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Research



# Artificial Selection to a Nonlethal Cold Stress in *Trogoderma variabile* Shows Associations With Chronic Cold Stress and Body Size

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## Abstract

Extreme temperature has been used as an alternative to chemical treatments for stored product pests for years. Resistance to heat or cold treatments has not been documented in stored product insects, but repeated use of ineffective treatments could lead to adaptive tolerance. *Trogoderma variabile* (Dermestidae) is a common pest of stored products, and the larval stage is highly resistant to cold and destructive. We artificially selected populations by inducing chill coma at four different cold temperature treatments: 3 and 5 h at –10°C and 3 and 5 h at 0°C. Recovery time was highly heritable after selection for seven generations for decreased recovery time (cold tolerance) and increased recovery time (cold susceptibility) at all time and temperature combinations. Three replicate populations for each time and temperature combination varied substantially, suggesting different mutations in each population were probably responsible for selected phenotypes. Body size decreased in populations selected for cold susceptibility compared with those selected for cold tolerance and survivorship to long-term cold stress increased in the cold-tolerant populations compared with the susceptible populations. After the cessation of the selection experiment, cold tolerance dissipated within four generations from the populations at –10°C, but was maintained in populations exposed to 0°C. Our results suggest that warehouse beetles can adapt to cold stress quickly, but in the absence of cold stress, the proportion of cold-tolerant/susceptible individuals is quickly reduced, suggesting that some of the mutations responsible for these phenotypes may be associated with fitness costs under normal conditions.

**Key words:** warehouse beetle, stored product pest, integrated pest management, chill-coma recovery, artificial selection

Thermal stresses due to extreme heat or cold can create dangerous environments for ectotherms, and they must evolve adaptive behaviors, physiological responses, or both to mitigate thermal stress (Brakefield 2003, Overgaard and Sørensen 2008, Angilletta 2009). Cold stress can affect cell membrane integrity and structure, ion homeostasis, and metabolism, which can ultimately result in cellular damage (Salt 1961, Fields 1992, Hazel 1995, Teets and Denlinger 2013). Response to thermal stress can occur via processes such as plasticity or acclimation on the short-term scale, but persistent exposures to thermal stresses can create selective pressures that lead to long-term adaptations in populations (Lee et al. 1987, Bowler and Terblanche 2008, Kellermann et al. 2009, Nyamukondiwa et al. 2011). Long-term adaptations to mitigate the effects of extreme temperature can come in a variety of forms including better sensing of cues associated with thermal changes and subsequent behavioral avoidance, changes in life-history parameters (Visser and Both 2005, Musolin 2007), shifts in thermal tolerance and plasticity where plasticity is the variation in response to different environments and tolerance is thermal limits for survival or reproduction (Sgro et al. 2016), or other behavioral modifications (May 1979, Casey 1981). Fitness costs can also accompany these adaptations, which can reduce a population's ability to exploit new environments or niches or cope with other changes to environmental conditions (Visser and Both 2005, Robinet and Roques 2010). The reduction in the ability to exploit new niches due to local adaptations can then be exploited for pest management when insect populations are subjected to temperatures outside of their adaptive range (Fields 1992, 2001, Mason and Strait 1998, Fields and White 2002).

Artificial selection experiments can be useful for studying physiological, biochemical, and genetic changes associated with repeated exposures to a stress and the occurrence of other phenotypes that may be correlated with these genetic changes (Hill and Caballero 1992). In a laboratory setting, artificial selection can force populations to undergo either favorable or unfavorable responses to thermal

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stress (Harshman et al. 1999, Brakefield 2003, Anderson et al. 2005, Gerken et al. 2016). This divergent selection regime can aid in mapping causative mutations and can be used to make inferences about the quantitative nature of a given trait or genes and alleles that are associated with certain environmental conditions (Hill and Caballero 1992). In many insect populations, selecting for a reduced tolerance to thermal stress may lead to large amounts of variation among individuals or replicates in the selected phenotype due to the relaxation of selective pressures on detrimental alleles (Frankham 1990, Gerken et al. 2016). In environments with a range of temperatures and extreme thermal events, individuals with alleles that confer greater susceptibility to cold stress will likely not be selected. Alleles associated with greater susceptibility may only persist in the population at low levels, in the absence of other factors such as random population bottlenecks. In contrast, populations that are adapted to thermal stress may have a less dramatic response to selection, since those alleles conferring tolerance are already more prevalent in the population (Frankham 1990). Studies artificially selecting in both directions can be used to understand beneficial alleles that help organisms tolerate or overcome a particular stress, alleles that reduce fitness to a given stress, and the functions of the genes associated with the mutations (Edwards et al. 2006, Kristensen et al. 2006).

Thermal stress can be used in insect pest management, and both cold and heat have been used for the management of insects pests of stored commodities (Dean 1911, Mathlein 1961, Burges and Burrell 1964, Bergh et al. 2006, Wilches et al. 2016). Structures used for processing and storage can be exposed to high temperatures by introducing heated air and raising the temperature to lethal levels to eliminate insect populations infesting the structure (Fields et al. 2012). Grain chillers can also be used to reduce growth rates in insects in stored grain and in climates where outside temperatures are low enough to induce mortality, cold treatments can be applied using outside ambient temperatures (Worden 1987, Fields 1992). Packaged goods can be treated using cold and heat treatments and are often used as alternatives to chemical pesticides or where chemical pesticides cannot be used (Mullen and Arbogast 1984, Fields 1992, Arthur 1996, Fields and White 2002, Fields et al. 2012, Flinn et al. 2015). Cold treatments are more favorable than heat treatments for direct application on commodities because heat can cause damage or favor the growth of mold or bacteria (Evans 1987, Mason and Strait 1998, Linnie 1999). Depending on the insect species and commodity, preventative treatments of –0.6 to 3.3°C for 7–90 d are commonly used for fresh commodities such as fruits and vegetables (Vincent et al. 2003). Freezing infested durable products for 4 d at –18°C is generally sufficient to kill most pests (Mason and Strait 1998, Johnson 2007, Andreadis and Athanassiou 2017) and typically does not cause substantial damage to most high-value, durable products (Fields et al. 2012, Throne et al. 2014).

Previous work on stored product pest insect response to cold has suggested high levels of cold tolerance in some species. Supercooling points for stored product pests range from –10 to –20°C (Evans 1987, Fields et al. 1998, Linnie 1999, Fields et al. 2012). The efficacy of cold treatments can also vary with duration and temperature (Salt 1961, Fields 1992, Strang 1992, Linnie 1999, Angilletta 2009). For example, similar mortality levels were observed in *Trogoderma variabile* Ballion (Coleoptera: Dermestidae) when insects were exposed to lower temperatures for shorter durations or to less extreme temperatures for longer durations (Abdelghany et al. 2015). Life stage also significantly influences the efficacy of cold treatments with eggs being the most susceptible and late-instar larvae being least susceptible in some species (Fields 1992, Mason and Strait 1998, Wang et al. 2004) but eggs being the most tolerant life stage in others (Loganathan et al. 2011, Arthur et al. 2015, Arthur et al. 2017). Response to cold is also affected by other factors such as diet (Mohammadzadeh and Izadi 2018), temperature during development, starvation, and short-term acclimation. For example, starvation has been previously shown to decrease cold tolerance while prior exposure to a nonlethal temperature (acclimation) before extreme cold improves cold tolerance in *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) (Scharf et al. 2016) and *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae) (Qiang et al. 2008). *Trogoderma granarium* Everts (Coleoptera: Dermestidae) reared on triticale had better survival at –10°C for 24 h than when raised on sorghum or rye (Mohammadzadeh and Izadi 2018). Cooler rearing temperatures were associated with increased recovery time from cold stress in *T. castaneum* (Scharf et al. 2015). Body size is also an important component of cold tolerance (Angilletta 2009) with larger species of *Tribolium* recovering quicker from cold stress than smaller species (Scharf et al. 2014). Despite the fact that numerous environmental factors have been associated with cold tolerance in stored product pests, the effect of long-term selection has not been quantified (Wilches et al. 2016) and genetic mutations associated with cold tolerance have yet to be identified in any stored product pest species.

