of the T allele on failure to right in the MA population was not significant after multiple test correction (χ^2 = 4.40, *P*-value = 0.29). Failure to right in the MA population was also not associated with genotype (χ^2 = 4.46, *P*-value = 0.86) or the presence of the C allele (χ^2 = 0, *P*-value = 1).

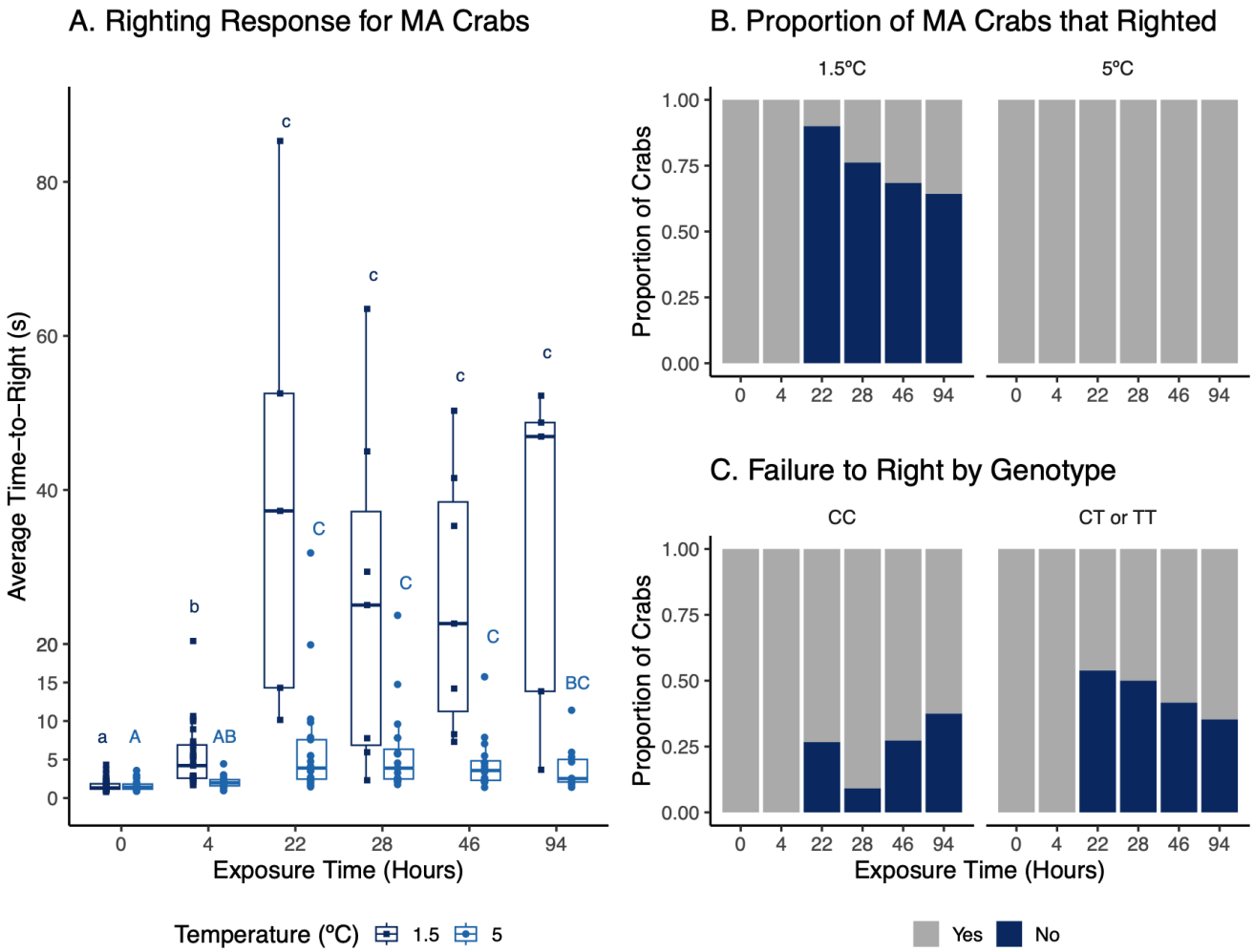

Figure 6. **A**) Average time-to-right for MA *C. maenas* in 1.5ºC (dark blue squares) and 5ºC (light blue circles) temperature treatments; crabs that failed to right in 90 s were not included in these data Righting response was significantly impacted by temperature, time, and their interaction. Pairwise differences between timepoints are indicated within 1.5ºC (dark blue lowercase) and 5ºC (light blue uppercase) treatments. **B**) Proportion of MA crabs that righted within 90 seconds. **C**) Proportion of MA crabs at 1.5ºC that failed to right with and without a T allele. In panels **B**) and **C**), the dark blue color represents crabs that failed to right in 90 s. Failure to right was significantly impacted by temperature and time.

Crabs from WA responded to 1.5ºC and 5ºC in a similar fashion to their MA counterparts. Temperature (χ^2 = 111.41, *P*-value < 0.0001), time (χ^2 = 284.36, *P*-value < 0.0001), and their interaction (χ^2 = 65.38, *P*-value < 0.0001) significantly impacted righting response in WA *C. maenas*. Righting response in 1.5ºC was significantly slower than TTR in 5ºC for all timepoints except hour 0, prior to the start of the cold ramp (**Table S3**). Within a

treatment, there were significant differences in righting response over time (**Table S4**). Righting response for WA crabs at 5ºC slowed from 1.35 s ± 0.56 s to 5.19 s ± 4.73 s at 22 hours, but was 4.34 s ± 3.37 s at 94 hours (**Figure 7A**). Righting response at 1.5ºC slowed and became more variable throughout the experiment. Crabs at 1.5ºC exhibited TTR of 15.94 s ± 10.27 s at 22 hours, and 33.59 s ± 22.29 s at 94 hours (**Figure 7A**). Genotype distribution in WA was similar to the MA population (**Figure S2E**). There was no significant impact of genotype (χ^2 = 1.02, *P*-value = 1), presence of the C allele (χ^2 = 0.36, *P*-value = 1), or presence of the T allele (χ^2 = 0.51, *P*-value = 1) on average TTR in WA crabs. Crab ID explained 4.74% of variance in the final model (temperature, time, and their interaction only).

A small proportion of *C. maenas* from WA failed to right at 1.5ºC: 34.5% WA crabs failed to right at hour 22 and 40% failed to right at hour 94 (**Figure 7B**). Failure to right in WA crabs was related to temperature (χ^2 = 27.35, *P*-value < 0.0001) and time (χ^2 = 22.17, *P*-value = 0.0001), but not their interaction ($\chi^2 = 7.07 \times 10^{-8}$, *P*-value = 1). There was also no impact of sex (χ^2 = 2.17, *P*-value = 0.99), genotype (χ^2 = 2.60, *P*-value = 1), presence of the C allele (χ^2 = 1.98, *P*-value = 1), or presence of the T allele (χ^2 = 0.90, *P*-value = 1) on failure to right in WA *C. maenas*.

Population (MA or WA) did not have a significant impact on average TTR (χ^2 = 0.47, *P*-value = 0.49) or overall failure to right (χ^2 = 0, *P*-value = 1). Population-specific impacts on failure to right at 1.5ºC were also examined for crabs at each timepoint. Population had a marginally significant impact on failure to right after 22 hours of exposure (z = 2.59; *P*-value = 0.06), but not prior to exposure ($z = -2.05 \times 10^{-11}$; *P*-value = 1), or at any other timepoint (*P*-value > 0.05 for all other comparisons).

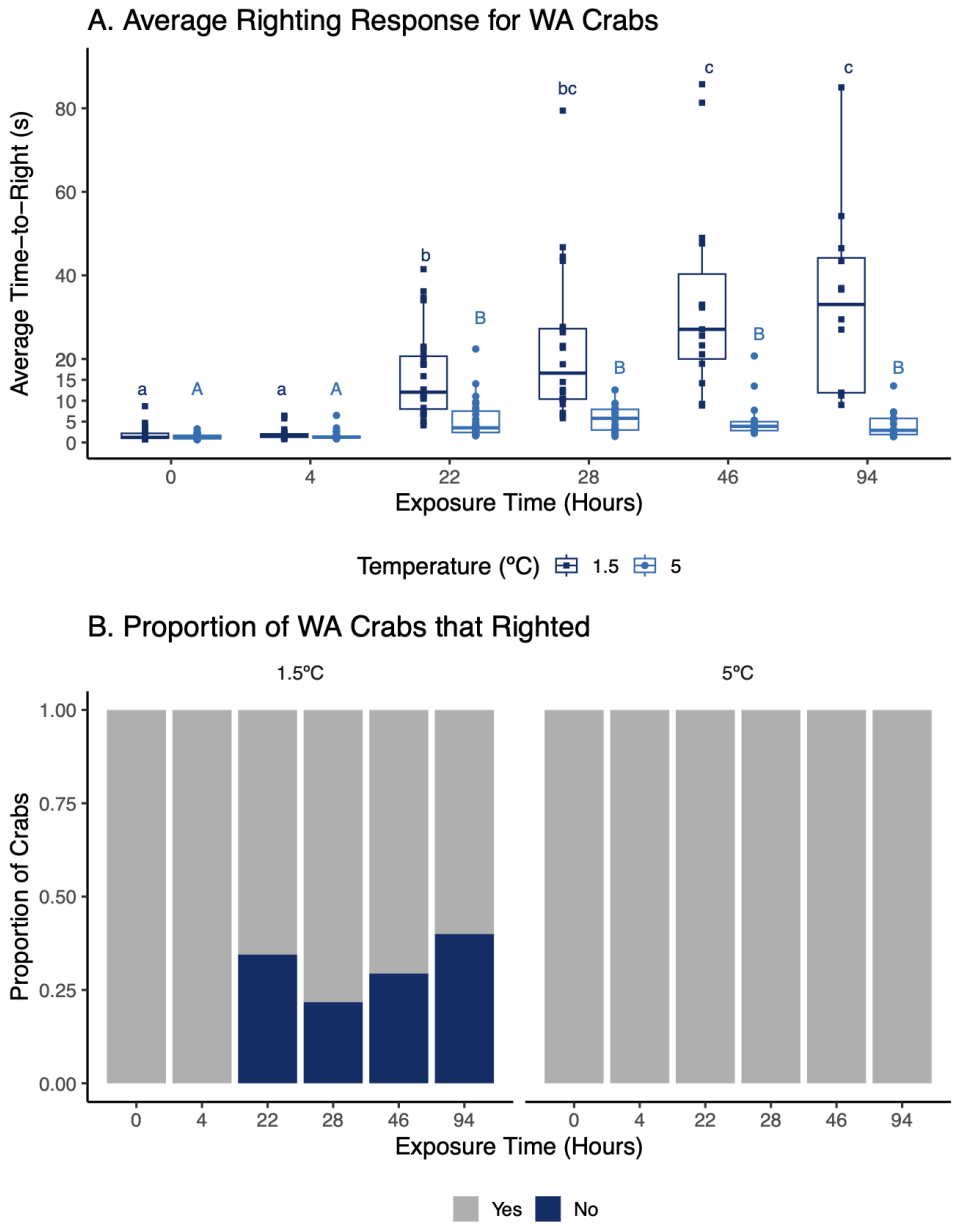

Figure 7. **A**) Average time-to-right for WA *C. maenas* in 1.5ºC (dark blue squares) and 5ºC (light blue circles) temperature treatments; crabs that failed to right in 90 s were not included in these data. Righting response was significantly impacted by temperature, timepoint, and their interaction. Pairwise differences between timepoints are indicated within 1.5ºC (dark blue lowercase) and 5ºC (light blue uppercase) treatments. **B**) Proportion of WA crabs that righted within 90 seconds. The dark blue color represents crabs that failed to right in the given threshold. Failure to right was significantly impacted by temperature and time.