*Trogoderma variabile* (Dermestidae; warehouse beetle) is a destructive stored product pest species found worldwide with a high tolerance to cold (Abdelghany et al. 2015, Wilches et al. 2016). Its close relative, *T. granarium*, the khapra beetle, is considered one of the most important quarantine pest species in the United States and several other countries (Abdelghany et al. 2015), but few studies have focused on understanding cold tolerance in *T. variabile* or on selection to cold in Dermestids. Stored product dermestids can undergo diapause in response to environmental pressures such as photoperiod, seasonal changes in temperature, food and resource availability, isolation, or disturbance (Loschiavo 1960, Burges 1961, Wright and Cartledge 1994, Abdelghany et al. 2015) or as a density-dependent phenomenon (Burges 1960, 1962a,b; Beck 1971; Nair and Desai 1973a,b) and this may affect their cold tolerance. For *T. granarium* larvae tested at –10°C, time to high mortality differed among studies ranging from >95% mortality after 30 d (Lindgren and Vincent 1959, Mathlein 1961) to 50% after 25 h (Strang 1992), with differences likely due to acclimation or diapause (Wilches et al. 2016). The larval stage also has the highest cold tolerance for most *Trogoderma* species (Mansbridge 1936, Solomon and Adamson 1955, Abdelghany et al. 2015), but quantitative mortality levels vary among species (Mathlein 1961, Reguzzi et al. 2011).

To understand the ability of *T. variabile* late-instar larvae to acclimate to cold stress, we performed artificial selection experiments by inducing chill coma by exposure to different temperature and exposure time combinations. Chill coma is the cessation of neuromuscular response in the insect that is reversible on warming and could be caused by a variety of metabolic mechanisms such as oxygen limitation or ion balance (MacMillan and Sinclair 2011). Depending on the duration and degree of cold stress, insects recovering from chill coma may or may not return to pre-stress fitness levels. We selected for individuals that quickly recovered (cold hardy) and individuals that did not recover quickly (cold susceptible) from chill coma after exposure to temperatures of 0 and –10°C for 3 or 5 h (Hazell and Bale 2011, Andersen et al. 2015, Gerken et al. 2016). We then tested the selected populations to determine whether tolerance for short exposure times conferred cross-tolerance to cold exposure for longer time periods (chronic cold stress; Bubliy and Loeschcke 2005, MacMillan et al. 2009). In later stages of selection, changes in body size were observed in some lines, so body size was also measured for each selected population as another metric of selection effects.

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## Materials and Methods

### Beetles

*Trogoderma variabile* used for selection experiments were from a laboratory population that has been maintained for ~30 yr. Beetles were reared in quart-sized (0.95 L) glass jars with approximately 400 ml of ground dog food topped with 50 ml of old-fashioned oats. Pieces of crumpled paper towel (2-Ply Perforated, Georgia Pacific, Atlanta, GA) placed on top of the diet were provided for the insects to climb and lay eggs on. Approximately 100 mixed sex individuals were subcultured for routine colony maintenance every 1–2 wk. The original population as well as subsequent selected populations were maintained at 30°C, 65% relative humidity, and a 16:8 (L:D) h cycle. All insects used for the selection experiments were randomly chosen from the initial population as late-instar larvae.

### Baseline Cold Tolerance Assays

To establish appropriate temperature and time for testing and selecting *T. variabile*, chill-coma recovery time assays were performed at 0, −5, and −10°C for 3, 5, and 8 h. Late-instar larvae chosen from the initial population were placed individually in one-ounce plastic cups, exposed to one of the nine temperature × time cold treatments listed above which immobilized the insects, and the time to become mobile (chill-coma recovery) following exposure was recorded in 30-s intervals. Recovery time was capped at 60 min for these assays. No larvae were saved from these trials, and each larva was only tested once. Three blocks of 30 insects each ($n$ = 90) were analyzed for each time and temperature combination.

### Selection Experiments

Four separate pools of 48 late-instar larvae were exposed to temperatures of −10 and 0°C for either 3 or 5 h (Fig. 1). These initial 48 larvae were the founding parental generation for each time and temperature combination. The temperature combinations were chosen as they represent a mild temperature that may be common in refrigeration storage and a more extreme temperature that approaches the temperature used in disinfestation methods, but is not lethal. Time was chosen based on the differences observed between 3 and 5 h in the baseline experiment (described above). The combination of selection direction, temperature, and time is further referred to as selection experiment.

Larvae were placed in individual wells of a plastic 24-well plate (Nunc Multidish, ThermoFisher Scientific, Waltham, MA) and were exposed to cold temperatures in a TH-030 Freeze/Thaw Chamber (Darwin Chambers, Saint Louis, MO) at the given test temperature and duration. Recovery time was recorded as above but capped at 120 min instead of 60 min. Recovery was defined for each individual as when it was moving any part of its body. The fifteen larvae that recovered from cold exposure in the shortest duration of time were selected as 'Quick' individuals and the last 15 larvae that recovered were selected as 'Slow' individuals. Quick selected individuals are hypothesized to contribute progeny to the population that will become more genetically tolerant to cold stress over time and slow selected individuals will contribute progeny that are hypothesized to become more genetically susceptible to cold. These two groups were separated and reared in 0.24-liter jars with approximately 200-ml dog food and 10 ml of old-fashioned oats applied to the surface. This selection process was performed with three unique parental generations for each selection experiment for a total of 24 individual selection populations, further referred to as lines.

For each selected population, the date of adult emergence (typically represented by the initial appearance of one to three adults) was recorded. The adults remained in the jars for 2 wk after this emergence date to provide ample time for mating and egg laying (Gerken and Campbell 2018). After this time point, the adults were collected from the diet and the sex ratios of each population were recorded. When lines had sufficient numbers of late-instar larvae, they were tested again for recovery time. Each selection line was tested

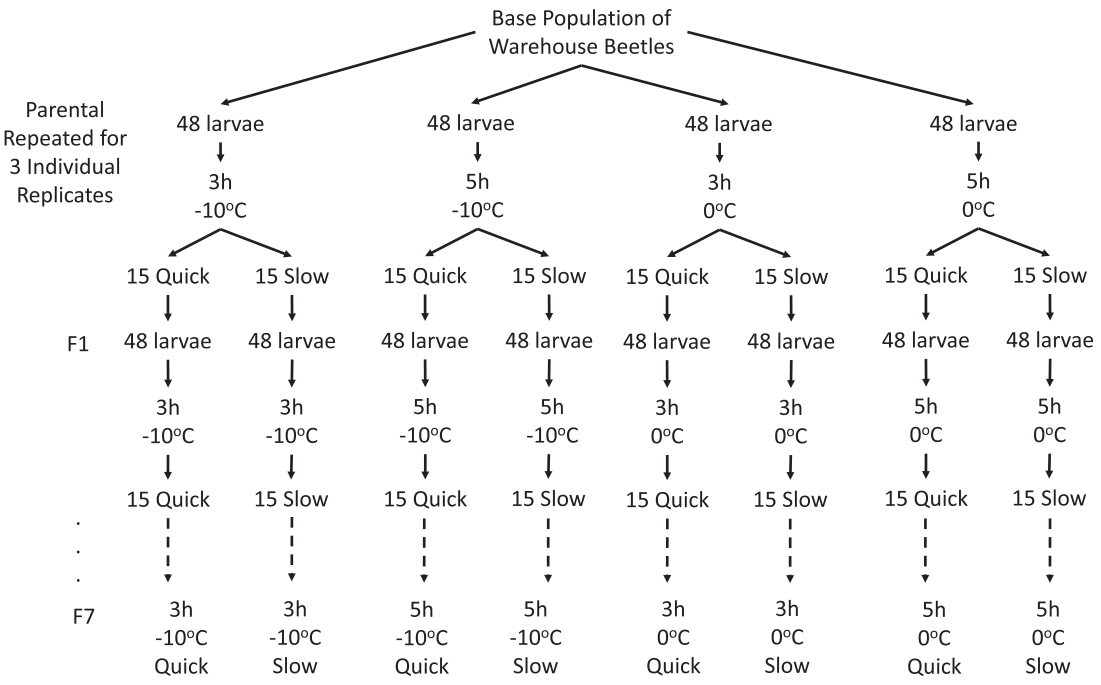


**Fig. 1.** Schematic of selection protocol. The original laboratory population of *T. variabile* (warehouse beetles) was maintained in laboratory culture for over 30 yr. Replicates were started on separate days over a 2-wk period. At each generation, 48 larvae were assayed for chill-coma recovery time and 15 larvae were used to start the next generation until seven generations of selection had been completed.

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only at the original time and temperature combination at which the selection was performed and the 15 individuals that responded the strongest in the given direction were selected for advancement. This process was continued for a total of seven generations of selection in addition to the initial selection on the parental generation or, if the population did not survive the full selection experiment, until the population collapsed. After selection ceased, populations were left to mate randomly for an additional 4 generations to test for retention of the cold selected phenotype.

### Body Size and Chronic Cold Tolerance Response in Selection Lines

We used survivorship to a chronic cold stress to understand the effect of selection on a different thermal tolerance trait or cross-tolerance. Chronic cold tolerance was assayed by placing late-instar larvae directly from their population jars into individual wells of a 24-well plate. The plate was then placed into the Watson freeze–thaw incubator at –10°C for 48 h. This assay was performed within five to six generations after the seven generations of selection with two replicates for each line and two for the original laboratory population. Survivorship was assayed 24 h after removal from –10°C by visual inspection of movement of the larvae after a light prodding.

Body length and width were measured for each line using a Nikon Stereomicroscope (SMZ18) with camera (DS Ri2) attachment. Measurements were performed in the NIS-Elements program (Melville, NY) and were taken from the tip of the head to the base of the abdomen (length) and across the thorax where the elytra connect to the thorax (width) for 20 males and 20 females from each line and from the general laboratory population. Measurements were taken four generations after the completion of the seven generations of selection.

### Analyses

Chill-coma recovery time for the base population was analyzed using SAS (version 9.4). A *proc glm* was run using chill-coma recovery time as the dependent variable and temperature (0, –5, and –10°C), exposure time (3, 5, and 8 h), and the interaction of time and temperature as main effects. If interaction or main effects were significant, a Tukey adjustment was used to calculate the LS-means.

A *proc glimmix* was run for each temperature of selection from generation 1 to generation 7. Chill-coma recovery time was the dependent variable and generation (1–7), exposure time (3 and 5 h), and selection direction (quick or slow) were used as main effects in the model, also testing for interaction effects among all terms. Parental data were not included in this model. The three biological replicates for each selection experiment were treated as random effects in the model and LS-means were calculated for significant effects with a Tukey adjustment. An additional post hoc *proc glimmix* model was run for each treatment combination (time × temperature) to explicitly test for differences between quick and slow selected populations each generation after selection with generation (parent, generations 1–7, and after four generations of no selection) and selection group (quick or slow) as main effects. LS-means were calculated, and Tukey adjustments were performed to test for the interaction of generation and selection group at each temperature. We also performed pairwise comparisons to test for differences in recovery time between the parental group (no selection) and each line after seven generations of selection and parental group to generation 7 plus four generations of random mating; differences between recovery time four generations after the selection experiments ceased was used to determine whether the differences in selection were retained in the population in the absence of selection. We also compared generation 1 to generation 7 with selection and generation 7 plus four generations of random mating with pairwise comparisons and all comparisons were Tukey adjusted.

Realized heritability estimates for narrow sense heritability ($h^2$; Falconer 1960) were calculated for each selection experiment using the breeder's equation with divergent selection design and a cumulative selection differential with each generation contributing to a pooled variance estimate (Hill 1972). Realized heritability uses an estimation of divergence between selection lines (quick vs slow) and the effects of selection accrued over time and narrow sense heritability is the proportion of the variation in the trait that is due to additive genetic effects by measuring phenotypic similarity of parents to offspring (Freeman and Herron 1998).

Survival after exposure to a chronic cold stress was analyzed separately for each selection temperature (–10 and 0°C) using *proc logitistic* with main effects of exposure time (3 or 5 h) and speed of recovery (quick or slow) and their interaction, with speed of recovery as the odds-ratio factor and survivorship as the dependent variable. Pairwise comparisons were calculated to assess differences in survival between the quick and slow lines at each exposure time as well as to beetles from the laboratory colony that had not been subjected to selection. A Tukey correction was used to correct for multiple comparisons.

Body size was analyzed using *proc glimmix* with independent models for body length or body width as the dependent variables and temperature (0 or –10°C), exposure time (3 and 5 h), and selection direction (quick or slow) as main effects in the model, also testing for interaction effects among all terms. Biological replicates for each selection experiment were considered as random effects, and LS-means were Tukey adjusted for significant effects.

## Results

### Baseline Cold Tolerance Assays

In the initial laboratory population, recovery time increased as temperature decreased from 0 to –10°C, but recovery time did not have a predictable trend at each temperature (Fig. 2). Results are reported with the subscript of *F*-values as the degrees of freedom for the predictor variable and based on the levels within that particular variable. Temperature ($F_2$ = 220.67, $P$ < 0.0001) and exposure time ($F_2$ = 2.99, $P$ = 0.051) were significant factors that influenced recovery time, and the interaction between temperature and exposure time was not significant ($F_4$ = 1.83, $P$ = 0.12). Recovery time for beetles exposed to each temperature differed significantly from one another ($P$ < 0.05) with beetles tested at –5°C having the longest average recovery time (41.3 ± 0.6 min; mean ± SE) and beetles tested at 0°C having the shortest average recovery time (19.1 ± 0.6 min). Interestingly, the recovery time of individuals exposed to –10°C was 37.9 ± 1.1 min, which fell between the recovery times of the beetles exposed to 0 and –5°C. Comparing cold exposure time as a factor in chill-coma recovery time, recovery at 3 h (31.6 ± 0.9 min) was significantly shorter than recovery at 5 h (33.8 ± 1.1 min; $P$ = 0.041), but did not differ from recovery at 8 h (32.9 ± 0.9 min; $P$ = 0.63). Additionally, recovery at 5 h did not significantly differ from recovery at 8 h ($P$ = 0.25).

### Selection Experiments

Because recovery times varied by temperature in the initial population, statistical analysis of the selected populations was performed separately for each temperature treatment (Fig. 3). At 0°C, the

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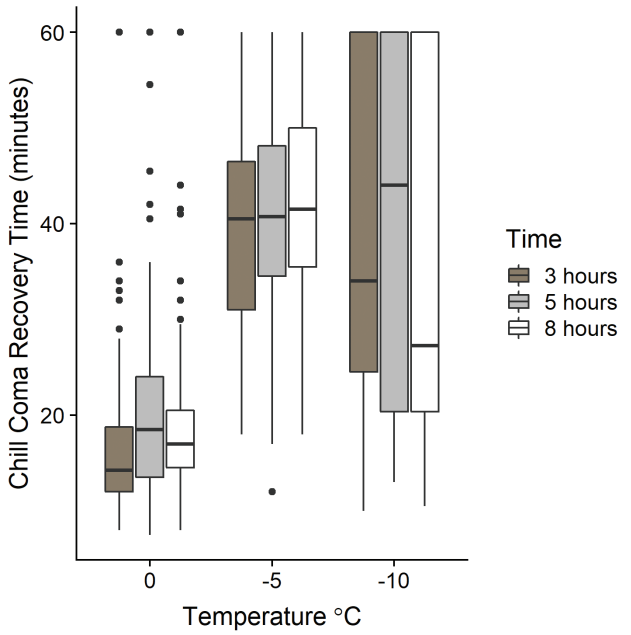


**Fig. 2.** Chill-coma recovery time for initial examination of population responses to cold stress. Each time and temperature combination was tested for three replicates with a total of 30 beetles in each replicate. Edges of the boxes represent the 25 and 75% distribution of the data (interquartile range or IQR) and ±1.58 × (IQR)/√(*n*) with outliers also represented by dots.

interaction of exposure time, selection direction, and generation was significant in our model ($F_6$ = 2.17, $P$ = 0.043) as were all other factors and interactions ($P$ < 0.003). When comparing the interaction LS-means, recovery times for populations selected for quick selected populations tended to cluster more closely together than slower recovery times and for slow populations had longer recovery times than the quick selected populations (Table 1). Similarly, for beetles selected at −10°C, the full interaction of exposure time, selection direction, and generation was significant ($F_6$ = 2.29, $P$ = 0.033) with all other factors and interactions significant ($P$ < 0.01) except for the interaction of exposure time and selection group ($F_1$ = 0.00, $P$ = 0.96). Populations selected for quick recovery times clustered closely together and had faster recovery times than slower populations, especially for later generations (Table 2) and replicates of slower recovery populations tended to be more variable. All replicate lines for quick selection survived while two of the replicates selected for slow recovery time did not make it the full seven generations of selection; replicate 1 selected at 0°C for 5 h did not survive past generation 6 and replicate 3 at −10°C for 5 h did not survive past generation 5 (Fig. 3).