Discussion

We examined how evolutionary adaptation and phenotypic plasticity interact to shape individual thermal phenotype in a highly successful non-indigenous species in two regions where it has been introduced. We focused on the impact on tolerance and plasticity of a specific genetic region (a putative supergene) strongly associated with both environmental temperature and thermal physiology in prior population-level studies. In experiments 1 and 2, we found that acute exposure to sub-lethal high temperature elicits a stress response, but this stress response could be ameliorated by repeated sub-lethal stress exposure. The presence of the warm-adapted supergene was only associated with faster righting response for one of the two tested populations, and genotype had no bearing on the efficacy of heat priming. In experiment 3, we saw that crabs in 5ºC sped up righting response with increased cold acclimation time, while some crabs at 1.5ºC failed to right; rates of failure-to-right differed among populations. The presence of the cold-adapted supergene did not impact cold acclimation or righting response, but there was a non-significant association between the warm-adapted supergene and failure to right in one population. Our work shows that *C. maenas* maintain broad thermal tolerance and plasticity despite the lack of genetic diversity. The maintenance of broad thermal tolerance and plasticity with little influence of supergene genotype suggests that adult tolerance is largely reliant on outcomes of selection during early life history in addition to phenotypic plasticity.

Green crabs display adaptive plasticity at sub-lethal temperatures

Our work investigating *C. maenas* response to heat shock adds to the large body of work highlighting the broad thermal tolerance of this species outside of its native range. We expected that *C. maenas* would speed up righting response during the heat shock because metabolic rates increase for ectotherms at warmer temperatures (Somero, Lockwood and Tomanek 2017). Instead, we found that crabs from ME and WA slowed TTR after heat shock. This contrasts with Jost et al. (2012), which reports TTR starting to slow at 34ºC for ME *C. maenas*, with no decrease at 32ºC. Methodological differences may be responsible for these contrasting results.

While our study held crabs at 30ºC for ~22 hours after a ~1ºC/hour heat ramp, Jost et al. (2012) used a 6ºC/hour increase in temperature. The faster temperature used in Jost et al. (2012) may activate different acute response pathways, leading to a different physiological outcome. Alternatively, the slower TTR observed in our study could indicate that thermal thresholds may be reduced with prolonged exposure to an elevated temperature.

Response to sub-lethal temperatures may also provide insight into *C. maenas*' global success. Previous research has shown that green crabs have a higher lethal thermal limit than many other crustaceans (Tepolt 2024). In previous studies, cessation of righting response has occurred between 31.3ºC-36ºC, depending on acclimation temperature, season, and population (Cuculescu, Hyde and Bowler 1998; Hopkin *et al.* 2006; Jost, Podolski and Frederich 2012). Sub-lethal temperature responses in other species have helped to highlight the mechanisms underlying their invasion potential. For example, sub-lethal temperature oxygen consumption and heart rate responses, but not upper thermal limit, provided a finer-scale understanding of *Mytilus galloprovincialis* distribution in its non-native South African range (Tagliarolo and McQuaid 2015). Future work should examine how chronic exposure to sub-lethal temperature alters crab physiological pathways in order to fully understand the thermal limits and invasion potential of this species.

The response of WA crabs to repeated heat stress demonstrates that negative impacts of acute sub-lethal stress may be ameliorated by adaptive plasticity. Most investigations of physiological plasticity in *C. maenas* have been conducted for heart rate and mortality after weeks or months of thermal acclimation (Cuculescu, Hyde and Bowler 1998; Kelley, de Rivera and Buckley 2011; Tepolt and Somero 2014). Previous research has also shown that thermal limits in *C. maenas* are plastic after short-term thermal acclimation: winter-caught crabs that experienced a 30ºC heat shock for 30 minutes increased T_{max} of righting response from 31.94ºC ± 0.72ºC to 33.13ºC ± 0.72ºC (Hopkin *et al.* 2006). In our study, the faster righting response of crabs that previously experienced heat stress suggests that exposure to sub-lethal

temperatures can physiologically “prime” crabs for subsequent stress. This adaptive plasticity may manifest in different ways. Heat shock may trigger production of heat shock proteins (HSPs), transcription factors, and other molecules necessary for maintaining homeostasis in elevated temperatures (Somero, Lockwood and Tomanek 2017). Exposure to heat stress can also alter regulatory pathways through differences in gene methylation, chromatin modifications that change transcription start site accessibility, or other epigenetic mechanisms (Eirin-Lopez and Putnam 2018; Baldanzi *et al.* 2022; Fernández *et al.* 2025). After the heat shock subsides, these heat shock-responsive molecules and regulatory pathways may remain active in the organism, preparing it for similar conditions within a certain timeframe. While not widely explored in crustaceans, beneficial priming (*ex*. heat hardening or hormetic priming) has been documented in arthropods such as *Drosophila melanogaster* (Sejerkilde, Sørensen and Loeschcke 2003; Sørensen *et al.* 2008; Moghadam *et al.* 2019), as well as a variety of marine taxa, including seagrasses (Pazzaglia *et al.* 2021), anemones (Glass *et al.* 2023), corals (Martell 2022), shellfish (Abbas *et al.* 2024), and sticklebacks (Spence-Jones *et al.* 2025). Studies have also shown that priming may be a contributing factor to increased thermal tolerance in non-indigenous species. Repeated heat stress increased thermal tolerance in *Semimytilus algosus* and *Mytilus edulis* mussels (Lenz *et al.* 2018) and priming improved heat tolerance in non-native *Bactrocera dorsalis* and *Bactrocera correcta* flies (Gu *et al.* 2019). Notably, priming in *B. dorsalis* was linked to upregulation of *heat shock protein 23*.

While our study did not identify genotype-specific trends in righting response due to priming, we do see that adaptive plasticity in response to heat stress is maintained even after a significant loss of genetic diversity. The maintenance of plasticity in *C. maenas* may be the product of plasticity being beneficial in spatially and temporally heterogeneous environments (Grenier, Barre and Litrico 2016; Hendry 2016). Conversely, modeling work shows that high plasticity can maintain limited genetic diversity and reduce diversity loss in changing environmental conditions, especially in the presence of environmental heterogeneity

(Gomez-Mestre and Jovani 2013). Epigenetic modifications (*ie.* epialleles) could also promote phenotypic diversity without changes to genetic diversity (Eirin-Lopez and Putnam 2018). These mechanisms do not preclude the possibility of genetic variation elsewhere contributing to thermal tolerance. A study of heat-stress related quantitative trait loci (QTL) in *D. melanogaster* found that the genetic basis of heat hardening and thermal tolerance were distinct (Arias *et al.* 2012). It is possible that additional investigation of the *C. maenas* genome will clarify if these traits have separate genetic bases in green crabs. Another complementary avenue for future work is the use of enzyme assays, particularly for HIF-1α. Future research could explore if the amino-acid changing mutations present in the different supergene variants of this gene alter the conformation or function of the enzyme in a way that it is more stable at higher temperatures, therefore impacting whole-organism physiology. This experiment should be repeated with a larger sample size of crabs to understand if these non-significant trends may be biologically relevant. Additionally, the conditions under which beneficial plasticity manifests should be evaluated by altering the intensity of heat shocks and the time in between the first and second heat shocks.

Righting response is plastic after short- and medium-term cold exposure

Albeit less frequently studied than heat tolerance, cold tolerance often plays an important role in dictating species distributions (Wasson *et al.* 2025). Organisms that are able to tolerate cooler conditions may be able to effectively colonize northern latitudes with limited competition (Walther *et al.* 2009; Christiansen *et al.* 2015; Dijkstra, Westerman and Harris 2017). Our work demonstrates adult green crabs can maintain motor function at 5ºC — with some maintaining motor function at 1.5ºC — which may allow them a competitive advantage in colder conditions. These findings are in line with previous research that highlights the extensive cold tolerance of adult *C. maenas*. For example, *C. maenas* successfully reproduce in sub-arctic conditions in Newfoundland (Best, McKenzie and Couturier 2017), and can survive at 2ºC for at

least two months (Rivers, McKenzie and McGaw 2025), and below 5ºC for over four months (Kelley, de Rivera and Buckley 2013). In prior work in a UK population, some crabs failed to right at 0ºC, while crabs that maintained movement had a ~5 second average righting response at 0ºC (Young, Peck and Matheson 2006). We observed an average righting response of 33.09 s ± 22.58 s (MA) or 33.59 s ± 22.29 s (WA) at 1.5ºC. These apparent differences in cold tolerance between crab populations may be due in part to genetic or environmental differences. However, it's likely that methodology plays a role. We included hesitation time as part of the righting response, while Young et al. (2006) does not, which likely increased our measurements relative to theirs. Nevertheless, it is clear that *C. maenas* has a robust cold tolerance, which may have facilitated northern range expansion into Newfoundland (Blakeslee *et al.* 2010) and Alaska (NOAA 2022).

In addition to a robust cold tolerance, *C. maenas* displayed some ability to acclimate to chronic cold stress. At 5ºC, righting response for MA and WA crabs sped up at the end of the experiment relative to earlier timepoints. We also observed some evidence of acclimation to 1.5ºC. While many crabs failed to right when initially exposed to 1.5ºC, some individuals "recovered" and were able to right at a later timepoint. This recovery suggests that failure to right is thermally plastic, and may be related to dormancy in this species. Historical work suggests that when temperature is ≤5ºC *C. maenas* enter torpor (Berrill 1982; Young, Peck and Matheson 2006), an energy conservation mechanism typically employed in colder conditions (Pörtner and Playle 1998). Since ectothermic body temperature, and metabolism, is positively correlated with metabolic function, movement and metabolic activity can cease in cold conditions (Pörtner and Playle 1998). Recent work suggests that crabs enter a dormant state (metabolic slowing with limited movement) as opposed to the true torpor exhibited by endothermic organisms (metabolic slowing with no movement, ie. hibernation) (Rivers, McKenzie and McGaw 2025). Dormancy, as opposed to a full torpor, may facilitate energy conservation while also allowing for opportunistic feeding and locomotive activity (*ex*. burying for

predator avoidance) (Rivers, McKenzie and McGaw 2025). The use of dormancy is also supported by prior research which found a steady decrease in heart rate with no cardiac breakpoint when cooling crabs from 15ºC to 0ºC, suggesting that *C. maenas* has sub-zero cold tolerance (Tepolt and Somero 2014). Adult locomotive plasticity in cold temperatures may be facilitated by additional mechanisms such as regulation of hemolymph magnesium to prevent calcium channel blockage and restore locomotor activity (Frederich *et al.* 2000; Aronson *et al.* 2015); increasing the number of polyunsaturated lipids present in muscle tissue to provide membrane fluidity (Chapelle 1978); and downregulating the cell cycle regulator *cyclin D1* to reduce cell proliferation (Kelley, de Rivera and Buckley 2013). In addition to exploring the mechanisms that govern adult dormancy in cold conditions, attention should be paid to cold tolerance mechanisms throughout life history. It is known that *C. maenas* larval development and survival are compromised when temperatures are below 10ºC (Hines *et al*. 2004; Nagaraj 1993; deRivera *et al.* 2007). Additional manipulative experiments with larval crabs may clarify differences in cold tolerance associated with ontogeny.