For each temperature and exposure time combination, the recovery times were compared between the populations selected for slow or quick recovery times at each generation to determine when the effects of selection were occurring. Divergent selection heritability was 0.32 (pooled variation = 40.04) for populations selected at 0°C for 3 h. The interaction of generation and selection direction was significantly different ($F_7$ = 35.51, $P$ < 0.0001) after selecting for 3 h at 0°C and recovery times between populations selected for slow and quick recovery times were significantly different (Supp Table 1 [online only]) after selection for three generations ($F_1$ = 3.89, $P$ = 0.049), six generations ($F_1$ = 63.76, $P$ < 0.0001), seven generations ($F_1$ = 332.24, $P$ < 0.0001), and four generations after cessation of selection experiments ($F_1$ = 129.49, $P$ < 0.0001). Heritability for populations selected at −10°C for 3 h was 0.17 (pooled variation = 449.25) and the interaction effect of generation and selection direction was also significant ($F_7$ = 5.25, $P$ < 0.0001). Recovery times of populations selected for quick or slow recovery were significantly different at generation 4 ($F_1$ = 10.35, $P$ = 0.0013), generation 7 ($F_1$ = 56.39, $P$ < 0.0001), and four generations after cessation of selection experiments ($F_1$ = 6.01, $P$ = 0.014).

For populations selected at 0°C for 5 h, heritability was 0.47 (pooled variation = 60.17), the interaction of generation and selection direction was significant ($F_7$=19.11, $P$ < 0.0001), and there were significant differences between populations selected for slow and quick recovery times in all generations (Supp Table 1 [online only]; $P$ < 0.006) except for generation 1 ($F_1$ = 0.41, $P$ = 0.52). For populations selected at −10°C for 5 h, heritability was 0.20 (pooled variation = 362.18), the interaction of generation and selection direction was also significant ($F_7$=5.49, $P$ < 0.0001), and populations selected for slow and quick recovery differed at generation 1 ($F_1$ = 18.98, $P$ < 0.0001), generation 4 ($F_1$ = 4.17, $P$ = 0.041), generation 5 ($F_1$ = 32.54, $P$ < 0.0001), generation 7 ($F_1$ = 34.96, $P$ < 0.0001), and four generations after the selection experiments were terminated ($F_1$ = 24.50, $P$ < 0.0001).

We also compared recovery times relative to the initial parental population after seven generations of selection and four generations after cessation of selection (Fig. 4). After seven generations of selection, recovery time for all the selected populations was different from the parental populations, except for those selected for quick recovery at −10°C for 3 h and −10°C for 5 h (Supp Table 2 [online only]). Only three populations maintained a significant difference from parental populations at four generations after the selections were terminated: quick recovery at 0°C for 3 h (14.3 ± 0.2 min compared with 19.1 ± 1.0 min for the parental population; mean ± SE, $t$ = −6.00, $P$ < 0.0001); quick recovery at 0°C for 5 h (18.3 ± 0.2 min compared with 22.4 ± 5.5 min; $t$ = −5.13, $P$ < 0.0001); and slower recovery for 5 h at −10°C (45.8 ± 1.7 min compared with 30.3 ± 1.3 min; $t$ = 5.96, $P$ < 0.0001).

In addition, we compared recovery times for the first generation of selection compared with recovery times after seven generations of selection and four generations after cessation of selection. Compared with the first generation of selection (Fig. 4), populations selected for slow recovery at 0°C for 3 h ($t$ = −12.09, $P$ < 0.0001), quick recovery at −10°C for 3 h ($t$ = 4.21, $P$ = 0.0031), and slow recovery at 0°C for 5 h ($t$ = −9.73, $P$ < 0.0001) were significantly different after seven generations of selection. Only populations selected for slow recovery at 0°C for 3 h ($t$ = −6.03, $P$ < 0.0001) and quick recovery at 0°C for 3 h ($t$ = 4.48, $P$ = 0.001) maintained this difference relative to the parental line four generations after cessation.

## Body Size and Chronic Cold Tolerance Response in Selection Lines

For evaluation of cross-tolerance of selection lines to a longer cold stress, we exposed all selection lines to −10°C for 48 h (Fig. 5). There was a significant effect of selection direction (quick vs slow) when populations were selected at 0°C (Wald $\chi^2$ = 9.49, $P$ = 0.0021) with quick selected lines having greater survivorship compared with slow selection lines. The main effect of selection exposure time and the interaction of selection exposure time and direction were not significant for lines selected at 0°C (Wald $\chi^2$ = 3.37, $P$ = 0.066; Wald $\chi^2$ = 2.82, $P$ = 0.093). At −10°C, selection direction (Wald $\chi^2$ = 0.76, $P$ = 0.38) and selection exposure time (3 vs 5 h; Wald $\chi^2$ = 0.51, $P$ = 0.47) were not significantly different but the interaction of exposure time and selection group was significantly different (Wald $\chi^2$ = 7.53, $P$ = 0.0061).

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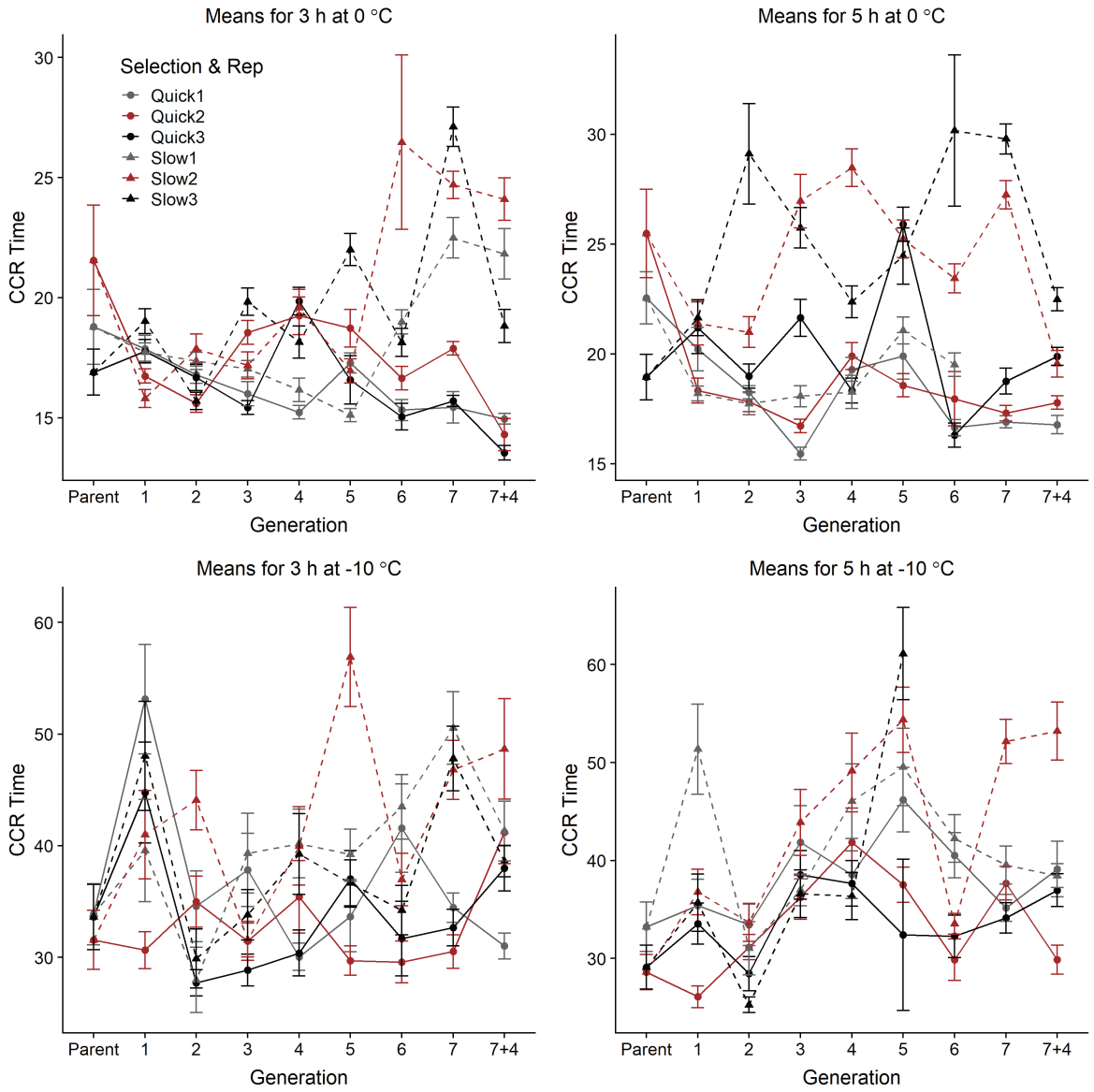