We explored the relationship between supergene and cold acclimation at the individual level because of a significant population-level association between supergene allele and cold tolerance (Tepolt and Palumbi 2020). Surprisingly, the cold-adapted supergene did not significantly impact TTR in 1.5ºC or 5ºC. However, we observed an association between the warm-adapted supergene and failure to right, but only in the northwest Atlantic population (MA). Although this association was not significant, it is suggestive, especially considering the heat shock data. While these differences among populations may be a product of the slightly different experimental temperatures for MA and WA crabs, it is unlikely that this ~0.5ºC temperature difference is solely responsible. The switch from an active to dormant state in *C. maenas* occurs between 4-6ºC (Young, Peck and Matheson 2006; Rivers, McKenzie and McGaw 2025), which is well above the coldest temperature used in this experiment. Another explanation may be the dual impacts of mitochondrial and nuclear diversity in MA crabs. Coyle et al. (2019) found that

failure to right after acute exposure to 4.5ºC was associated with mitochondrial haplotype in male crabs from populations in the Gulf of Maine. Multiple mitochondrial haplotypes are present in the northwest Atlantic, but to our knowledge, there is limited mitochondrial haplotype diversity within the MA population (Darling *et al.* 2014; Jeffery *et al.* 2017; Lehnert *et al.* 2018). While there are also differences in nuclear diversity in the northwest Atlantic, increased diversity in this region is concentrated in Maritime Canada and Newfoundland, much further north than MA (Darling *et al.* 2014; Jeffery *et al.* 2017; Lehnert *et al.* 2018). Instead, we suggest that crabs in the northwest Atlantic may develop more pronounced dormancy to deal with colder winters and to conserve energy while food is limited (**Figure 1**).

Interactions between genetics and plasticity in populations with low genetic diversity

Population-level association studies frequently identify genetic markers with tantalizing links to environmental factors or physiological traits (Rellstab *et al.* 2015; Riginos *et al.* 2016; Bay *et al.* 2017), but it can be difficult to bridge the gap between these markers and their potential impacts on physiological mechanisms at the individual level. We examined the impact of this supergene on thermal tolerance and plasticity because of strong correlations between the supergene and both cold tolerance and sea surface temperature in previous population-level studies (Tepolt and Palumbi 2020). Within a population, we hypothesized that individual-level variation in thermal tolerance and plasticity would be associated with supergene genotype. Aside from a significant association between the warm-adapted supergene and heat shock response in the ME population, the majority of the variation individual crabs exhibited in righting response was not associated with supergene genotype. Here, we discuss additional non-exclusive hypotheses: 1) natural temperature gradients impact both selection and plasticity; and 2) the supergene may be operating at a different level of biological organization and/or point in life history.

While there is a strong population-level association between supergene genotype and both cold tolerance and temperature (Tepolt and Palumbi 2020; Tepolt *et al.* 2022), this association is likely driven in large part by adaptive constraints at the larval stage. In species like *C. maenas* with extensive larval dispersal across a broad environmental gradient, genotype distributions in the adult population often reflect selection during larval dispersal, metamorphosis, and settlement (Koehn, Newell and Immermann 1980; Sanford and Kelly 2011; Tigano and Friesen 2016). Larvae of *C. maenas* are more thermally sensitive than adults, with limited survival below 10ºC (Hines *et al*. 2004; Nagaraj 1993; deRivera *et al.* 2007). Given that the minor allele frequency of the supergene is strongly and consistently correlated with sea surface temperature in the northeast Pacific population less than 30 years after initial introduction (Tepolt *et al.* 2022), it is likely that temperature-associated mortality at the larval and/or early juvenile stage shapes the distribution of the supergene in adult populations. Temperature is a known driver of year-to-year population dynamics in both northwest Atlantic and northeast Pacific populations, with warmer winters leading to larger populations (Welch 1969; Yamada and Kosro 2010; Behrens Yamada, Peterson and Kosro 2015). Comparing supergene distributions in larvae and newly-settled crabs with ocean conditions on a finer temporal and spatial scale may elucidate this connection further.

Above, we suggest that the different temperature profiles experienced by larvae in the northwest Atlantic and northeast Pacific may select for individuals with a supergene allele that “matches” local environmental conditions (Tepolt *et al.* 2022; Thia 2022). However, phenotypic plasticity at the larval and adult stages likely also shapes thermal tolerance. The large larval dispersal distances in this species mean that larvae likely experience spatially variable environments, which has been associated with greater developmental plasticity in other systems (Hendry 2016; Giménez 2020). This larval plasticity can increase phenotypic variation, leading to changes in recruitment and survival in subsequent life stages (Giménez 2020).

Impacts of temperature stress can also manifest across ontogenetic stages (*ie*. within-generation carryover effects) and generations (*ie*. intergenerational carryover effects) (Donelson *et al.* 2018; Byrne *et al.* 2019). Recent work in the species' native range has shown that environmental conditions during embryonic brooding can impact temperature and salinity tolerance (Torres *et al.* 2020; Šargač *et al.* 2021, 2022). Similar intergenerational effects have been explored in other species: maternal investment in the kelp crab *Taliepus denatus* is correlated with seasonality and predictability of environmental conditions (Fernández *et al.* 2025); slipper limpet (*Crepidula fornicata*) larvae brooded at lower temperatures took longer to reach competency than counterparts brooded at higher temperatures (Meyer-Kaiser 2020); adult mussels (*Mytilus californianus*) exposed to higher temperatures had less tolerant offspring (Waite and Sorte 2022); and brooding temperature in an invasive tunicate impacts juvenile thermal tolerance (Tobias, Solow and Tepolt 2024). Given the complicated relationship between plasticity and selection (Hendry 2016), it is possible that both of these mechanisms are important in shaping thermal tolerance, and should be explored in future studies.

In addition to environmental conditions shaping genotype and plasticity at different life stages, it is possible that examining different physiological metrics could detect a greater role for the supergene in thermal tolerance (ex. heart rate, respiration rate, gene expression). Since the association was established using heart rate at 0ºC (Tepolt and Palumbi 2020), it may be illustrative to examine the relationship between genotype and heart rate plasticity on an individual level. For example, heart rate is associated with genotype in *Drosophila melanogaster* (Robbins *et al.* 1999). Molecular pathway regulation may also clarify the role of the supergene in adult phenotype. Flies (*D. suboscura*) with a warm-adapted supergene exhibit higher basal abundance of Hsp70 proteins than counterparts with the cold-adapted supergene version (Calabria *et al.* 2012), and experimentally altering the expression of *estrogen receptor α* in a supergene changed aggression in the white-throated sparrow (Merritt *et al.* 2020). Since we

observed no supergene-associated difference in whole-organism response, it is possible that the supergene genotype is influencing physiology at a lower level of biological organization. For example, a study in coral larvae detected metabolic reprogramming under elevated temperature without a decrease in survival (Huffmyer *et al.* 2024), and American lobster (*Homarus americanus*) exposure to ocean acidification resulted in broad metabolic reprogramming that was not associated with changes to resting metabolism (Noisette *et al.* 2021). Future work should examine if the warm- and cold-adapted supergene alleles are associated with differences in metabolic pathways and gene expression that allow for physiological homeostasis.

Conclusion

We sought to elucidate the role of genotype in shaping individual-level thermal tolerance based on information from population-level association studies. Previous work demonstrates a strong association between supergene allele frequency and both cold tolerance and sea surface temperature (Tepolt and Palumbi 2020). While we did not see strong evidence of supergene impacting individual thermal tolerance, we found that adult *C. maenas* maintains broad thermal tolerance despite lack of genetic diversity. In particular, we found that crabs exhibit potentially beneficial plasticity in response to sub-lethal heat stress, and can acclimate to cold temperatures within 94 hours. Future work — from enzyme assays to metabolomic investigations — should elucidate the mechanisms that underpin this exceptionally robust thermal tolerance. Our work underscores the importance of pairing individual-level physiological assessments with genetic association studies to contextualize population-level trends. Although this association was characterized in adult crabs (Tepolt and Palumbi 2020), environmental conditions like temperature are likely shaping both supergene distribution and phenotypic plasticity throughout life history, including during larval dispersal and settlement. In order to

clarify the relationship between population- and individual-level tolerance, future work should explore if and how the supergene influences thermal tolerance in planktonic larvae, young-of-the year, and adult crabs. Additionally, the development of the *C. maenas* genome will facilitate a fuller understanding of the genomic content of the supergene and permit more mechanistic examination of its contribution to thermal plasticity. Our work highlights how thermal plasticity contributes to the broad thermal tolerance of successful non-indigenous species, even after a substantial loss of genetic diversity.

Data availability

All raw data and scripts are available at https://github.com/yaaminiv/cold-green-crab.

Author contributions

YRV, JCK, and CP conceived, designed, and carried out all experiments with assistance from HLF and input from CKT. YRV, JCK, and JK conducted molecular assays. JCK, CP, and HLF conducted preliminary data analysis. YRV refined all data analysis. YRV wrote the initial draft with input from JCK and CP; and YRV and CKT revised and edited this manuscript. All authors reviewed and approved the final manuscript.

Conflict of Interest Statement

The authors have no conflicts of interest to report.

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Supplementary Information

Appendix S1: Genotype impacts on righting response in New Hampshire population

The influence of supergene genotype on righting response in room temperature conditions was evaluated using *C. maenas* trapped at New Castle, NH, USA on July 22-24, 2024 under NH permit MFD 2426 (n_{NH} = 15; carapace $width_{NH}$ = 69 mm ± 7 mm, $weight_{NH}$ = 73.25 g ± 21.16 g). Crabs were acclimated at room temperature (~22ºC) for five days before experimentation.

Righting response was measured after acclimation (**Figure S3**), and averaged 6.23 seconds ± 10.43 seconds. Genotype significantly impacted TTR (t = 10.84, *P*-value = 8.96 x 10^{-3}), with CC crabs having faster TTR than CT crabs ($mean_{NH, CC}$ = 4.41 seconds ± 6.67 seconds; $mean_{NH, CT}$ = 14.76 seconds ± 14.27 seconds), but the importance of these effects may be impacted by low sample size of CT crabs. No TT crabs were collected from the field for this experiment.

Crabs were dissected per approach in Experiment 1 to characterize the macroparasite community in this population. Crabs from NH were parasitized by either trematode metacercaria, acanthocephalans, or both. Individual crabs contained between 0-471 trematode metacercarial cysts and 0-19 acanthocephalans. Parasite intensities were 1.06 ± 2.20 cysts/mm and 0.04 ± 0.07 parasites/mm for trematode metacercaria and acanthocephalans, respectively. Neither trematode metacercarial cyst infection intensity (t = 0.283, *P*-value = 0.79) nor acanthocephalan infection intensity (t = -1.76, *P*-value = 0.12) had a significant impact on righting response.

Appendix S2: Oxygen consumption for repeated heat shock experiment

A pilot experiment on the rate of oxygen consumption was additionally used to measure metabolic performance of *C. maenas* to understand how repeated heat shocks influenced physiology. After obtaining righting response measurements on day 4 (after the second heat shock), crabs ($n_{control}$ = 6 and $n_{treatment}$ = 4) weighing ~20g were placed in 90 mm x 50 mm (270 mL) glass respirometry chambers with FireSting oxygen sensor spots (PS-OXSP5). These glass chambers were cleaned with 70% ethanol, covered with dark material to prevent light from entering, and covered with mesh to allow for water exchange. Crabs in the chambers were acclimated for one hour in an aerated tank at the crab's treatment temperature. Respirometry chambers were refilled with aerated water at the treatment temperature, sealed with parafilm, a glass lid, and a weight to prevent oxygen intrusion, and then placed in a water bath at the treatment temperature. Percent oxygen saturation was measured with a 4-channel FireSting PRO (PyroScience) until readings reached 75% saturation. Respirometry chambers were then flushed and resealed without the crab to measure background respiration for at least 20 minutes. Bare optical fibers for the FireSting devices (PS-SPFIB-BARE) were calibrated using ambient air as the upper limit.

Oxygen consumption rates were compared between control and treatment crabs. To obtain rates for each individual crab, dissolved oxygen (µmol/L) was first calculated from percent air saturation, temperature (ºC), and pressure (mbar) provided by the FireSting and a salinity measurement obtained from each tank with a refractometer. These calculations were performed using the `conv_o2` function from the R package `respirometry` v.2.0.0 (Birk 2023). Linear models were fitted to data when air saturation was between 80-100% saturation. Regression slopes from these models were then obtained for dissolved oxygen (`fit` from the R package `generics` v.0.1.3 (Wickham, Kuhn and Vaughan 2022)) and adjusted R-squared values (`glance` from `generics` package). Similarly, the last ten minutes of background respiration measurements were used to obtain a "blank" oxygen consumption rate. The slopes

from the blank were subtracted from the crab respiration slope to obtain a blank-corrected oxygen consumption slope. A linear model (`lm` in R) was used to evaluate how treatment impacted oxygen consumption. Genotype effects were not tested due to low sample size.

Oxygen consumption on day 4 was slower for crabs that experienced a prior heat shock (7475.60 nmol/hr ± 4671.50 nmol/hr) than for those that did not (15394.55 nmol/hr ± 8506.37 nmol/hr) (**Figure S4**). However, this trend was not significant ($t = -1.68$, $p = 0.13$).

Tables

Table S1. PCR primers and amplification methods for SMC gene. A total of 12.5 µL of GoTaq® Master Mix (Promega, USA), 2.5 µL of the forward primer (10 µM), 2.5 µL of the reverse primer (10 µM), and 5.5 µL nuclease-free water was used with 2 µL of DNA for each sample. Additional information on SMC F and R primers can be found in Coyle et al. (2019).

Year used	Primers	Sequence	PCR product length (bp)	PCR profile
2023 (Experiment 2)	SMC_F SMC_R	Forward: 5' - AGCACAGGAAGGCTGTGG - 3' Reverse: 5' - ACGAAATCATAAGCCTCTTCACG - 3'	126	3 min. 94ºC; 17 cycles (30 sec. at 94ºC, 30 sec. at 65ºC [-1ºC per cycle], 1.5 min. at 72ºC). 20 cycles (30 sec. at 94º, 30 sec. at 48ºC 1.5 min. at 72ºC). 10 min. at 72ºC
2024 (Experiments 1 and 3)	SMC_long_F SMC_long_R	Forward: 5' - TGATGCTCAGCACAGGAAGG - 3' Reverse: 5' - CTTCCATACTTTAACTTCATGAGAACA - 3'	690	3 min. 95ºC; 35 cycles (30 sec. at 95ºC, 30 sec. at 60ºC, 1 min. at 72ºC). 5 min. at 72ºC

Table S2. Experimental temperatures used for all populations and conditions. Average temperatures are reported for all populations and conditions, except for the second heat shock in experiment 2, where individual tank temperatures are reported due to significant differences in tank temperatures.

Experiment	Population	Condition	Temperature (ºC; Mean ± SE)
1	WA	Heat shock	30.04 ± 0.30
	ME	Heat shock	29.91 ± 0.30
2	WA	First Heat Shock	Control: 15.43 ± 0.46 Treatment: 31.55 ± 1.15
	WA	Second Heat Shock	$Control_{Tank1}$: 32.14 ± 0.10 $Control_{Tank\ 2}$: 32.13 ± 0.07 $Treatment_{Tank\ 1}$: 32.09 ± 0.17 $Treatment_{Tank\ 2}$: 29.46 ± 0.16
3	MA	Cold	5.39 ± 0.30
	MA	Colder	1.66 ± 0.32

	WA	Cold	5.20 ± 0.22
	WA	Colder	2.14 ± 0.35

Table S3. Pairwise statistical test results examining treatment differences at specific timepoints for Experiment 3. All reported statistics are for the 1.5ºC - 5ºC contrast.

Hour	$t_{MA, 1.5ºC - 5ºC}$	$P\text{-value}_{MA, 1.5ºC - 5ºC}$	$t_{WA, 1.5ºC - 5ºC}$	$P\text{-value}_{WA, 1.5ºC - 5ºC}$
0	0.35	0.73	0.41	0.68
4	4.34	< 0.0001	4.82	< 0.0001
22	7.84	< 0.0001	9.79	< 0.0001
28	9.09	< 0.0001	11.97	< 0.0001
46	9.21	< 0.0001	12.03	< 0.0001
94	9.04	< 0.0001	11.60	< 0.0001

Table S4. Pairwise statistical test results examining differences in TTR at each timepoint for specific treatments for Experiment 3.

Contrast	$t_{MA, 1.5ºC}$	$P\text{-value}_{MA, 1.5ºC}$	$t_{MA, 5ºC}$	$P\text{-value}_{MA, 5ºC}$	$t_{WA, 1.5ºC}$	$P\text{-value}_{WA, 1.5ºC}$	$t_{WA, 5ºC}$	$P\text{-value}_{WA, 5ºC}$
Hour 0 - Hour 4	-7.39	< 0.0001	-2.03	0.33	-1.26	0.80	-0.96	0.93
Hour 0 - Hour 22	-9.77	< 0.0001	-7.69	< 0.0001	-16.66	< 0.0001	-8.34	< 0.0001
Hour 0 - Hour 28	-10.50	0	-7.41	< 0.0001	-17.04	< 0.0001	-9.37	< 0.0001
Hour 0 - Hour 46	-11.79	0	-5.67	< 0.0001	-17.84	< 0.0001	-7.88	< 0.0001
Hour 0 - Hour 94	-10.60	< 0.0001	-4.44	0.0002	-16.29	< 0.0001	-6.19	< 0.0001
Hour 4 - Hour 22	-6.10	< 0.0001	-5.25	< 0.0001	-15.08	< 0.0001	-7.27	< 0.0001
Hour 4 - Hour 28	-5.71	< 0.0001	-5.02	< 0.0001	-15.62	< 0.0001	-8.37	< 0.0001
Hour 4 - Hour 46	-6.92	< 0.0001	-3.66	0.005	-16.62	< 0.0001	-7.04	< 0.0001
Hour 4 - Hour 94	-6.52	< 0.0001	-2.77	0.07	-15.21	< 0.0001	-5.43	< 0.0001

Hour 22 - Hour 28	1.38	0.74	0.007	1	-2.07	0.31	-1.59	0.61
Hour 22 - Hour 46	0.51	1	1.19	0.84	-4.30	0.0003	-1.01	0.91
Hour 22 - Hour 94	0.16	1	1.51	0.66	-3.87	0.002	0.25	1
Hour 28 - Hour 46	-0.99	0.92	1.15	0.86	-2.33	0.18	0.40	1
Hour 28 - Hour 94	-1.28	0.80	1.48	0.68	-2.05	0.31	1.55	0.63
Hour 46 - Hour 94	-0.38	1	0.44	1	0.13	1	1.10	0.88

Figures

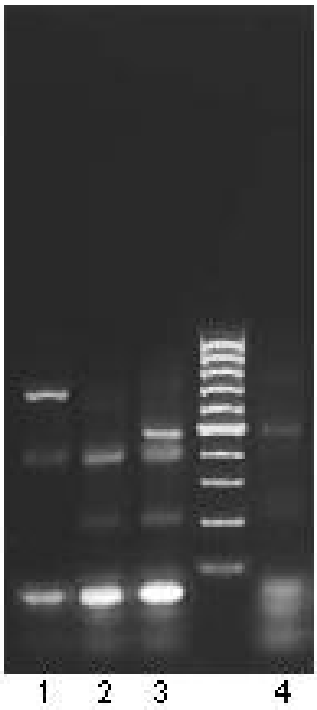


Figure S1. Example gel for genotyping with AluI restriction digest. **1**) Undigested PCR product (690 bp). A faint band is also present between 300-400 bp that is likely a result of non-target amplification. Presence of this non-diagnostic band can vary across CC, CT, and TT samples. **2**) CC band (389 bp). **3**) CT bands (477 bp and 389 bp). **4**) TT band (477 bp). Primer-dimer bands for all samples and the non-diagnostic 88 bp bands for CC and CT genotypes are present in all digested samples. Shown with a 100 bp ladder for reference.

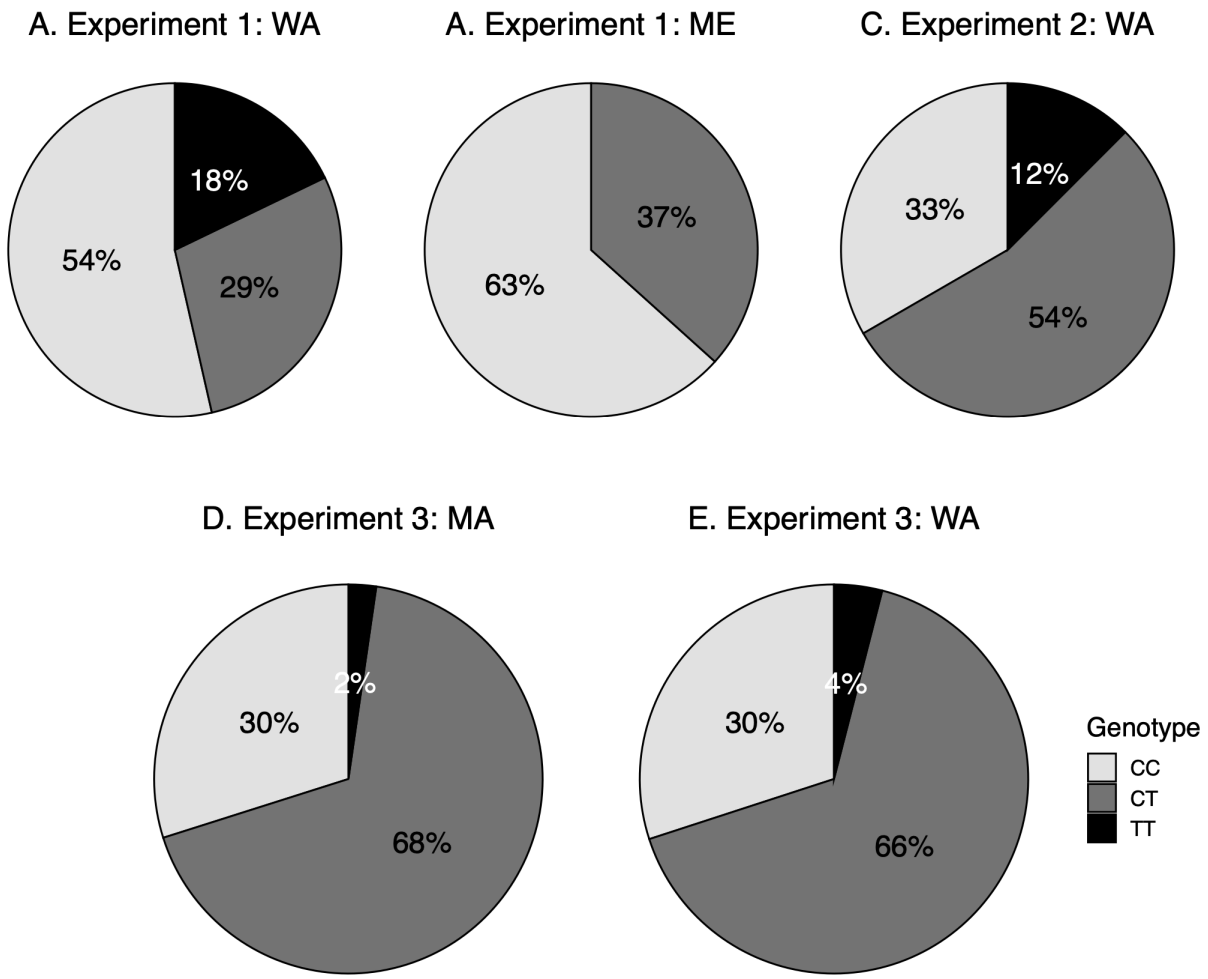


Figure S2. Pie charts for supergene genotypes in **A**) WA and **B**) ME populations used in experiment 1, **C**) WA population used in experiment 2, and **D**) MA and **E**) WA populations used in experiment 3. Percentages for each genotype are presented.