**Fig. 3.** Line graph of mean chill-coma recovery time of selected population replicates for seven generations. Chill-coma recovery times of parent populations selected for quick (decreased recovery time) and slow (increased recovery time) populations and populations after seven generations of selection and four additional generations without (7 + 4) were recorded under four selection regimes. Quick selected populations are represented by solid lines with circle symbols, and slow selected populations are represented by dashed lines with triangle symbols. Error bars represent SE.

Assessing specific pairwise comparisons showed that populations selected for quick recovery at 0°C for 3 h had significantly higher survival to longer-term exposure to cold temperatures than the populations selected for slow recovery at 0°C for 3 h (Table 3; $z$-value= −3.52, $P$ = 0.0039). However, when compared to the original laboratory population of unselected beetles, survivorship of the selected lines was not different.

No differences in survival to the long-term cold exposure were observed in beetles selected at −10°C in terms of selection direction (quick vs slow) or in comparison with control beetles (Table 3) with Tukey correction, although survivorship for lines selected for slow recovery at 5 h at −10°C did show an upward trend in survival in comparison to the quick lines (Fig. 5). Interestingly, beetles that underwent selection, particularly for quick recovery at −10°C, had less variation in survivorship compared with base population (Fig. 5).

When comparing body size changes among selected populations, populations selected for quick recovery tended to have larger body sizes compared to slow selected populations and lines selected at 3 h tended to be larger than populations selected at 5 h (Fig. 6; Supp Fig. 1 [online only]). Specifically, for females, populations selected for quick recovery at −10°C had significantly larger body lengths than populations selected for slow recovery at this temperature in both the 3- and 5-h exposures but only differed in body width when 3-h exposures were used for selection (Fig. 6; Supp Tables 3 and 4 [online only]). For males, lines selected for quick recovery at 0°C for 5 h and at −10°C for both 3 and 5 h had larger body lengths than populatiohns selected for slow recovery times under the same selection conditions. Body width was greater in populations selected for quick recovery compared with slow recovery only at −10°C for 5 h. For beetles selected at 0°C, both females and males selected for slow recovery had longer body lengths in the 3-h exposure compared with

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**Table 1.** Effect of selection at 0°C at each generation

| Time | Selection direction | Generation | LS-mean | Tukey groups |
|---|---|---|---|---|
| 3 h | Quick | 1 | 17.46 | H I J |
| | | 2 | 16.35 | J |
| | | 3 | 16.66 | I J |
| | | 4 | 18.10 | G H I J |
| | | 5 | 17.53 | H I J |
| | | 6 | 15.67 | J |
| | | 7 | 16.19 | J |
| | Slow | 1 | 17.52 | H I J |
| | | 2 | 16.98 | I J |
| | | 3 | 18.02 | G H I J |
| | | 4 | 17.97 | G H I J |
| | | 5 | 18.02 | G H I J |
| | | 6 | 21.20 | C D E F |
| | | 7 | 24.77 | B |
| 5 h | Quick | 1 | 19.91 | E F G H |
| | | 2 | 18.35 | G H I J |
| | | 3 | 17.94 | G H I J |
| | | 4 | 19.18 | F G H I |
| | | 5 | 21.46 | C D E F |
| | | 6 | 17.10 | I J |
| | | 7 | 17.82 | H I J |
| | Slow | 1 | 20.41 | D E F G |
| | | 2 | 22.61 | B C D E |
| | | 3 | 23.59 | B C |
| | | 4 | 23.01 | B C D |
| | | 5 | 23.59 | B C |
| | | 6 | 24.37 | B |
| | | 7 | 27.74 | A |

LS-mean is in minutes. Tukey adjusted groups are significant at $P < 0.05$.

**Table 2.** Effect of selection at −10°C at each generation

| Time | Selection direction | Generation | LS-mean | Tukey groups |
|---|---|---|---|---|
| 3 h | Quick | 1 | 42.84 | B C D |
| | | 2 | 32.43 | F |
| | | 3 | 32.75 | E F |
| | | 4 | 31.96 | F |
| | | 5 | 33.47 | E F |
| | | 6 | 34.28 | D E F |
| | | 7 | 32.93 | E F |
| | Slow | 1 | 42.86 | B C D |
| | | 2 | 33.92 | E F |
| | | 3 | 34.90 | D E F |
| | | 4 | 39.81 | C D E F |
| | | 5 | 44.26 | B C |
| | | 6 | 38.23 | C D E F |
| | | 7 | 48.43 | A B |
| 5 h | Quick | 1 | 31.68 | F |
| | | 2 | 31.00 | F |
| | | 3 | 38.88 | C D E F |
| | | 4 | 39.35 | C D E F |
| | | 5 | 40.81 | B C D E F |
| | | 6 | 34.22 | D E F |
| | | 7 | 35.81 | D E F |
| | Slow | 1 | 41.31 | B C D E |
| | | 2 | 30.68 | F |
| | | 3 | 39.16 | C D E F |
| | | 4 | 43.86 | B C |
| | | 5 | 55.05 | A |
| | | 6 | 37.19 | C D E |
| | | 7 | 46.67 | A B C |

LS-mean is in minutes. Tukey adjusted groups are significant at $P < 0.05$.

the 5-h exposure. Similarly, females from populations selected for quick recovery at both 0 and −10°C for 3 h had larger body lengths than those selected at the same temperature for 5 h. Males from populations selected for slow recovery at −10°C for 3 h had larger body lengths than those selected for 5 h.

For body width, males and females selected at a 5-h time point tended to have smaller body widths than those selected for 3 h. Females and males of populations selected for 3 h at 0°C for both quick and slow recovery also had significantly larger body widths than those selected for 5 h at 0°C. Females from populations selected for slow recovery at 0°C for 3 h also had higher body widths than those selected for 5 h at 0°C. For populations selected at −10°C, only males selected for slow recovery at 3 h had significantly larger body widths than those selected at 5 h. Compared with unselected populations one trend is that female body width was smaller in female beetles from some of the selected populations compared to laboratory population of unselected females. Females selected for quick or slow recovery at 0°C for 5 h or selected at −10°C for 5 h had smaller body widths than unselected beetles. Unselected beetles had larger body length than males only when selected for slow recovery at −10°C for 5 h (Fig. 6; Supp Tables 3 and 4 [online only]).