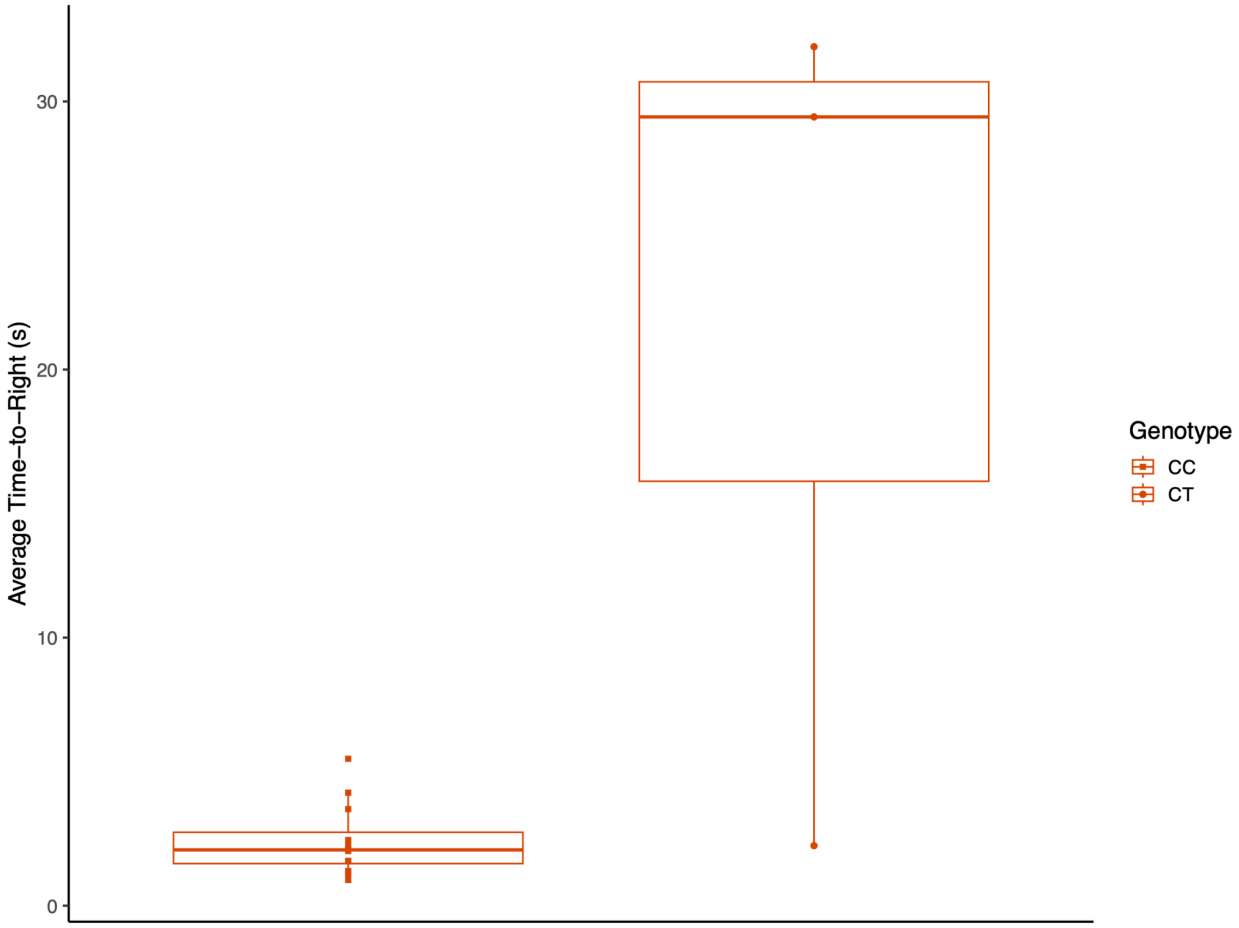


Figure S3. Righting response in NH *C. maenas* by genotype. CT crabs had significantly slower righting response at room temperature than CC crabs (t = 10.84, P-value = 8.96 x 10^{-3}).

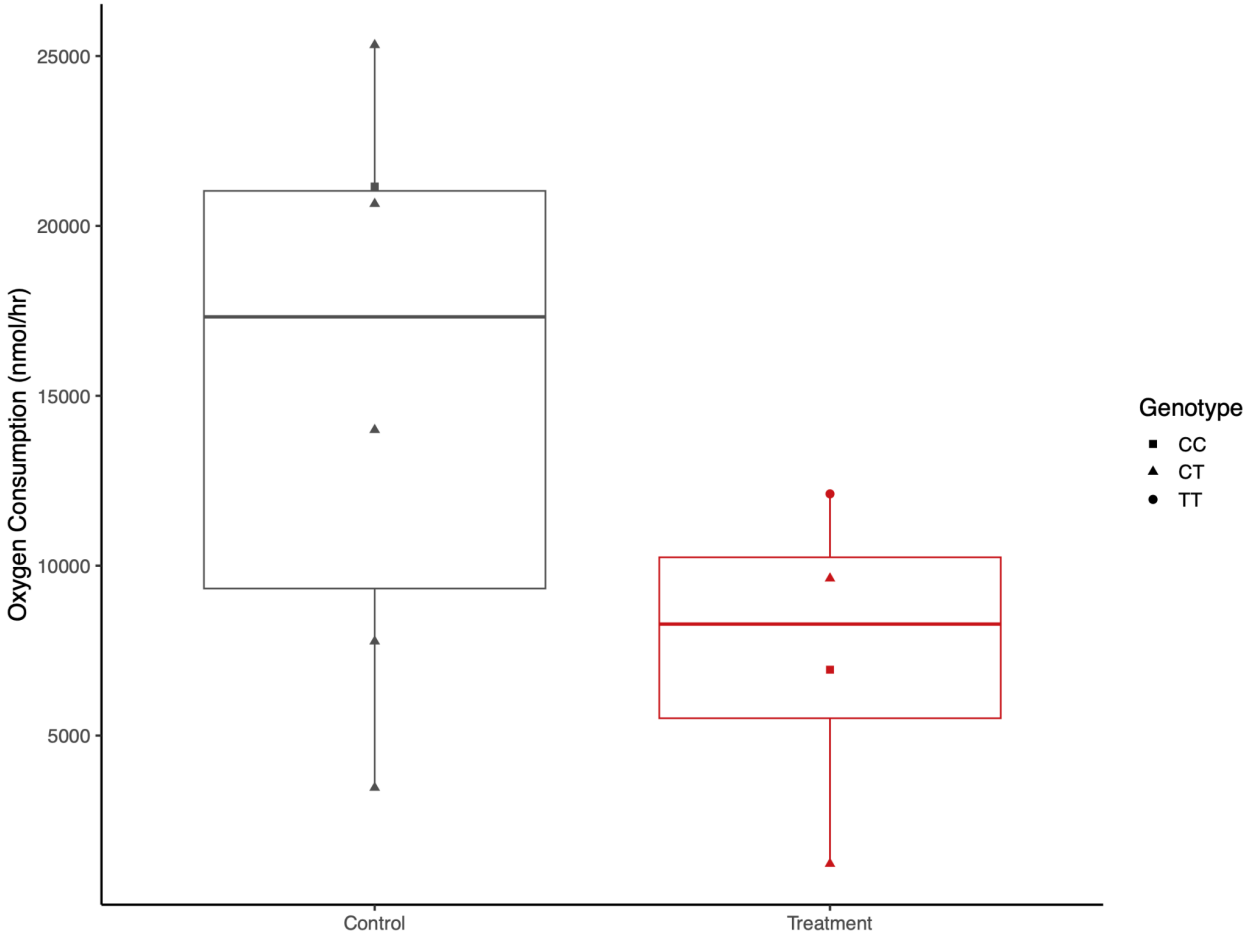
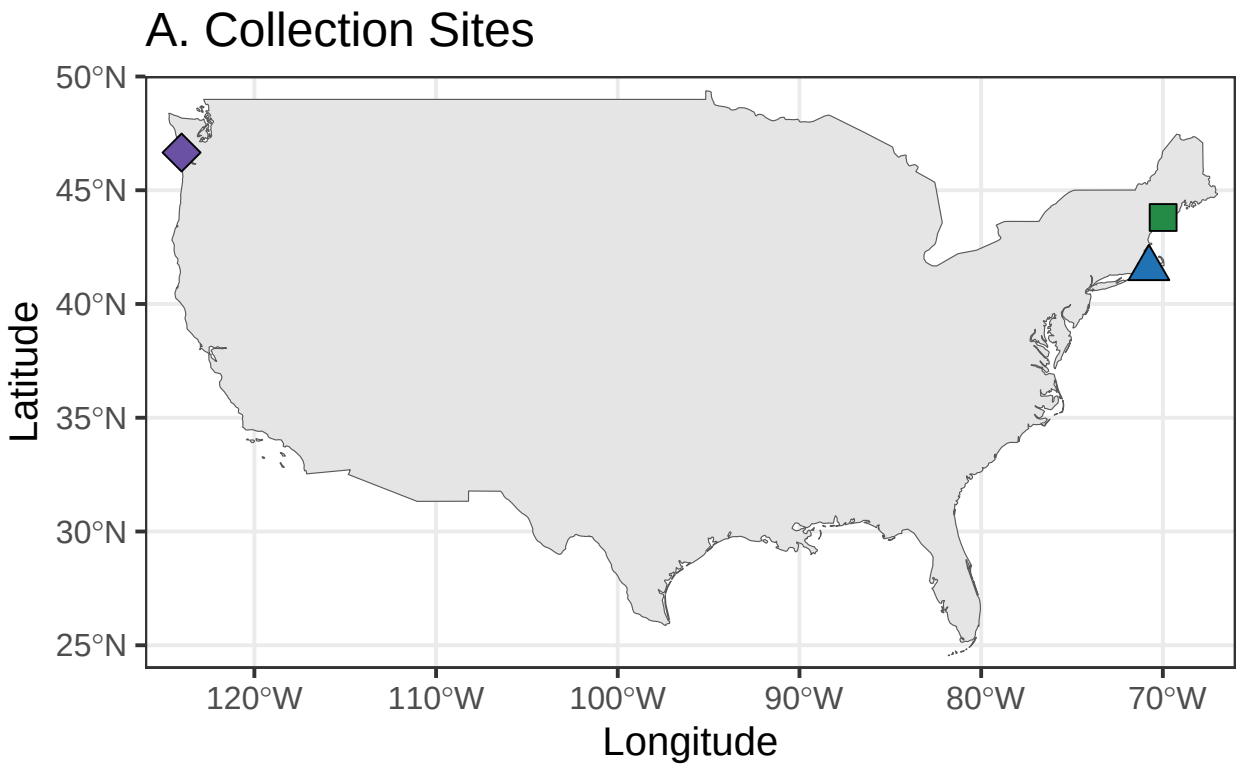
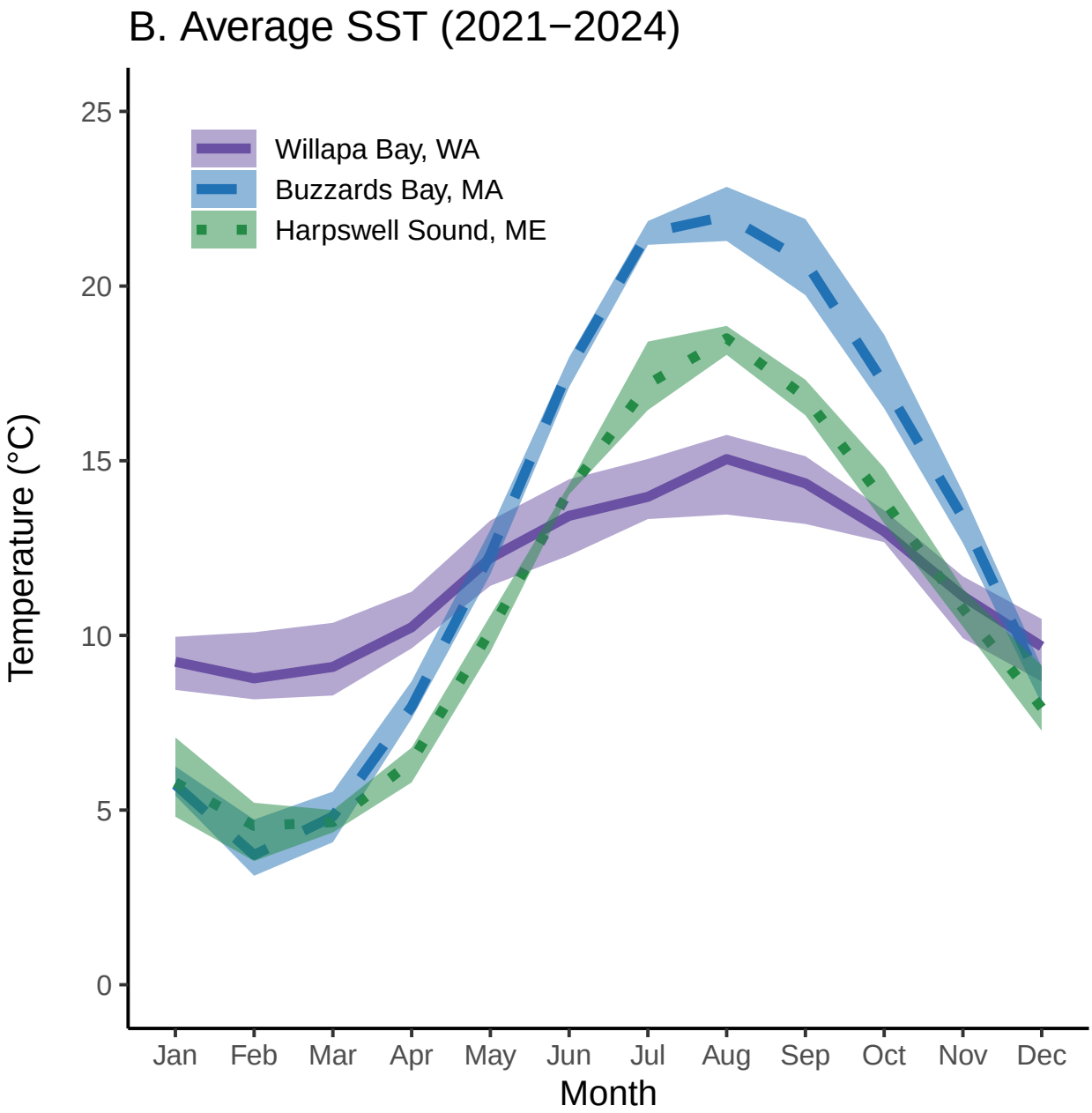
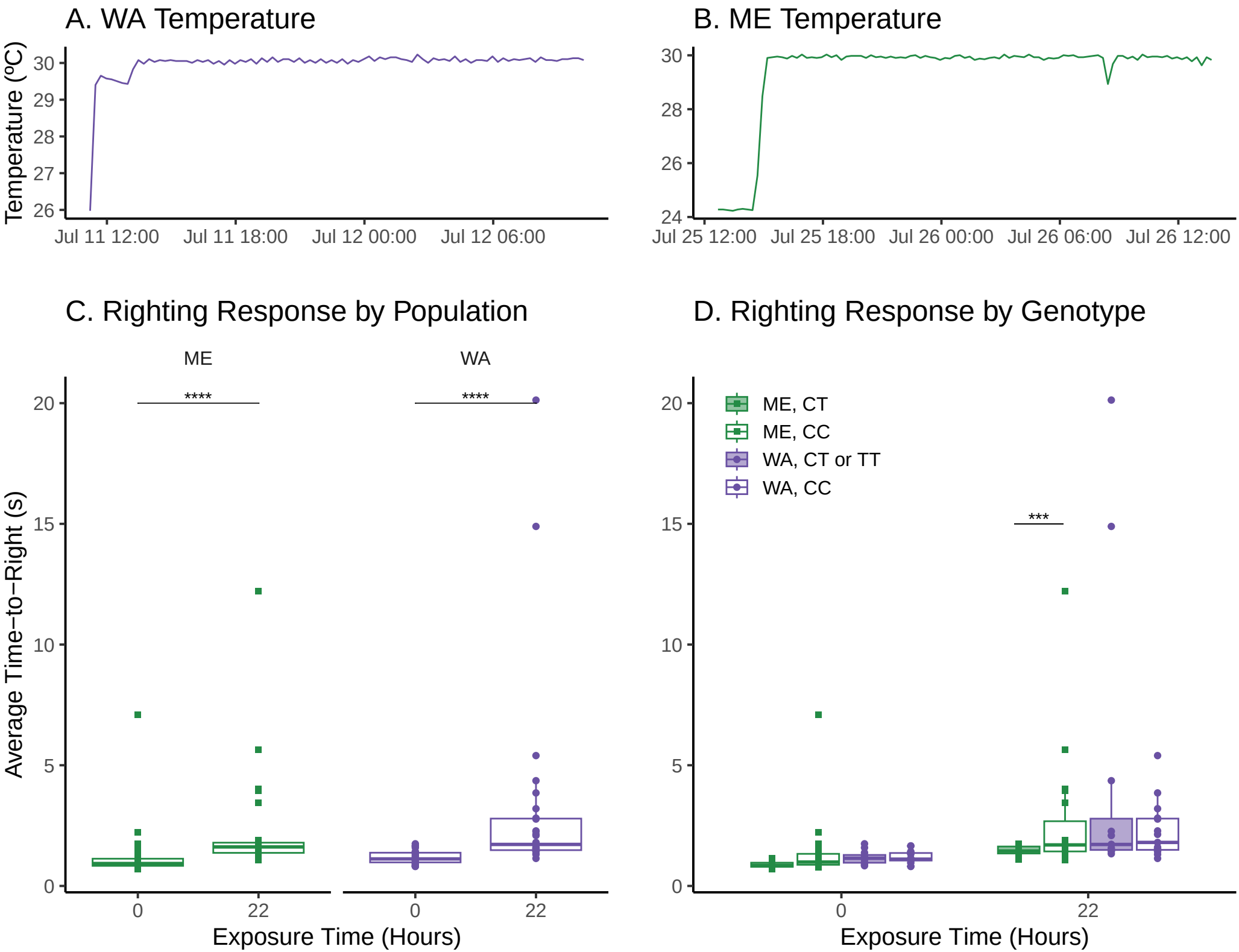
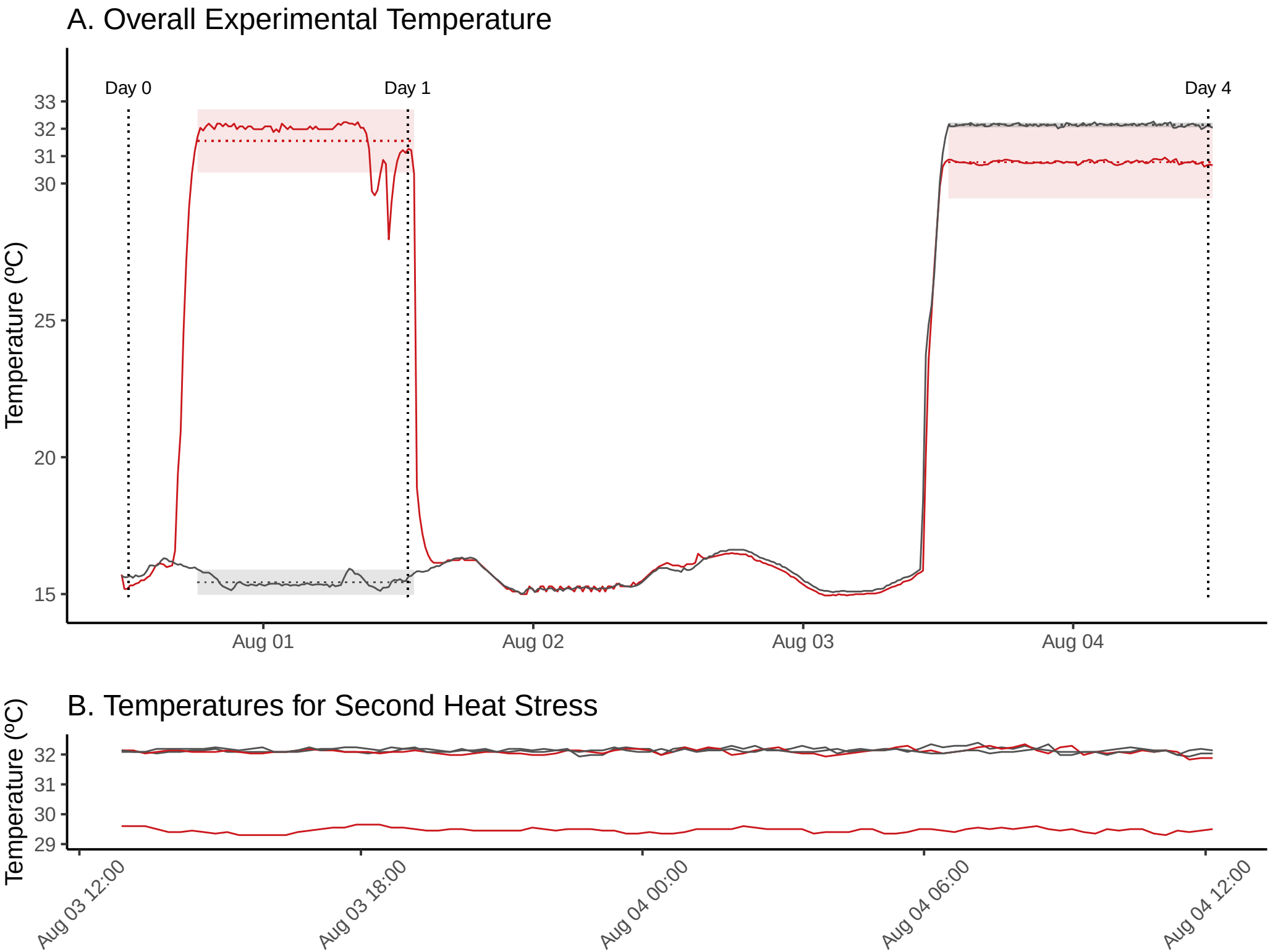