## Discussion

Stored product insect pests are often sheltered from extreme thermal stress because they spend significant time in human-made, thermally controlled environments. By using artificial selection, we can assess if these insects can maintain the ability to adapt to extreme cold stress and we will enhance our understanding of their ability for further range expansion and potential to develop resistance to any cold stress management treatments. Under artificial selection, *T. variabile* can undergo selection for cold tolerance at all time and temperature combinations assayed. Our initial screening of the population showed substantial variation in recovery time and the influence of exposure time and temperature on recovery from cold stress (MacMillan and Sinclair 2011). In our selection experiment, we found that the most consistent response to selection was for a quick recovery time after exposure to 0°C for 3 h, the least extreme of the time-temperature combinations tested. Under these conditions, populations selected for quick recovery also showed lower variability in recovery times among replicates in comparison to the other selection experiments; this may reflect the inherent underlying allelic capacity for cold tolerance in this laboratory population or that this population is primed to recovery more quickly after cold exposure. In contrast, when selection was for a slower recovery, there is not a constitutive path to adaptation or there may be more polygenic variation in selection for cold susceptibility. The differences in variation of recovery time between the quick (low variation) and slow (higher variation) is typical of asymmetric selection, especially in laboratory reared populations (Frankham 1990, Brakefield 2003, Edwards et al. 2006, Gerken et al. 2016). Laboratory conditions can often select for reduced ranges in thermal tolerance, but we see the capacity for decreased chill-coma recovery time maintained in this population. This suggests that higher thermal tolerance may not be costly to maintain, unlike some other thermal tolerance traits including plasticity which requires insects to maintain positive responses to a broader range of temperatures (Kleynhans et al. 2014), or there are other factors such as directional dominance or cold

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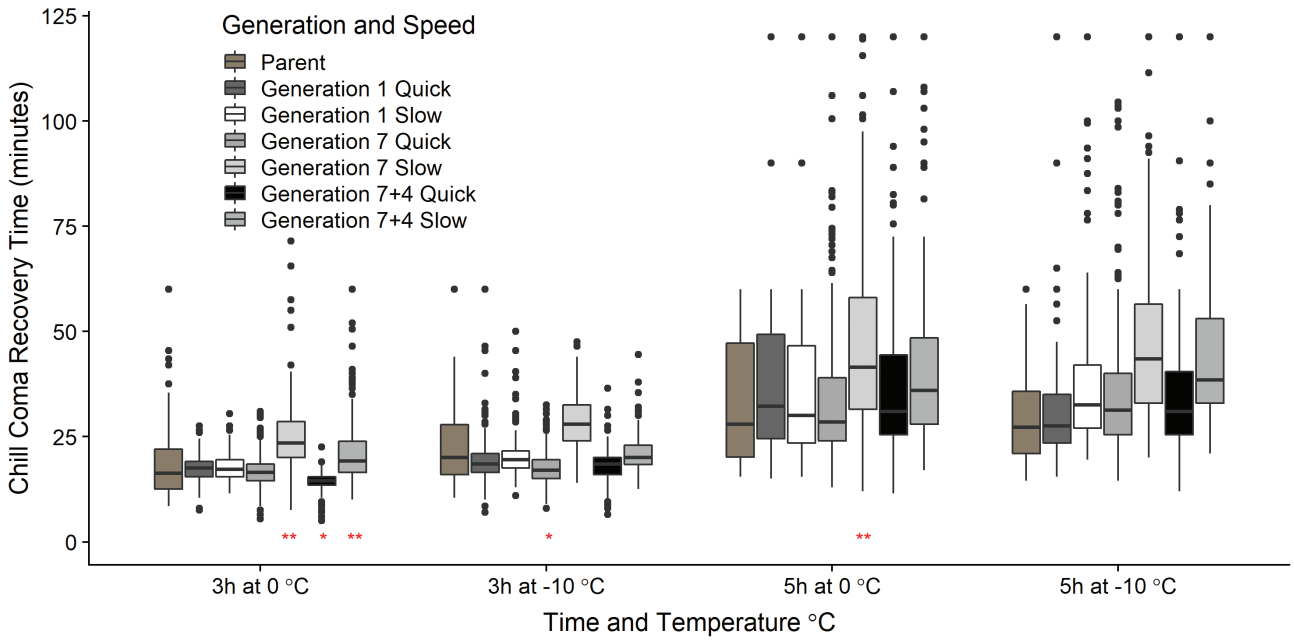


**Fig. 4.** Box plot of chill-coma recovery times of beetles post-selection compared with parental populations. Chill-coma recovery times were recorded for the parental groups, after one generation of selection, after seven generations of selection, and four generations after selection were stopped. Tukey adjusted *P*-values of <0.01 and <0.0001 are represented by * and **, respectively. Edges of the boxes represent the 25 and 75% distribution of the data (interquartile range or IQR) and ±1.58 × (IQR)/√(*n*) with outliers also represented by dots.

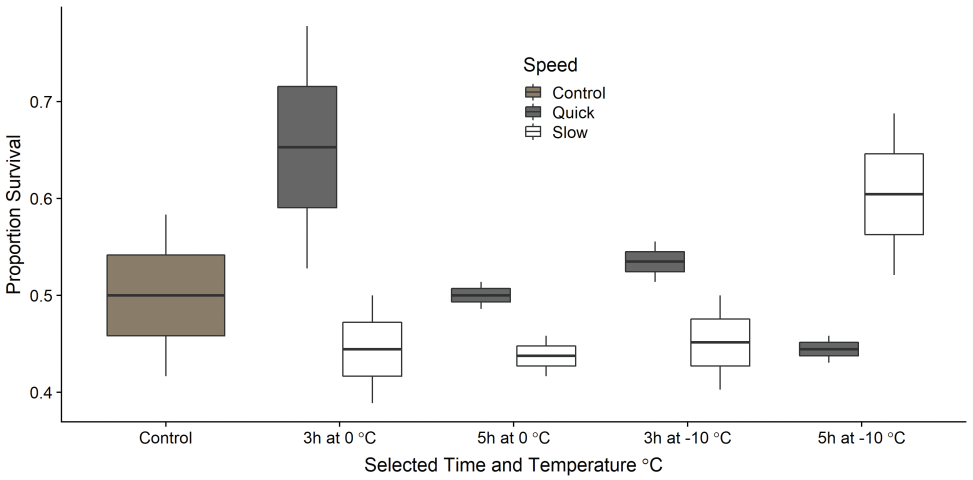


**Fig. 5.** Survival after exposure at −10°C for 48 h. Edges of the boxes represent the 25 and 75% distribution of the data (interquartile range or IQR) and whiskers are ±1.58 × (IQR)/√(*n*).

susceptibility being a recessive trait found in low-enough frequencies to show asymmetrical responses (Hill and Caballero 1992).

*Trogoderma variabile* have high heredity in their response to selection on cold tolerance, suggesting that they may be primed to respond to different thermal conditions in an adaptive manner. In comparison, heritability in *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) selected for quick and slow chill-coma recovery time ranged from 0.07 to 0.11 (Gerken et al. 2016), which is much lower than we observed for *T. variabile* in this study. This strong heritability is an important factor to consider when using cold treatments for disinfestation; not only does *T. variabile* have a high tolerance to cold stress (Abdelghany et al. 2015, Wilches et al. 2016) that is maintained even in the laboratory, but insects that survive cold treatments can quickly breed resistance into populations in as few as 1–3 generations. However, heritability did decline in the more extreme temperature treatments suggesting that *T. variabile* is may be reaching its physiological limits to tolerating cold temperatures.