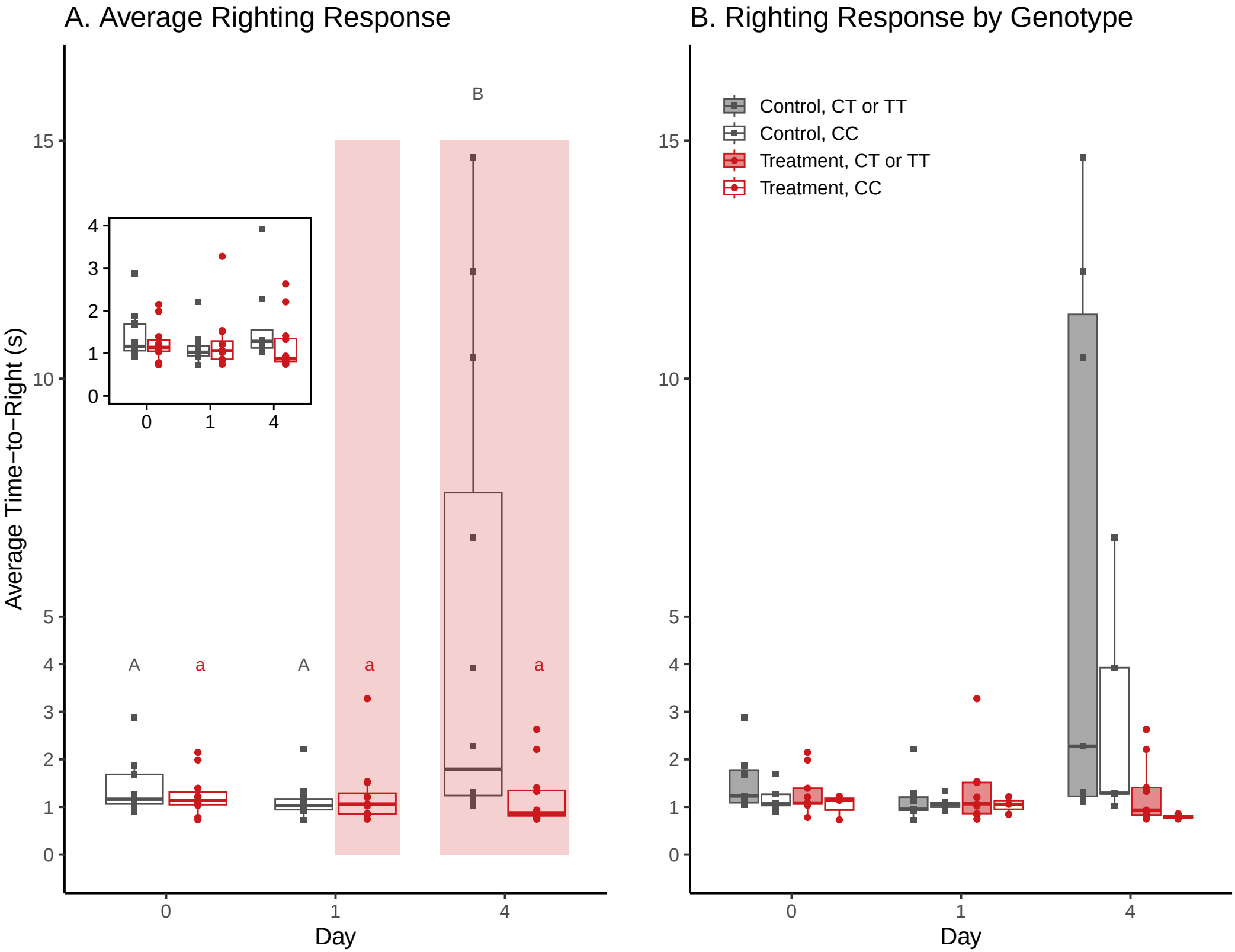
Figure S4. Oxygen consumption (nmol/hr) after the day 4 heat shock. Crabs that did not experience a prior heat shock are in grey, while those that did are in red. Different shapes indicate crab genotype. There was no significant impact of prior heat shock exposure on oxygen consumption.

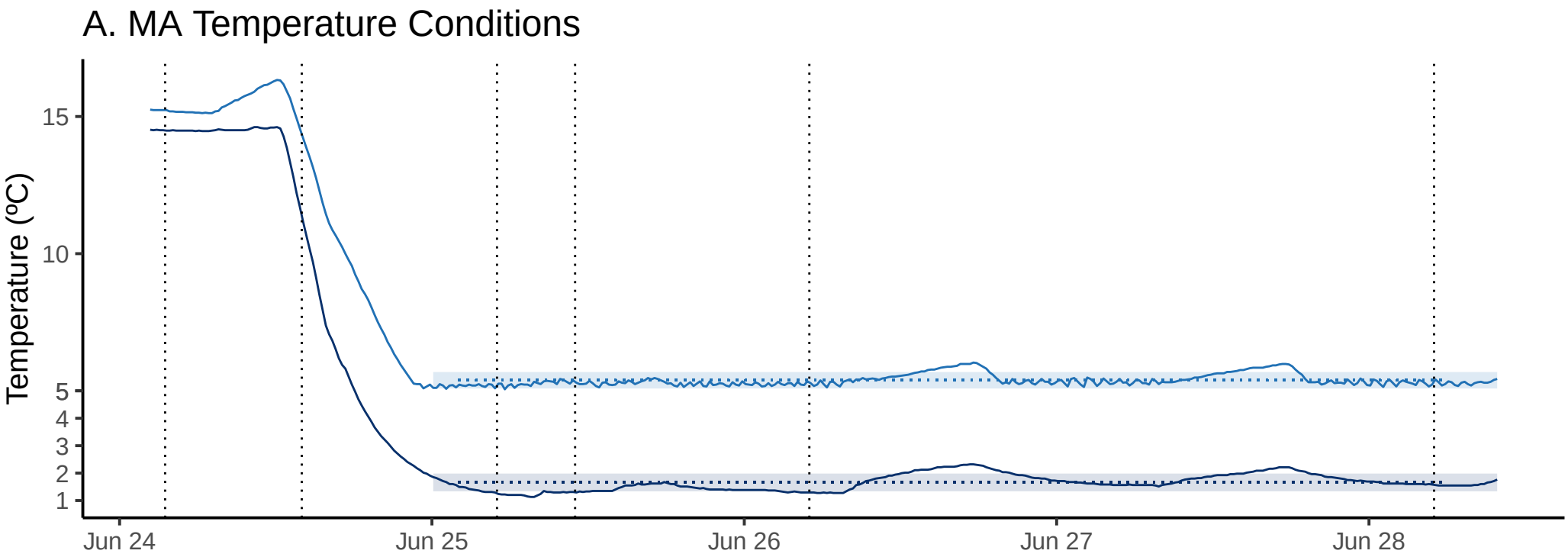

A. Collection Sites
50°N
45°N
40°N
35°N
30°N
25°N
Latitude
120°W
110°W
100°W
90°W
80°W
70°W
Longitude

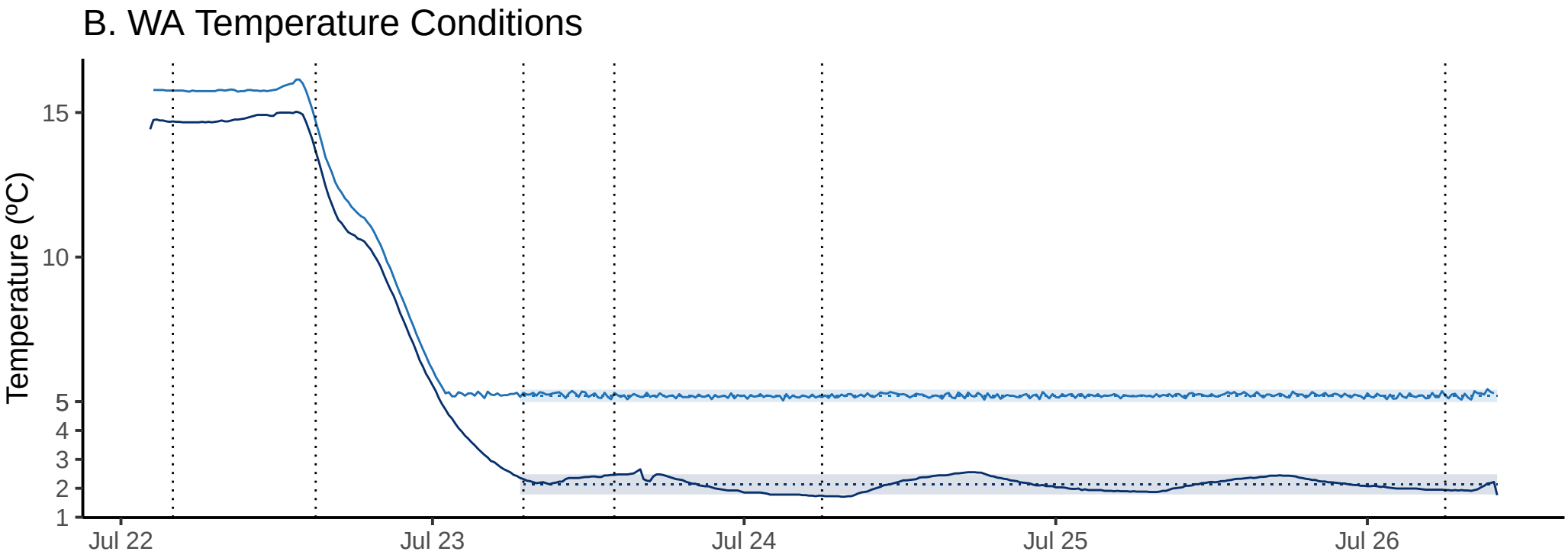

B. Average SST (2021−2024)
Willapa Bay, WA
Buzzards Bay, MA
Harpswell Sound, ME
25
20
15
10
5
0
Temperature (°C)
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month