Not only do *T. variabile* show a strong response to artificial selection for recovery from chill coma, but some evidence for cross-tolerance to a more chronic cold stress was also observed, especially in the population that was selected at 0°C for 3 h for seven generations. In this study, populations selected for quicker recovery to chill coma for 3 h at 0°C also showed increased survival to −10°C for 48 h, compared with those selected for slow recovery to chill coma; however, survival did not differ from control populations that were unselected for chill coma recovery time, but we did show a

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**Table 3.** Logistic differences in survival to chronic cold stress

| Comparison | Time–Temperature | Estimate | SE | *z*-value | Unadjusted *P*-value | Adjusted *P*-value |
|---|---|---|---|---|---|---|
| Quick versus slow | 3 h at 0°C | −0.85 | 0.24 | −3.52 | 0.0004 | 0.0039 |
| | 3 h at −10°C | −0.33 | 0.24 | −1.41 | 0.16 | 0.62 |
| | 5 h at 0°C | −0.25 | 0.26 | −0.95 | 0.34 | 0.88 |
| | 5 h at −10°C | 0.65 | 0.27 | 2.41 | 0.016 | 0.11 |
| | 3 h quick vs 5 h slow at 0°C | −0.88 | 0.27 | −3.27 | 0.0011 | 0.0096 |
| | 3 h slow vs 5 h quick at 0°C | 0.22 | 0.24 | 0.94 | 0.35 | 0.88 |
| | 3 h quick vs 5 h slow at −10°C | 0.28 | 0.27 | 1.06 | 0.29 | 0.83 |
| | 3 h slow vs 5 h quick at −10°C | −0.028 | 0.24 | −0.12 | 0.91 | 1.00 |
| Quick vs quick | 3 h vs 5 h at 0°C | −0.63 | 0.24 | −2.61 | 0.009 | 0.068 |
| | 3 h vs 5 h | −0.36 | 0.24 | −1.53 | 0.13 | 0.54 |
| | 3 h vs 5 h at −10°C | | | | | |
| Slow vs slow | 3 h vs 5 h at 0°C | −0.028 | 0.27 | −0.11 | 0.92 | 1.00 |
| | 3 h vs 5 h at −10°C | 0.62 | 0.27 | 2.31 | 0.021 | 0.14 |
| Quick vs control | 3 h at 0°C | −0.63 | 0.34 | −1.87 | 0.062 | 0.33 |
| | 3 h at −10°C | −0.14 | 0.33 | −0.42 | 0.68 | 0.99 |
| | 5 h at 0°C | 0.00 | 0.33 | 0 | 1.00 | 1.00 |
| | 5 h at −10°C | −0.22 | 0.33 | −0.67 | 0.50 | 0.96 |
| Slow vs control | 3 h at 0°C | 0.22 | 0.33 | 0.67 | 0.50 | 0.96 |
| | 3 h at −10°C | 0.19 | 0.36 | 0.58 | 0.24 | 0.76 |
| | 5 h at 0°C | −0.25 | 0.35 | −0.71 | 0.48 | 0.95 |
| | 5 h at −10°C | 0.42 | 0.33 | 1.19 | 0.23 | 0.75 |

Adjusted *P*-values are Tukey–Kramer adjusted for multiple comparisons.

trend for increased survival in quick recovery lines compared with control. Lines selected for quick recovery at 0°C for 3 h and slow recovery at −10°C for 5 h did show a trend for higher proportions of survival at a long-term stress, which could indicate some response to selection on chronic cold stress survival. The increase in survival for slow recovery lines at −10°C for 5 h may seem counter-intuitive but suggests that this population may have evolved a trade-off response that confers tolerance to long-term cold stress compared to their susceptibility to shorter-term treatments. This result also indicates that *T. variabile* may use different mechanisms, physiological strategies, or mutations to adapt to short- and long-term exposure to cold (Teets and Denlinger 2013, Gerken et al. 2015). These slow recovery populations also show smaller body sizes compared their quick recovery counterparts and smaller body size could contribute to an increased ability to survive long-term stress following selection at the most extreme experimental levels. Cross-tolerance to different biotic and abiotic stresses has been observed in other insect species previously, but this tolerance among insects can be inconsistent at times depending on methodology (e.g., knockdown versus chill-coma vs survival; MacMillan et al. 2009, Andersen et al. 2015) as well as the mutations that are driving the selection within the adapted populations.

Changes in body sizes were also observed in the selected populations. Smaller body sizes were observed in populations that were selected for slow recovery time compared to quicker recovery time and these differences were more striking in individuals that had been exposed to more extreme conditions; e.g., −10°C or 5 h. Males and females tended to be smaller in terms of width and length when selected at 5 h compared to 3 h regardless of temperature (0 or −10°C) or selection direction. For these selected populations, it appears that longer selection exposure time to cold stress is associated with smaller body size. This change in body size could be due to population bottlenecks from selection or increased physiological demands experienced during exposure to cold (Williams 1966, Rosenheim et al. 2008, Berger et al. 2012). There is also some variation among replicates of the selected populations, which indicates that drift could still be a contributing factor but is not the single overarching driver of changes in body size, but there may be some effect over time (Supp Fig. 1 [online only]). Additionally, changes in body size may serve as the adaptive mechanism by which these insects recover from exposure to cold. Body size affects surface area, which in turn can affect temperature regulation, contact with the external environment, and recovery time after exposure to cold. Thus, exposure to a colder temperature at 5 h compared with 3 h may be selecting for those that will ultimately be smaller in size. Presumably, those individuals may be able to warm back up after cold exposure more quickly and thus, they will preferentially be selected for the next generation based on our selection protocol. However, other research has shown that in mealworms larger body sizes increase energy reserves, leading to greater cold tolerance (Renault et al. 2003), whereas in *D. melanogaster*, there was no correlation of body size to overall survival (MacMillan et al. 2009).

Lines selected for slow recovery time also had smaller body sizes than quick recovery time lines. In this case, the decrease in body size may be due to individuals with poorer fitness being selected for the next generation, which would perpetuate a reduction in body size. Additional support for this size change being due to a loss of fitness is that two of the populations selected for slow recovery at 5 h died out, one after five generations and one after six generations. Selecting for slow recovery or longer durations may also increase the impacts of inbreeding depression, accumulation of deleterious mutations due to population bottlenecks, or selection may have been linked to mutations with some fitness costs. Genotypes could also be affected by linkage drag, where recombination is unlikely to break linkage disequilibrium from traits associated with poorer fitness or developmental difficulties. Changes could also be due to a change in metabolic or reproductive capacity of parents such as reduced egg size (Renault et al. 2003). Populations selected at the 5-h time point may need to devote more metabolic responses to maintaining basal metabolism, which may result in a decline in energy devoted to reproductive capacity, leading to smaller progeny (Schaffer 1974, Rosenheim et al. 2008, Berger et al. 2012). Even though the stress

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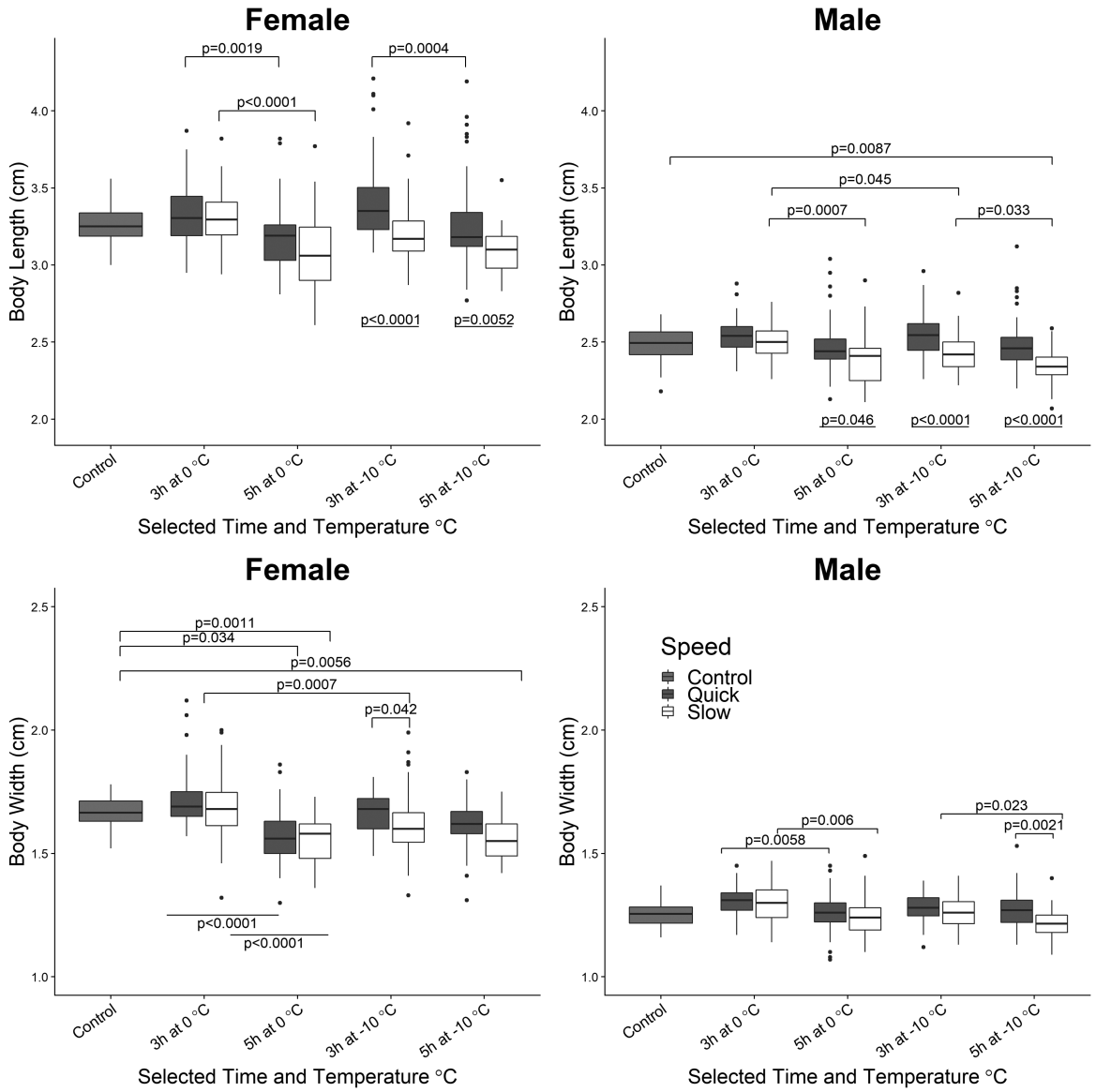