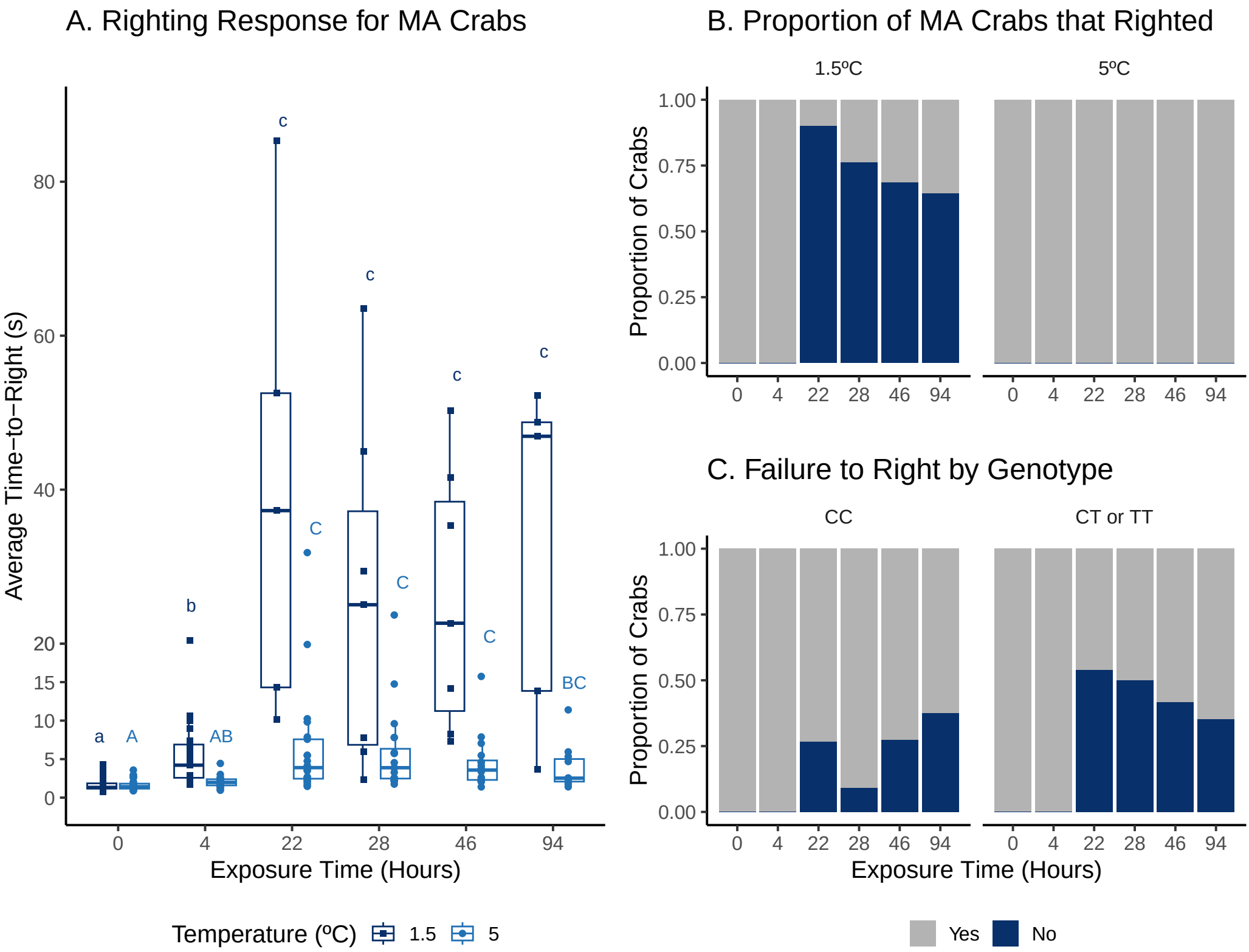

A. WA Temperature
Temperature (ºC)
30
29
28
27
26
Jul 11 12:00
Jul 11 18:00
Jul 12 00:00
Jul 12 06:00
B. ME Temperature
30
28
26
24
Jul 25 12:00
Jul 25 18:00
Jul 26 00:00
Jul 26 06:00
Jul 26 12:00
C. Righting Response by Population
ME
WA
****
****
Average Time−to−Right (s)
20
15
10
5
0
0
22
0
22
Exposure Time (Hours)
D. Righting Response by Genotype
ME, CT
ME, CC
WA, CT or TT
WA, CC
***
20
15
10
5
0
0
22
Exposure Time (Hours)

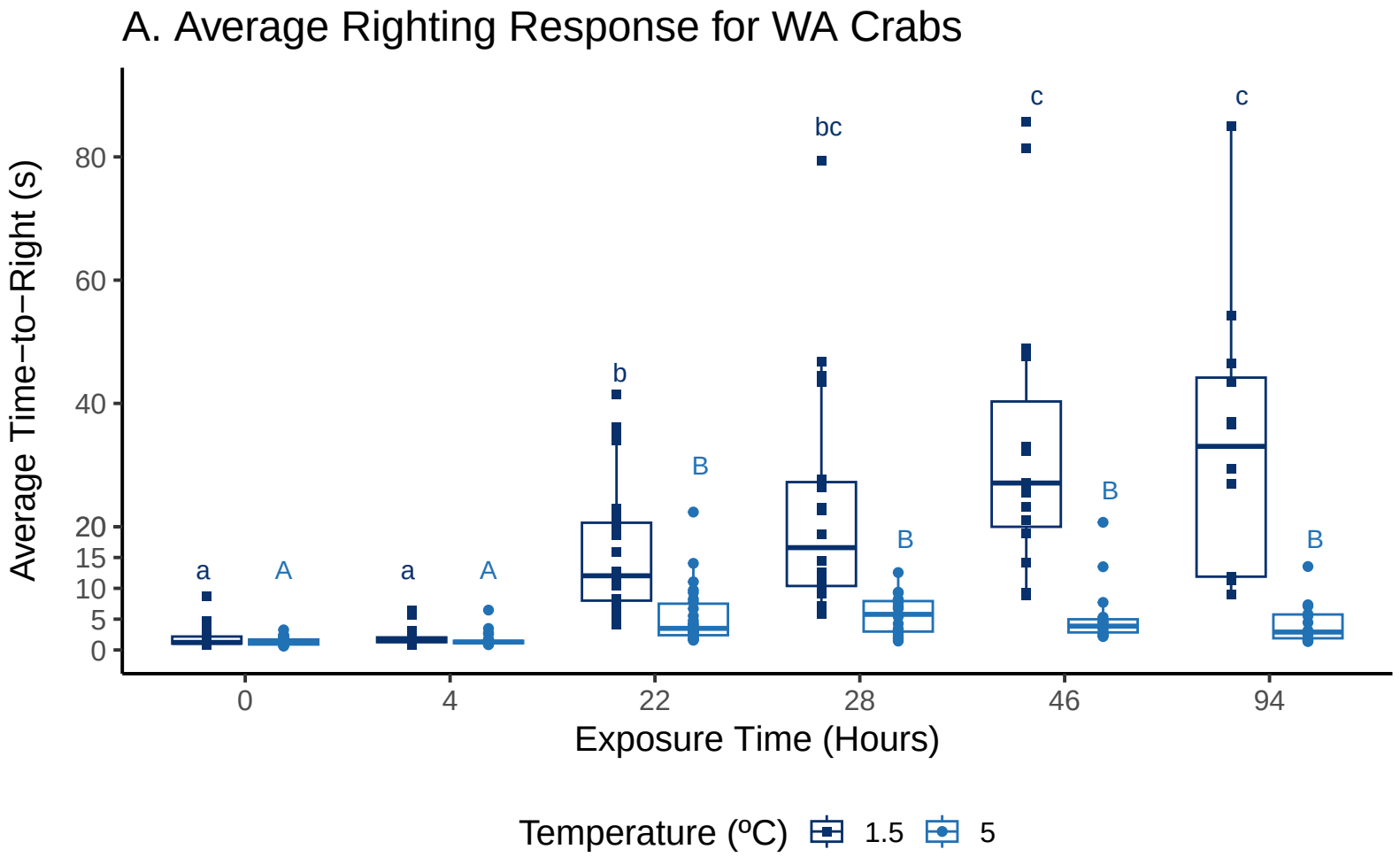

A. Overall Experimental Temperature
Day 0
Day 1
Day 4
Temperature (ºC)
33
32
31
30
25
20
15
Aug 01
Aug 02
Aug 03
Aug 04
B. Temperatures for Second Heat Stress
Temperature (ºC)
32
31
30
29
Aug 03 12:00
Aug 03 18:00
Aug 04 00:00
Aug 04 06:00
Aug 04 12:00

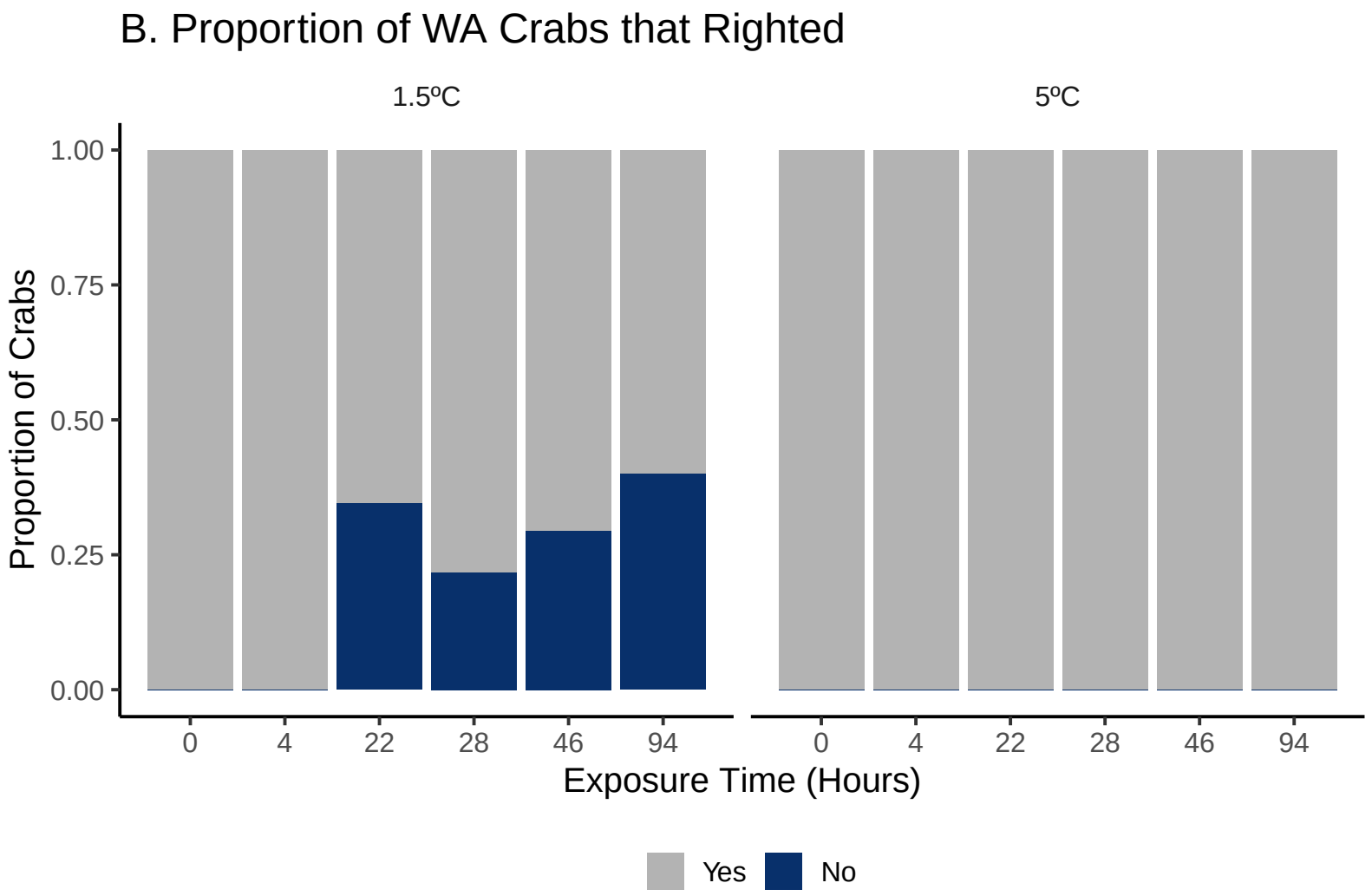

A. Average Righting Response
B. Righting Response by Genotype
Average Time−to−Right (s)
Day
0
1
4
A
a
A
a
B
a
Control, CT or TT
Control, CC
Treatment, CT or TT
Treatment, CC


A. MA Temperature Conditions
Temperature (ºC)
15
10
5
4
3
2
1
Jun 24
Jun 25
Jun 26
Jun 27
Jun 28


B. WA Temperature Conditions
Temperature (ºC)
15
10
5
4
3
2
1
Jul 22
Jul 23
Jul 24
Jul 25
Jul 26


A. Righting Response for MA Crabs
Average Time−to−Right (s)
Exposure Time (Hours)
0
4
22
28
46
94
a
A
b
AB
c
C
c
C
c
C
c
BC
Temperature (ºC)
1.5
5
B. Proportion of MA Crabs that Righted
1.5ºC
5ºC
Proportion of Crabs
1.00
0.75
0.50
0.25
0.00
C. Failure to Right by Genotype
CC
CT or TT
Yes
No


A. Average Righting Response for WA Crabs
Average Time−to−Right (s)
80
60
40
20
15
10
5
0
a
A
a
A
b
B
bc
B
c
B
c
B
0
4
22
28
46
94
Exposure Time (Hours)
Temperature (ºC)
1.5
5


B. Proportion of WA Crabs that Righted
1.5ºC
5ºC
Proportion of Crabs
1.00
0.75
0.50
0.25
0.00
0
4
22
28
46
94
Exposure Time (Hours)
Yes
No