**Fig. 6.** Body length and body width comparisons of selected and unselected beetles. Relevant significant pairwise comparisons are represented by bars above and below the boxplots, with relevant comparisons being by exposure time and selection direction, temperature-selection direction, exposure time-temperature, or relative to unselected beetles. Edges of the boxes represent the 25% and 75% distribution of the data and whiskers represent ±1.58 × (interquartile range)/√(*n*), with outliers also represented as dark circles.

experienced by these populations is short term, these effects are long-lasting and persist over the entire adult lifespan.

Although a relationship between exposure time and body size was noted, no relationships between exposure times and recovery times were noted within the experiment. For example, in the initial population, exposure time did not appear to affect recovery time and in the selected populations, recovery times often did not differ between populations that were selected at 3 and 5 h. Previous research has suggested that lethal time of 50% of a population ($LT_{50}$) of *T. variabile* adults is 6.3 d at 0°C and 15 d for $LT_{95}$ (Abdelghany et al. 2015), so the time points used in our study are relatively short in comparison and may not lead to conditions that would lead to differences in recovery time.

Diapause in this species has been shown to increase cold tolerance when applied with acclimation to a mild cold temperature. The populations used in the current experiments were given ample amounts of food and were held with conspecifics in uncrowded conditions, so diapause should not have been a factor in their response to chill coma recovery, although it may have led to some of the outliers at each generation. Further studies examining how diapause affects selection for cold tolerance may provide further insight into how diapause interacts with cold tolerance.

Selected populations were all initiated from the same *T. variabile* laboratory population that has been kept in culture for almost 30 yr. They were also all raised in the same environmental chamber and subjected to stress in the same cold chamber. However, there were often differences in recovery time among the three replicates for each selection regime, which could indicate different adaptation mechanisms for responding to or tolerating the cold stress. This is likely, as there are multiple mutations that can lead to cold tolerance or susceptibility. For example, several different physiological processes are associated with response to cold stress as outlined by

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Teets and Denlinger (2013) and sources therein. Ion balances can become perturbed when insects are exposed to cold treatment, which impairs neuromuscular function and induces the chill-coma response (Koštál et al. 2004, Koštál et al. 2006, MacMillan and Sinclair 2011, MacMillan et al. 2012). Prolonged exposure to cold can cause irreversible damage to ion regulation, cellular metabolism, and ATP stores, which can lead to an accumulation of toxic by-products (Dollo et al. 2010, Koštál et al. 2011, Teets and Denlinger 2013). At extremely low temperatures, rapid membrane fluidity can also be affected (Steponkus 1984, MacMillan and Sinclair 2011), damage to the cytoskeleton can occur (Kim et al. 2006), and proteins and enzymes can be denatured. Additionally, reorganization of membrane structure (Russell 1997, Lee Jr et al. 2006) and changes in fatty acid composition and metabolism (Storey 1983, Michaud and Denlinger 2006) are induced by exposure to cold and may confer tolerance to this thermal stress. Selection could act on any of these processes, which could result in different mechanisms and adaptation for responding to cold in our selection lines. This would explain the variability in response times among the replicates within the same selection regime.

With a slew of physiological processes implicated in the response to cold stress, genetic changes for cold tolerance can manifest throughout the genome. In *D. melanogaster*, several gene expression studies have implicated stress proteins known as heat shock proteins as well as proteins associated with membranes (Qin et al. 2005) that transcriptionally respond to cold, whereas other studies have identified genes associated with muscle structure and function, immune response, stress response, carbohydrate metabolism, and egg production that were responsive to this stress (Zhang et al. 2011). Genes associated with transcriptional regulation, apoptosis, membranes, and calcium ions were enriched for quantitative trait loci variation in cold tolerance in *D. melanogaster* (Gerken et al. 2015). Selection for cold tolerance in *D. melanogaster* also shows varying responses in expression levels of genes involved in tolerance. For example, very few differences in gene expression were observed in *D. melanogaster* after selection for 10 generations indicated in one study (Sørensen et al. 2007), but 94 genes were differentially expressed between selected and unselected lines in another study in the absence of cold stress (Telonis-Scott et al. 2009). Heat shock proteins have also been implicated in changes in temperature and stress responses in stored products pests such as *Tribolium castaneum* (Mahroof et al. 2005, Zhao and Jones 2012). Surveying of genes in response to cold stress or selection has not been done in *Trogoderma* spp., and a list of candidate genes for temperature can provide a better understanding of genes that can evolve in response to cold tolerance or that may be targets of a gene-specific pesticide that uses genetic knockdown or knockouts to manage insect populations (Whyard et al. 2009, Gu and Knipple 2013, Zhang et al. 2013).

Interestingly, even though populations selected for quick recovery time had a smaller difference in mean recovery time among the three replicates after seven generations of selection (range 1.85–3.5 min) than did those selected for slow recovery time (range 2.5–12.6 min), variation in recovery time increased for individuals selected for both quick recovery (range 1.4–10.2 min) and slow recovery times (range 2.9–14.8 min) four generations after the selection experiments were stopped. This suggests that immediately following multiple generations of selection pressures, greater variation in the phenotype among replicates may be a signal of the detrimental phenotype or direction of selection (Frankham 1990) or mutations favorable under the right experimental conditions may carry fitness costs under normal environmental conditions and may not persist at high levels in the population in the absence of selection. When forced to undergo artificial selection, the populations selected for slow recovery may respond with more variation in random mutations than the tolerant lines, creating a more variable response in recovery time compared with the more tolerant selection response (Brakefield 2003). Further dissecting the underlying genetic differences among the replicates will provide additional information on the processes used to respond to artificial selection in a detrimental direction.

Stored product pests can survive under a variety environmental conditions, including human-made and thermally regulated facilities. Cold treatments may represent viable control tactics for some stored product species (Andreadis and Athanassiou 2017). However, this study shows that *T. variabile* populations can quickly adapt to nonlethal cold temperatures and that these adaptions could lead to cross-tolerance to more chronic cold stress, which has direct implications for pest control and disinfestation. We also observe that temperature has the most dramatic effects on chill-coma recovery time and that exposure time has very little effect on this phenotype. Furthermore, we also observed a greater variation in recovery time in the populations selected for slow recovery time, suggesting that slow recovery time is not a prevalent phenotype in this population and may be maladaptive and selected against in natural populations. Future genetic analyses will highlight the underlying changes to adaption to cold stress. Managers that use cold stress as a management tactic must use appropriate guidelines for temperature of exposure to ensure that the product cools to an appropriate temperature to induce lethality to prevent or reduce the likelihood of cold tolerance in a population because these insects can so quickly adapt to cold stress.

## Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

## Acknowledgments

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